ISSN 1300 - 6045

KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

The Journal of the Faculty of Veterinary Medicine, University of Kafkas

(Yılda Altı Sayı Yayımlanır)

(Published Bi-monthly)

http://vetdergi.kafkas.edu.tr

(ISSN: 1309-2251)

ÖZEL SAYI - A : GIDA ve HALK SAĞLIĞI
SUPPLEMENT - A : FOOD & PUBLIC HEALTH

Cilt / Volume : 16

Sayı / Number : SUPPLEMENT - A

Yıl / Year : 2010

ISSN 1300 - 6045

KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

THE JOURNAL OF THE FACULTY OF VETERINARY MEDICINE UNIVERSITY OF KAFKAS

ÖZEL SAYI-A: **GIDA ve HALK SAĞLIĞI**

SUPPLEMENT-A: **FOOD & PUBLIC HEALTH**

CİLT VOLUME 16 | SAYI NUMBER SUPPLEMENT-A | HAZİRAN JUNE 2010

This journal is indexed and abstracted by Thomson Reuters Services beginning with Volume 13 (1) 2007 in the followings:

- Science Citation Index Expanded (also known as SciSearch®)
- Journal Citation Reports/Science Edition

This journal is also indexed and abstracted in:

- CAB Abstracts
- ULAKBIM-TUBITAK

YAZIŞMA ADRESİ (ADDRESS FOR CORRESPONDENCE)

Kafkas Üniversitesi Veteriner Fakültesi Dergisi Editörlüğü
36040 - Kars / TÜRKİYE
Phone: +90 474 2426807-2426836/1244
Fax: +90 474 2426853
E-mail: vetdergi@kafkas.edu.tr

ELEKTRONİK BASKI (ELECTRONIC EDITION) **ISSN: 1309-2251**

http://vetdergi.kafkas.edu.tr

ONLINE MAKALE GÖNDERME (ONLINE SUBMISSION)

http://vetdergikafkas.org

Bu dergi Kafkas Üniversitesi Veteriner Fakültesi tarafından iki ayda bir yayımlanır.
This journal is published bi-monthly, by the Faculty of Veterinary Medicine, University of Kafkas.

BASKI (Print)

ESER OFSET MATBAACILIK
Tel: +90 442 2334667
ERZURUM

Bu Sayının Hakem Listesi *(alfabetik sıra)*
The Referees List of This Issue *(in alphabetical order)*

AKGÜN Sadi	Ankara Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
AKSU Harun	İstanbul Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
ATASEVER Mustafa	Atatürk Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
AYDIN Ali	İstanbul Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
AYDIN Berna Duman	Kafkas Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
AYGÜN Osman	Fırat Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
BOSTAN Kamil	İstanbul Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
ÇAKMAK Ayşe	Ankara Üniversitesi Veteriner Fakültesi Parazitoloji Anabilim Dalı
ÇELEBİ Selahattin	Atatürk Üniversitesi Tıp Fakültesi Mikrobiyoloji Anabilim Dalı
ÇELİK T. Haluk	Ankara Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
ÇIBIK Recep	Uludağ Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
ÇİFTÇİOĞLU Gürhan	İstanbul Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
DİK Bilal	Selçuk Üniversitesi Veteriner Fakültesi Parazitoloji Anabilim Dalı
DOĞAN Abdullah	Kafkas Üniversitesi Veteriner Fakültesi Farmakoloji Anabilim Dalı
DOĞRUER Yusuf	Selçuk Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
ERGUN Sebahattin	Çanakkale Onsekiz Mart Üniversitesi Su Ürünleri Fakültesi Su Ürünleri
EYİGÖR Ayşegül	Uludağ Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
GÜLMEZ Murat	Kafkas Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
GÜNER Ahmet	Selçuk Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
GÜRBÜZ Ümit	Selçuk Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
İNCİ Abdullah	Erciyes Üniversitesi Veteriner Fakültesi Parazitoloji Anabilim Dalı
KAYA Mükerrem	Atatürk Üniversitesi Ziraat Fakültesi Gıda Mühendisliği Bölümü
KAYAARDI Semra	Celal Bayar Üniversitesi Mühendislik Fakültesi Gıda Mühendisliği Bölümü
KILIÇ Birol	Süleyman Demirel Üniversitesi Mimarlık Mühendislik Fakültesi Gıda Mühendisliği Bölümü
KUBİLAY Ayşegül	Süleyman Demirel Üniversitesi Eğirdir Su Ürünleri Fakültesi Su Ürünleri Bölümü
KUMLAY Ahmet Metin	Iğdır Üniversitesi Ziraat Fakültesi
KÜPLÜLÜ Özlem	Ankara Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
NALBANTOĞLU Serpil	Ankara Üniversitesi Veteriner Fakültesi Parazitoloji Anabilim Dalı
NİZAMLIOĞLU Mustafa	Selçuk Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknoloji Anabilim Dalı
OĞUZ Halis	Selçuk Üniversitesi Veteriner Fakültesi Farmakoloji ve Toksikoloji Anabilim Dalı
ORAL Nebahat BİLGE	Kafkas Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
ÖZER Barbaros	Abant İzzet Baysal Üniversitesi Mühendislik-Mimarlık. Fakültesi Gıda Mühendisliği Bölümü
SAĞUN Emrullah	Yüzüncü Yıl Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
SERVİ Kadir	Fırat Üniversitesi Veteriner Fakültesi Farmakoloji ve Toksikoloji Anabilim Dalı
SEZER Çiğdem	Kafkas Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
ŞİRELİ Tansel	Ankara Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
TANYILDIZI Sadettin	Fırat Üniversitesi Veteriner Fakültesi Farmakoloji ve Toksikoloji Anabilim Dalı
TAYAR Mustafa	Uludağ Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
TRAŞ Bünyamin	Selçuk Üniversitesi Veteriner Fakültesi Farmakoloji Anabilim Dalı
UÇAR Gürkan	Selçuk Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı
ÜNLÜSAYIN Mustafa	Akdeniz Üniversitesi, Su Ürünleri Fakültesi Su Ürünleri İşleme Teknolojisi Anabilim Dalı
ÜNVER Ahmet	Kafkas Üniversitesi Veteriner Fakültesi Mikrobyoloji Anabilim Dalı
VATANSEVER Zati	Kafkas Üniversitesi Veteriner Fakültesi Parazitoloji Anabilim Dalı
YALÇIN Sakine	Ankara Üniversitesi Veteriner Fakültesi Hayvan Besleme ve Beslenme Hastalıkları Anabilim Dalı
YARSAN Ender	Ankara Üniversitesi Veteriner Fakültesi Farmakoloji ve Toksikoloji Anabilim Dalı
YAZAR Enver	Selçuk Üniversitesi Veteriner Fakültesi Farmakoloji ve Toksikoloji Anabilim Dalı
YILDIRIM Murat	İstanbul Üniversitesi Veteriner Fakültesi Farmakoloji ve Toksikoloji Anabilim Dalı

İÇİNDEKİLER
(CONTENTS)

ARAŞTIRMA MAKALELERİ
(RESEARCH ARTICLES)

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S1-S5, 2010
DOI:10.9775/kvfd.2009.744

RESEARCH ARTICLE

Isolation of Staphylococci from Food Handlers and Investigation of Their Enterotoxigenicity and Susceptibility to Some Antibiotics

Haluk ÇEPOĞLU * Leyla VATANSEVER ** Nebahat BİLGE ORAL **

* TSK KBRN Okulu Eğitim ve Merkezi Komutanlığı, 34840 Küçükyalı, İstanbul - TÜRKİYE
** Kafkas Üniversitesi, Veteriner Fakültesi, Besin Hijyeni Ve Teknolojisi Anabilim Dalı, 36100 Paşaçayırı, Kars - TÜRKİYE

Makale Kodu (Article Code): KVFD-2009-744

Summary

A total of 92 isolates of staphylococcal species consisting of 7 coagulase positive staphylococci (CPS) and 85 coagulase negative staphylococci (CNS) were isolated from hands of the 25 food handlers in different restaurants. Similarly, 13 coagulase positive staphylococci and 96 coagulase negative staphylococci isolates were cultured from the nasal cavity of the workers. Only one isolate of all the hand isolates was resistant to Vancomycin. Nine of all the coagulase negative staphylococci isolate including 4 hand and 5 nasal cavity samples were resistant to Methicillin. Four of 20 coagulase positive staphylococci isolate produced staphylococcal enterotoxins (SE). Only one hand isolate of all the coagulase negative staphylococci isolates produced staphylococcal enterotoxins E. These results indicate, like before, that the food handlers would have been the main source of the staphylococcal contamination of food. It is important to note that coagulase negative staphylococci can produce staphylococcal enterotoxins and they can also cause to food poisoning.

Keywords: *Food handlers, Staphylococci, Enterotoxin, Antibiotic susceptibility*

Gıda Çalışanlarından İzole edilen Stafilokokların Enterotoksijenitelerinin ve Bazı Antibiyotiklere Duyarlılıklarının Araştırılması

Özet

Farklı restoranlarda çalışan 25 gıda personelinin ellerinden, 85 koagulaz negatif stafilokok (KNS), 7 koagulaz pozitif stafilokok (KPS) olmak üzere 92 adet stafilokok izolatı elde edilmiştir. İşçilerin burun deliklerinden 13 koagulaz pozitif stafilokok ve 96 koagulaz negatif stafilokok izolatı kültüre edilmiştir. El izolatlarından bir tanesinin Vancomycin'e, 4'ü el, 5'i de burun örnekleri olmak üzere toplam 9 koagulaz negatif stafilokok'un Methicillin'e dirençli olduğu saptanmıştır. Yirmi koagulaz pozitif stafilokok izolatından 4'ünün stafilokokkal enterotoksin (SE), ellerden izole edilen koagulaz negatif stafilokok'lardan 1'inin de stafilokokkal enterotoksin E ürettiği gözlenmiştir. Ulaşılan bulgular, gıda çalışanlarının gıdaların kontaminasyonunda önemli bir kaynak oluşturabileceğini birkere daha doğrulamıştır. Ayrıca koagulaz negatif stafilokok izolatlarının da stafilokokkal enterotoksin üretebileceği ve dolayısıyla gıda zehirlenmesi oluşturabileceği, önemli bir veri olarak belirlenmiştir.

Anahtar sözcükler: *Gıda Çalışanları, Stafilokoklar, Enterotoksin, Antibiyotik duyarlılığı*

INTRODUCTION

Staphylococcal food poisoning is caused by the ingestion of foods containing enterotoxins produced by some species of staphylococci [1,2]. It is one of the most economically important diseases in the United States, costing approximately $1.5 billion each year [3]. The disease is characterized by sudden onset of symptoms, including; nausea, vomiting, abdominal cramps, and diarrhea within 1 to 6 h after ingestion of toxin-contaminated foods. The duration is short, generally lasting from 24 to 48 h and complete recovery usually occurs within 1 to 3

İletişim (Correspondence)
☎ +90 474 2426807/1123
✉ lvatansever@hotmail.com

days. Staphylococcal food poisoning is generally considered a mild, self-limited illness with low mortality rate. However, the hospitalization rate has been reported to be as high as 10% [4]. The diagnosis of this food-borne illness is based primarily on recovering enterotoxigenic staphylococci and enterotoxins from leftover foods.

Staphylococci are widespread in nature. They can be found in the air, in dust, in water, and on humans and animals. The main human reservoirs of these organisms are the skin and nasal cavity [5]. About 40 to 44% of healthy humans carry staphylococci in the nose [6]. Strains present in the nose often contaminate the back of hands, fingers and face and so, nasal carriers can easily become skin carriers. Although it is difficult to determine the origin of the strains involved in staphylococcal food poisoning outbreaks, food handlers are usually regarded as one of the primary source of these organisms [7,8]. It has been reported that, one of the important pathogens often transmitted via food contaminated by infected food handlers is Staphylococcus aureus [9]. For many years, *S. aureus* was the only staphylococcal species known to produce enterotoxins [1]. An important characteristic that differentiates *S. aureus* from most staphylococcal species is its ability to produce coagulase, an enzyme that clots blood plasma. Other coagulase - positive species such as *S. hyicus* [10] and *S. intermedius* [11] have been also identified. These and several coagulase - negative species including *S. epidermidis* [12] and *S. xylosus* [13,14] have been shown to produce low levels of enterotoxins. Among them, *S. epidermidis* and *S. intermedius* were reported to be the causative agents in food-borne outbreaks [2]. Therefore, while a high correlation between coagulase production and enterotoxigenicity has been reported [12,15,16], the ability to produce coagulase should not be considered the only indication for enterotoxin production. Although several staphylococcal species have been implicated in food - poisoning incidents, *S. aureus* remains as the predominant species.

The purpose of the present study was to determine whether the food handlers from different restaurants carried coagulase positive and negative staphylococci in their nasal cavity and hands. It was also aimed to investigate enterotoxigenicity and resistance of the isolates to some antibiotics.

MATERIAL and METHODS

Sample Collection

Samples from the nasal cavity and hands of the 25 food handlers working 5 different restaurants were obtained using sterile swabs which moistured using sterile saline (0.9% NaCl) solution. Samples were taken only ones during May.

Isolation of Staphylococci from Food Hhandlers

One swab was used to swab areas in between fingers and the wrist area of the hand and another swab was used to swab the nasal cavity. Each swab collected from the nasal cavity and hands was streaked on Baird Parker Agar (BP, Merck 1.05406.0500 + egg yolk-telluride emulsion, Merck 1.03785.0100) plates and incubated at 37°C for 48 h. Total of five colonies, two typical colonies of *S. aureus* are black, shiny, convex and surrounded by clear zones of approximately 2- 5 mm and three coagulase negative staphylococci are black, shiny colonies but clear zones are absent were selected. Five colonies were transferred in to tubes containing 5 ml of Brain Heart Infusion Broth (BHI, Oxoid CM375). The tubes were incubated at 37°C for 24 h and transferred to BP agar incubated at 37°C for 48 h. Then were transferred to Nutrient Agar slants (NA, Oxoid CM3) (stock culture) for further testing.

Identification of Isolated Colonies

Each colony was transferred from Nutrient Agar to two separate test tubes containing 1 ml of BHI broth and incubated at 37°C for 24 h. Catalase and tube coagulase tests were carried out. Gram stain and staphytect plus test, latex slide agglutination test (Oxoid DR 850 B), were also performed.

Tube coagulase and staphytect plus test negative, catalase positive, gram positive coccal isolates were further analysed to differentiate between coagulase negative staphylococci (CNS) and micrococci isolates. For this purpose, glucose fermentation (GF), acid production from glycerol (GA) and response to Furazolidone and Bacitracin antibiotics were used. Glucose fermentation and GA was determined by the method described by Baker [17]. Susceptibility to a 100µg Furazolidone disk (Oxoid CT0448B) and 10 units Bacitracin (Oxoid CT0005B) were determined using the standardized CLSI 2006 disc diffusion method [18]. The method was performed using Mueller Hinton Agar (Oxoid CM0337) with 5% defibrinated sheep blood which is prepared in the lab. Zone sizes at growth inhibition were measured in millimeters after 24 h of incubation at 37°C.

Susceptibility of Identified Colonies to Some Antibiotics

Susceptibility to antibiotics was tested by the disc diffusion method as described above with Mueller Hinton Agar. The following antibiotic discs were used;

Erythromycin (15 µg, Oxoid CT0020B), Gentamycin (10 µg, Oxoid CT0024B), Methicillin (5 µg, Oxoid CT0029B), Tetracycline (30 µg, Oxoid CT0054B) and Vancomycin (30 µg, Oxoid CT0058B). The plates were incubated at 37°C for 24 h. Zone size at growth inhibition were measured and determined according to Gür [19].

Enterotoxin Testing

All the CPS isolates and selected CNS isolates from hands and nasal cavity swabs were analysed for detecting staphylococcal enterotoxins. Selected colonies were inoculated 5 ml of BHI broth and incubated at 37°C for 24 h. and then centrifuged for 15 min at 5.000 g. Supernatant of culture extracts were filtered using sterile filter (0.2 µm, Sartorius CE-0297). A 100 µl of filtrate from each culture extracts were used staphylococcal enterotoxin analysis. Staphylococcal enterotoxins were detected by the sandwich enzyme immunoassay test kit RIDASCREEN SET A, B, C, D, E (R-Biopharm AG, D-64293, Germany). The test was performed by following the manufacturer's instructions.

RESULTS

A total of 250 isolates, 125 isolates from hands and 125 isolates from nasal cavity swabs, were examined. The 20 isolates were identified as CPS including 13 (10.4%) isolates from the nasal cavity and 7 (5.6%) isolates from the hands of workers. The CPS isolates were found to be egg yolk reaction positive except 3 isolates (2 isolates from nasal, one isolate from hand samples). 96 (76.8%) of 125 isolates from nasal swabs and 85 (72.8%) of 125 isolates from hand swabs were identified as CNS.

Resistance to different antimicrobial agents was detected in all CPS and CNS isolates. Five isolates (5.21%) were resistant to Methicillin, 27 (28.42%) to Erythromycin, 34 (36.17%) to Tetracycline and one isolate expressed medium level resistance to Vancomycin and Gentamicin. The results of antibacterial susceptibility of nasal CNS were shown in *Table 1*.

The results of antibacterial susceptibility of hand CNS isolates were shown in *Table 2*. While 4.70% isolates were resistant to Methicillin, 1.19% to Vancomycin, 33.33% to Erythromycin and 29.11% to Tetracycline, 3.53% isolates showed medium resistance to Methicillin, 2.38% to Vancomycin.

All the nasal and hand CPS isolates were found to be susceptible to Methicillin, Vancomycin and Gentamicin. One nasal CPS isolate and two hand CPS isolates were found to be resistant to Erythromycin and Tetracycline.

All the CPS (n:20) isolates and 52 (28.73%) of 181 CNS isolates from hands and nasal cavity swabs were analysed for detecting staphylococcal enterotoxins. Only one CNS isolate from hand swabs produced SEE. However, 4 CPS isolates 2 from nasal cavity and 2 from hand swabs, produced SE. One isolate from nasal cavity swabs produced SEA, SEC, SED and SEE. One isolate from hand swabs produced SEE and others produced SEC. None of them produced SEB.

Table 1. *Antibacterial susceptibility of nasal CNS isolates*
Tablo 1. *Nasal KNS izolatlarının antibakteriyel duyarlılığı*

Antibiotics	Numbers of Isolates	Susceptible (%)	Resistant (%)	Medium Resistant (%)
Methicillin	96	87 (90.63)	5 (5.21)	4 (4.16)
Vancomycin	96	95 (98.96)	0	1 (1.04)
Gentamicin	95	94 (98.95)	0	1 (1.05)
Erythromycin	95	68 (71.58)	27 (28.42)	0
Tetracycline	94	59 (62.77)	34 (36.17)	1.(1.06)

Table 2. *Antibacterial susceptibility of hand CNS isolates*
Tablo 2. *El KNS izolatlarının antibakteriyel duyarlılığı*

Antibiotics	Numbers of Isolates	Susceptible (%)	Resistant (%)	Medium Resistant (%)
Methicillin	85	75 (91.77)	4 (4.70)	3 (3.53)
Vancomycin	84	81 (96.43)	1 (1.19)	2 (2.38)
Gentamicin	83	82 (98.8)	0	1 (1.2)
Erythromycin	84	55 (65.48)	28 (33.33)	1 (1.19)
Tetracycline	79	55 (69.63)	23 (29.11)	1 (1.26)

DISCUSSION

In recent years, much attention has been given to food production, processing, packaging, transportation and storage. Therefore when foods are not produced and stored in proper conditions and or if any kind of damage occurs, they may be contaminated with infectious or toxigenic microorganisms, thus becoming a source of illness for humans. One aspect in the investigations of food poisoning outbreaks is to determine how the implicated food becomes contaminated. It is recognized that food handlers are the major source of contamination with staphylococci. High frequency of carrier status among food handlers has been identified by several investigators and many investigation studies conducted on staphylococci carrier status in humans in many countries [20-25], showed that 30 to 50% of them were carriers at any given time. Pereira et al.[25] examined 55 healthy food handlers in a large industrial kitchen in Belo Horizonte (Brazil) and found that 32 (58.2%) were carriers of *S. aureus* and 17 (30.9%) carried enterotoxigenic strains in their nasal cavity, throat and under fingernails. In the present study, 20 of 25 food handlers were found to be colonized by staphylococci.

In a study conducted by Udo et al.[26], the researchers found that 81.61% CNS from hand and only 7% CNS from the nasal cavity of the same workers. Francisco Polledo et al.[16] also found that CNS constituted 39.3% and CPS 27.6% of the nasal flora of food handlers. Our results from hands were similar to Udo et al.[26] with 72.8% CNS while results of nasal cavity (76.8% CNS) of the same restaurant workers was higher than Udo et al.[26] and Francisco Polledo et al.[16]. Differences from results may reflect differences in different populations living in different geographical regions as mentioned by Udo et al.[26]. At the same time, workers work in different foods so it is possible that, they introduce a lot of flora to their fauna of microorganisms from these foods.

Udo et al.[26] found that all CNS isolates (n: 155) were susceptible to Vancomycin, Gentamicin, Streptomycin. In our study one (1.19%) hand isolate were resistant to Vancomycin while two (2.38%) isolates were shown medium resistance to Vancomycin. However, all the CPS isolates were found to be susceptible to Vancomycine. Ligozzi et al.[27] was conducted a study to evaluate the VITEK 2 system for identification and antimicrobial susceptibility testing of medically relevant gram positive cocci and they used 100 clinical isolates of CNS. Their results also showed that all CNS isolates were susceptible to Vancomycin. Similarly to results of Udo et al.[26], our results also showed that the incidence of antibiotic resistance was lower than that obtained from the skin flora of hospitalized patient and clinical speciment [28-30].

Investigation of SE production from food served in restaurants was shown that five different SE can be detected from those foods and these SE are SEA, SEB, SEC, SED and SEE [31]. It is important to know source and distribution of these enterotoxigenic staphylococci to protect from food poisoning. Francisco-Polledo et al.[16] investigated 201 staphylococci isolates from food workers' nasal cavity for production of enterotoxins. They found that 36 CPS strains produced enterotoxins and distribution of enterotoxins were SEA (12 isolates), SEB (8 isolates), SEC (7 isolates), SED (2 isolates), SEE (2 isolates), SEA + SED (4 isolates) and SEB+SEC (one isolate). In their study, none of the coagulase negative isolates produced enterotoxin. In another study, 207 isolates of *S. aureus* from nasal cavity of restaurant workers were investigated for staphylococcal enterotoxin production and found that 55 isolate produced SE. They found that 18 strains produced SEA, 14 strains produced SEC, 13 strains produced SED and 9 strains produced SEB and SEE [4]. Udo et al.[26] also found that 8% of the CNS (including one nasal isolate) and 12.5% of *S. aureus* from the hands of the food handlers produced one or combination of staphylococcal enterotoxins. In the present study only one hand isolate (1.9%) of 52 CNS isolates and four (2 hand, 2 nasal cavity) isolates (20%) of 20 CPS was produced staphylococcal enterotoxin.

Despite the fact that only one of the CNS produced SE, its detection was significant because it confirms that CNS from different sources can produce SE [32].

The search for food borne pathogenic microorganisms is a common practice at the Public Health Laboratory, but examination for food handlers is sometimes neglected during the investigation of an outbreak. Food handlers must be considered a potential source of enterotoxigenic staphylococci, and the identification of the enterotoxin produced by strains isolated from both food handler and incriminated food will help trace the agent's profile. Although in some countries individuals colonized with staphylococci are not allowed to handle food, this is not a practical solution to the problem, because it is difficult to control. The best solution is the proper training of food handlers in order to prevent the contamination of vulnerable foods, and to instruct them on the need of proper storage of such foods. It is recommended that three approaches to reduce the incidence of food borne disease attributed to food handlers can be used: conducting training and education programs, implementing a Hazard Analysis and Critical Control Points system, and supporting certification of food service manager [9]. In conclusion it is important to know that CNS can also be cause of staphylococcal food poisoning along with *S. aureus* and food handlers could

be contaminate the food easily, if the food not handle carefully.

REFERENCES

1. Bergdoll MS: Staphylococcus aureus. **In,** Doyle MP (Ed): Foodborne bacterial pathogens. pp. 463-523, Marcel Dekker, Inc., New York, 1989.

2. Breckinridge JC, Bergdoll MS: Outbreak of foodborne gastroenteritis due to a coagulase-negative enterotoxin-producing *Staphylococcus. N Engl J Med,* 284, 541-543, 1971.

3. Todd ECD: Preliminary estimates of costs of foodborne disease in the United States. *J Food Prot,* 52, 595-601, 1989.

4. Holmberg SD, Blake PA: Staphylococcal food poisoning in the United States: New facts old misconceptions. *JAMA*, 251, 487-489, 1984.

5. Jay JM: Staphylococcal gastroenteritis. **In,** Jay JM (Ed): Modern Food Microbiology. 3rd ed., pp. 437-458, Van Nostrand Reinhold Company Inc., New York, 1986.

6. Williams REO: Healthy carriage of *Staphylococcus aureus*: its prevalence and importance. *Bacteriol Rev,* 27, 56-71, 1963.

7. Bryan FL: Factors that contribute to outbreaks of food borne disease. *J Food Prot,* 41, 816-827, 1978.

8. Genigeorgis CA: Present state of knowledge on staphylococcal intoxication. *Int J Food Microbiol,* 9, 327-360, 1989.

9. Ollinger-Snyder P, Matthews ME: Food safety: Review and implications for dietitians and dietetic technicians. *J Am Dietetic Assos,* 96, 163-171, 1996.

10. Devriese LA, Hájek V, Oeding P, Meyer SA, Schleifer KH: *Staphylococcus hyicus* (Sompolinsky 1953) comb. *nov*. and *Staphylococcus hyicus* subsp. *chromogenes* subsp. *nov*. *Int J Syst Bacteriol,* 28, 482-490, 1978.

11. Hájek V: *Staphylococcus intermedius*, a new species isolated from animals. *Int J Syst Bacteriol,* 26, 401-408, 1976.

12. Alişarlı M, Sancak YC, Akaya L, Elibol C: Investigation of *Staphylococcus aureus* isolation and thermonuclease activity and enterotoxin formation in some dairy desserts. *Turk J Vet Anim Sci,* 27, 1457-1462, 2003.

13. Bautista LP, Gaya M, Medina M, Nunez M: A quantitative study of enterotoxin production by sheep milk staphylococci. *Appl Environ Microbiol,* 54, 4799-4806, 1988.

14. Orden JA, Goyache J, Hernandez A, Domenech A, Suarez G, Gomez-Lucia E: Production of staphylococcal enterotoxins and TSST-1 by coagulase negative staphylococci isolated from ruminant mastitis. *J Vet Med B,* 39, 144-148, 1992.

15. Adesiyun AA, Raji I, Yobe V: Enterotoxigenicity of *Staphylococcus aureus* from anterior nasal cavity of dining hall workers. *J Food Protect,* 49, 955-957, 1986.

16. Francisco-Polledo JJ, Garcia ML, Moreno B, Menes I: Importance of food handlers as a source of enterotoxigenic staphylococci. *Zbl Bakt Hyg I Abt Org B,* 181, 364-373, 1985.

17. Baker JS: Comparison of various methods for differentiation of staphylococci and micrococci. *J Clin Microbiol,* 19, 875-879, 1984.

18. CLSI: Clinical and laboratory standards Institute. Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard - Ninth ed. Clinical and Laboratory Standards Institute document M2-A9. Wayne, PA, 2006

19. Gür D: Antimikrobik duyarlılık testleri için uygulama standartları. Onikinci Bilgi Eki. *Türk Mikrobiyoloji Derneği,* 52, Ocak 2002.

20. Carmo LS, Bergdoll MS: Staphylococcal food poisoning in Belo Horizonte (Brazil). *Rev Microbiol,* 21, 320-323, 1990.

21.Carmo LS, Vieira V, Reis J, Pereira ML, Santos EJ, Bergdoll MS: *Staphylococcus aureus* and *Salmonella enteritidis* present in food implicated in food poisoning. *Rev Microbiol,* 27, 122-125, 1996.

22. Iaria ST, Furllanetto SMP, Campos MLC: Pesquisa de *Staphylococcus aureus* enterotoxigênico nas fossas nasais de manipuladores de alimentos em hospitais. *Rev Saúde Pública,* 14, 93-100, 1980.

23. Mori M., Kato E, Hamada S: Distribution of enterotoxigenic staphylococci in healhty food handlers and biological properties of isolates. *Japan J Bacteriol*, 32, 501-508, 1977.

24. Oliveira Santos B, Aguillar M, Tanaka OMU: Behaviour of *Staphylococcus aureus* strains from simultaneous areas of healthy carriers working in different nursing category in a general teaching hospital. *Rev Microbiol,* 21, 309-310, 1990.

25. Pereira ML, Carmo LS, Lara MA, Silva SO, Dias RS, Bergdoll MS: Enterotoxigenic Staphylococci from food handlers working in an industrial kitchen in Belo Horizonte-MG (Brazil). *Rev Microbiol,* 25, 161-165, 1994.

26. Udo EE, Al-Bustan MA, Jacob LE, Chugh TD: Enterotoxin production by coagulase-negative staphylococci in restaurant workers from Kuwait City may be a potential cause of food poisining. *J Med Microbiol,* 48, 819-823, 1999.

27. Ligozzi M, Bernini C, Bonora MG, Fatima M, Zuliani J, Fontana R: Evaluation of the VITEK 2 system for identification and antimicrobial susceptibility testing of medically relevant gram-positive cocci. *J Clin Microbiol,* 40, 1681-1686, 2002.

28. Kloos WE, Bannerman TL: Update on clinical significance of coagulase negative staphylococci. *Clin Microbiol Rev,* 7, 117-140, 1994.

29. Larson RA, Burke JP: The epidemiology and risk factors for nosocomial catheter-associated bacteriuria caused by coagulase-negative staphylococci. *Infect Cont,* 7, 212-215, 1986.

30. Ug A, Ceylan Ö: Occurrence of resistance to antibiotics, metals and plasmids in clinical strain of *Staphylococcus* spp. *Arch Med Res*, 34, 130-136, 2003.

31. Soriano MJ, Font G, Molto C, Manes J: Enterotoxigenic staphylococci and their toxins in restaurant foods. *Trends Food Sci Technol,* 13, 60-67, 2002.

32. Hoover DG, Tatini SR, Maltais JB: Characterization of staphylococci. *Appl Environ Microbiol,* 46, 649-660, 1983.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S7-S11, 2010
DOI:10.9775/kvfd.2009.792

RESEARCH ARTICLE

The Determination of Dioxin and Dioxin-like Compound Levels in Beef and Chicken Meat Samples Consumed in Turkey [1]

Sadettin TANYILDIZI * Osman ÇİFTÇİ **

[1] *This study was supported by the Scientific and Technological Research Council of Turkey (TUBITAK), Project no: 105O244*

* Fırat Üniversitesi, Veteriner Fakültesi Farmakoloji ve Toksikoloji Anabilim Dalı, TR-23100 Elaziğ - TÜRKİYE
** İnönü Üniversitesi, Eczacılık Fakültesi, Farmasötik Toksikoloji Anabilim Dalı, TR-44280 Malatya - TÜRKİYE

Makale Kodu (Article Code): KVFD-2009-792

Summary

The polychlorinated dibenzo-p-dioxins (PCDDs), polyclorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyles (PCBs) residues in beef and chicken samples obtained from the different cities of Turkey were investigated. For this purpose, 120 beef and 120 chicken samples collected from different regions of Turkey between February 2006 and May 2008 and analyzed for dioxin and dioxin like compounds. The results showed that the mean levels of PCDD/Fs in lower bound of toxic equivalent concentrations (TEQ) were 2.83 and 3.17 pg TEQ/g fat for beef and chicken, respectively and in upper bound were 3.45 and 4.21 pg TEQ/g fat for beef and chicken, respectively. PCDDs were more dominant congeners according to PCDFs in both beef and chicken. Furthermore, the concentration of PCB congeners in lower bound were found 0.0108 and 0.0085 pg TEQ/g fat in order of beef and chicken, respectively and in upper bound were determined 0.0572 and 0.0595 pg TEQ/ g fat in beef and chicken, respectively. According to the obtained findings, on a lipid basis, levels of dioxins in beef and chicken were slightly higher than other countries and World Health Organization (WHO) limits. However, mean values of PCBs were lower than the other countries at all samples. In conclusion, the findings of this study showed that the TEQ levels of PCDD/Fs are slightly higher in beef and chicken samples consumed in Turkey and long-term consumption of these foods may cause health risk for human and animals.

Keywords: *Dioxin, PCDD/Fs, PCBs, Beef meat, Chicken meat*

Türkiye'de Tüketilen Kırmızı Et ile Tavuk Eti Örneklerinde Dioksin ve Dioksin Benzeri Bileşik Düzeylerinin Belirlenmesi

Özet

Yapılan bu çalışmada, Türkiye'nin değişik bölgelerinden toplanan tavuk eti ve kırmızı etlerde Poliklorludibenzo-p-dioksin (PCDD), Poliklorludibenzo furan (PCDF) ve dioksin benzeri bileşikler olan Poliklorlubifenil (PCB) bileşik düzeyleri araştırıldı. Bu amaçla, Şubat 2006 ile Mayıs 2008 tarihleri arasında Türkiye'nin değişik illerinden toplanan 120'şer adet tavuk eti ve kırmızı et numunesinde dioksin ve benzeri bileşiklerin kalıntı düzeyleri tespit edildi. Analizler sonucunda, PCDD/F bileşiklerinin toksik eşdeğer konsantrasyonları (TEQ) için alt sınırın kırmızı et ve tavuk etinde sırası ile 2.83 ve 3.17 pg TEQ/g yağ, aynı değerin üst sınırının ise yine sırası ile 3.45 ve 4.21 pg TEQ/g yağ olduğu belirlendi. Ayrıca PCDD bileşiklerinin miktar olarak PCDF bileşiklerine göre daha yüksek düzeyde olduğu saptandı. Buna ilaveten, PCB bileşiklerinin alt sınır düzeyleri kırmızı et ve tavuk etinde sırasıyla 0.0108 ve 0.0085 pg TEQ/g yağ iken, üst sınır değeri yine sırasıyla 0.0572 ve 0.0595 pg TEQ/ g yağ olduğu belirlendi. Elde edilen bulgular ışığında, yağ dokusu bazında, ülkemizde tüketilen kırmızı et ve tavuk etindeki PCDD/F bileşik düzeylerinin diğer ülkeler ve Dünya Sağlık Örgütünün (WHO) belirlediği limitlere göre çok az miktarda yüksek olduğu bununla birlikte, PCB bileşik düzeylerinin ise daha düşük olduğu tespit edildi. Sonuç olarak, Türkiye'de tüketilen kırmızı et ve tavuk etinde PCDD ve PCDF'lerin toksik eşdeğer konsantrasyonlarının az miktarda yüksek olduğu ve bu gıdaların uzun süreli tüketiminde insan ve hayvan sağlığı açısından risk oluşturabileceğini göstermektedir.

Anahtar sözcükler: *Dioksin, PCDD, PCDF, PCB, Kırmızı et, Tavuk eti*

İletişim (Correspondence)
☎ +90 422 3411216
✉ osmciftci@gmail.com

INTRODUCTION

Dioxins, which included polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (PCBs) are highly toxic chemicals and occur as widespread, low-level in animal feeds, human foods and environment [1,2]. The hydrophobic properties of these compounds cause their accumulation in the fatty content of food products [3]. The consumption of animal fats is the primary pathway for human exposure [4,5]. These compounds can induce various toxic responses including immuno-toxicity, carcinogenicity, and adverse effects on reproduction, development, and endocrine functions [6,7]. Dioxins caused significant food safety crises in 1996 in United States and Europe. The Belgian crises in 1999 is one of them, involved mineral oil containing high PCDD/F levels as a contaminant for poultry feed [8]. These incidents showed that the monitoring of dioxin concomitants in foodstuffs is required for human and animal health. For this reason, the toxicity and pollution risks of dioxin compounds have been investigated for 20 years in the world. However, there is no investigation about PCDD/Fs and PCBs levels in foods till now in Turkey.

The objective of this study is to determine the some dioxin and dioxin-like polychlorinated biphenyls compounds levels in beef and chicken samples consumed in Turkey between February 2006 and May 2008 and to compare these levels with international standards. This paper is also the first study about the residue levels of dioxin and dioxin-like compounds animal originated foods, chicken and beef meat, consumed in Turkey.

MATERIAL and METHODS

Sampling

Samples of beef and chicken were randomly purchased from the local market from thirteen major cities of Turkey (Ankara, Istanbul, Afyon, Kayseri, Gaziantep, Van, Konya, Bursa, Şanlıurfa, Elazığ, Hatay, Adapazarı, Izmit). The samples were stored at -20°C in the dark and extracted within 30 days. Every year, 40 beef and 40 chicken samples were bought from markets. Finally, we used 240 individual food samples in the 3 years for this investigation.

Materials

All chemicals and silica gel, type 60, 70/230 mesh were analytical grade for dioxin analyses and purchased from Merck (Germany). PCDDs, PCDFs and dioxin-like PCBs standards were bought from AccuStandard (New Haven, USA). $^{13}C_{12}$- labeled standards were obtained from Wellington Laboratories (Canada).

Extraction

The extraction was done according to modified method 8290 and 1613 of United States Environment Protection Agency (USEPA) [9]. A 20 g homogenized sample mixed with anhydrous sodium sulfate was spiked with $^{13}C_{12}$ - labeled standards (100 µl of 4 ng/ml), allowed to dry and then extracted for 16 h using methylene chloride: hexane (1:1) in a soxhalet extractor. The solvent was evaporated and the lipid content was determined gravimetrically.

The lipid residue was dissolved in toluene and the mixture was placed onto an acidic silica column. The column was prepared with a glass wool plug, 20 ml toluene and 1 g silica gel and then 4 g of 40% (w/w) sulfuric acid-impregnated silica gel was added. This column was washed twice with toluene and refluxed with toluene for 30 min. Finally, the eluate was evaporated to dryness and redissolved in toluene containing injection standards.

Analyses

Ten PCDD/Fs and three PCBs congeners were analyzed by PTV-LV-GC/MS, GC (Schimadzu QP 2010 plus) coupled with a Plus Mass Spectrometer operating the EI mode 70 eV and with a resolution of 10.000. The capillary column was a TRB5 MS capillary column (60 m x 0.32 mm I.D., 0.25 µm film thickness, Teknokroma S. Coop. C. Ltda, Barcelona, Spain) connected to a BEST PTV injector and PTV-LV 2.75×2TRC for PCDD/Fs and PCBs verification. The oven temperature was maintained at 100°C for 6 min, ramped at 52°C min^{-1} to 200°C; ramped at 2.9°C min^{-1} to 250°C for 6 min; ramped at 2.9°C min^{-1} to 260°C and finally ramped at 10°C min^{-1} to 300°C for 5 min. The equipment conditions were designed for Eppe et al. [10]. During the analysis the injection volumes were increased from 1 µl to 4 µl with the PTV-LV inlet. At the result, analytical sensitivity is greatly enhanced for analysis of samples.

Calculations

The TEQ values of PCDD/Fs and PCBs were calculated using the toxic equivalent factor (TEFs) according to World Health Organization [11]. The data below the detection limit (LOD) were calculated as lower, and upper bound levels, assuming that all levels of the different congeners were equal to zero or equal to their LOD, respectively. The recovery always ranged from 60% to 120%.

RESULTS

The metabolism and lifetime of an animal might affect the amount of residue and congener patterns of dioxin compounds in meat tissues. Totally, 240 beef and chicken samples were analyzed for PCDDs, PCDFs and non-ortho dioxin-like PCBs in this study. The minimum, maximum and mean concentration values for individual congeners and the fat content are showed in *Table 1*, the sum of all congeners are also included.

The results indicated that the concentrations of PCDDs were higher than PCDFs in beef and chicken samples. The highest PCDD/Fs levels were found in chicken samples. These levels were raised depending on the presence of 2,3,7,8 TCDD and 1,2,3,4,7,8 HxCDD. However, The Congeners 1,2,3,4,6,7,8 HpCDD, 1,2,3,4,6,7,8 HpCDF and 1,2,3,4,7,8,9 HpCDF were not determined in beef and chicken samples consumed in Turkey. The average lower and upper bound WHO-TEQ values for PCDD/Fs and non-ortho PCBs are given in *Table 2*. The TEFs recommended by the World Health Organization (WHO) in 1998 were used to calculate the toxicological concentration as TEQ. The Congeners 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD with the highest toxic equivalent factors (TEFs) values were determined in

Table 1. *Mean, minimum and maximum concentration values of PCDD, PCDFs and PCBs (pg/g fat) in the samples beef and chicken consumed in Turkey*

Tablo 1. *Türkiye'de tüketilen kırmızı et ve tavuk etinde PCDD, PCDF ve PCB bileşik kalıntı düzeylerinin ortalama, minimum ve maksimum konsantrasyonları. (pg/g yağ)*

Congeners	Beef (n = 120) Percent Fat (%) 4.96			Chicken (n = 120) Percent Fat (%) 16.3		
	Min	Max	Mean	Min	Max	Mean
PCDDs						
2,3,7,8 TCDD	0.43	3.16	1.56	0.93	4.5	2.10
1,2,3,7,8 PeCDD	0.12	2.23	0.92	0.16	3.2	1.0
1,2,3,4,7,8 HxCDD	0.40	3.80	2.17	0.43	3.10	1.42
1,2,3,6,7,8 HxCDD	0.20	2.70	1.40	0.32	2.82	1.28
1,2,3,7,8,9 HxCDD	0.25	2.45	1.10	0.25	2.12	0.95
1,2,3,4,6,7,8 HpCDD	nd	nd	nd	nd	nd	nd
OCDD	1.43	5.10	3.65	2.85	9.05	5.01
Sum PCDD	**2.83**	**19.44**	**11.1**	**4.94**	**26.79**	**11.76**
PCDFs						
2,3,7,8 TCDF	0.09	1.02	0.20	0.11	1.12	0.30
1,2,3,7,8 PeCDF	0.20	2.70	1.00	0.40	2.5	1.2
2,3,4,7,8-PeCDF	0.11	1.24	0.20	0.14	1.38	0.21
1,2,3,4,7,8 HxCDF	0,04	1.04	0.14	0.10	1.20	0.24
1,2,3,6,7,8 HxCDF	0.05	0.95	0.12	0.15	1.25	0.21
1,2,3,7,8,9 HxCDF	0.03	0.90	0.11	0.09	0.98	0.15
2,3,4,6,7,8 HxCDF	0.04	0.84	0.07	0.07	0.90	0.10
1,2,3,4,6,7,8 HpCDF	nd	nd	nd	nd	nd	nd
1,2,3,4,7,8,9 HpCDF	nd	nd	nd	nd	nd	nd
OCDF	1.05	4.86	2.22	1.15	5.24	3.11
Sum PCDF	**1.61**	**13.55**	**4.06**	**2.21**	**14.57**	**5.52**
Sum of PCDD/Fs	**4.44**	**32.99**	**15.16**	**7.15**	**41.36**	**17.28**
Non-ortho PCBs						
3,3',4,4'TCB	0.6	2.1	1.31	0.9	4.1	1.98
3,4,4',5 TCB	0.7	2.8	1.20	0.9	3.3	1.42
3,3',4,4',5PeCB	0.01	0.34	0.09	0.03	0.41	0.11
3,3',4,4',5,5' HxCB	0.9	6.8	2.52	1.1	7.1	2.38
2,3,3',4,4',5,5' HpCB	1.5	7.4	4.46	0.9	8.3	3.42
Sum PCB	**3.71**	**19.44**	**9.58**	**3.83**	**23.21**	**9.31**

n *= number of samples;* ***nd*** *= not detected*

both beef and chicken samples. The TEQ levels of PCDD/Fs increased depending on the high TEF values of 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD congeners. The congener profiles of TEQ levels are showed in *Fig. 1*.

Table 2. *Lower and upper bound concentrations of PCDD/Fs and PCBs in beef and chicken samples (pg TEQ/g fat)*

Tablo 2. *Kırmızı et ve tavuk eti örneklerinde PCDD, PCDF ve PCB bileşiklerinin toksik eşdeğer konsantrasyon olarak alt ve üst sınırı (pg TEQ/g yağ)*

Sample	PCDD/Fs pg TEQ/g		PCBs TEQ pg TEQ/g Fat	
	Lower Bound	Upper Bound	Lower Bound	Upper Bound
Beef	2.83	3.45	0.0108	0.0572
Chicken	3.17	4.21	0.0085	0.0595

The toxicity of the individual congeners may vary orders of magnitude. The TEQ concept has been developed to facilitate risk assessment and regulatory control [11].

There is no investigation about the concentrations of PCDDs, PCDFs and PCBs in food, water, animals, soil and air in Turkey. However, there is only a study about measurement of dioxin compounds in the soil and air of Kocaeli, a city of Turkey. Bakoglu and coworkers [13] reported that PCDD/Fs concentrations in surface soils were between 0.4 and 4.27 pg TEQ/kg. Besides, the concentrations of these toxic compounds in ambient air were between 23 and 563 fg/m³ in Kocaeli. These ambient air concentrations are higher compared to those monitored in European countries ranging about 100 fg/m³ [14,15]. Dioxin compounds enter atmospheric air

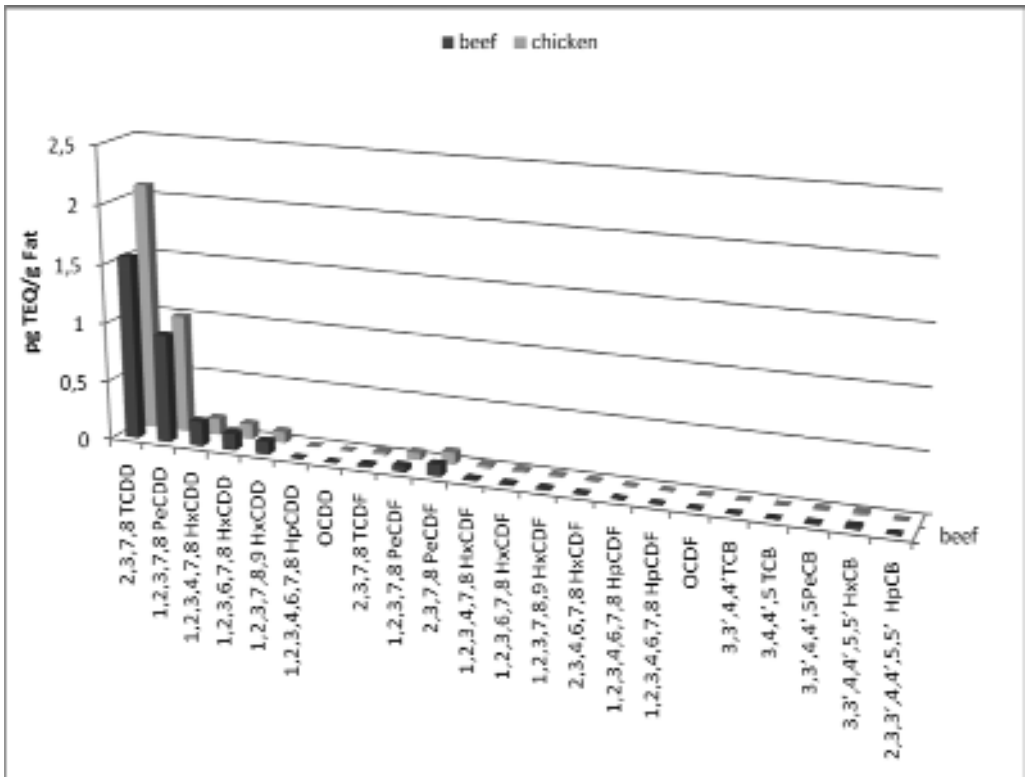

Fig 1. Congener profiles for TEQ levels of PCDD/Fs in beef and chicken meat

Şekil 1. Kırmızı et ve tavuk etinde bulunan PCDD ve PCDF bileşiklerinin toksik eşdeğer konsantrasyon düzeylerine göre profilleri

DISCUSSION

The exposure of human to PCDDs, PCDFs and PCBs occur through environmental, and accidental and occupational contamination. Over 90 percent of human exposure is determined to occur through the diet from animal origin. The sources of PCDD and PCDF contamination of animal foods are contaminated feed for animals, chicken and farmed fish, improper of sewage sludge, garden composts flooding of pastures and certain types of food processing [12]. Toxic equivalent (TEQ) measures all toxic dioxins, furans and PCBs in terms of the most toxic form of dioxin, 2,3,7,8-TCDD. from different sources and diffuse to the ground then directly transferred to animals through inhalation and indirectly taken through crops by animals [16]. The fats and proteins obtained from these animals contain PCDD/Fs residues are also used as feed. The consumption of these contaminated feed by animals cause accumulation of dioxin compounds in the body of animals. However, the investigation of the sources of dioxin contaminations is a very complex subject because Turkey is importing 60% of consumed feed and their additives.

It was reported that the values of PCDD/Fs TEQ were between 0.89 and 2.26 pg/g fat for beef and were

between 1.36 and 8.92 pg/g fat for chicken. Additionally, non- ortho PCB TEQ levels ranging from 0.15 to 0.44 pg/g fat for beef and were between 0.56 and 3.74 pg/g fat for poultry [17]. Guruge et al.[18]. suggested that the concentrations of PCDD/FS in the fat of chicken samples in Japan were 1.71 pg TEQ/g lipid. Besides, Kim et al.[19] reported that the mean values of PCDD/Fs in upper bound were 0.21 and 0.04 pg WHO TEQ/g fat for beef and chicken in South Korea. The findings of this study revealed that the mean values of PCDD/Fs in lower bound were 2.83 and 3.45 pg WHO- TEQ/g fat for beef and chicken, respectively. Additionally, the same values in upper bound were 3.17 and 4.21 pg WHO- TEQ/g fat for beef and chicken, respectively *(Table 2)*. It is very difficult the comparison of the levels of PCDD/Fs and PCBs with reported concentrations in the other countries. Because all samples were obtained from livestock had different environmental conditions and feed regime. Although, in this study, PCDD/Fs concentrations of beef and chicken samples were higher than the levels in Japan and South Korea, these values were similar levels reported in European countries (PCDD/Fs TEQ 3-4.5 pg/g fat for beef) [20].

The human and animals receives its major exposure to PCDD/Fs through the intake food. The findings of this study showed that the levels of PCDD/Fs TEQ are high in beef and chicken samples consumed in Turkey. For this reason, the consumption of these foods for a long time can cause health risks in human. To avoid dioxin contamination, the dioxin residues in food, air, soil and other environmental factors could be monitored in Turkey and continuous efforts are required to identify and decrease the sources of PCDD/Fs and PCBs release into the food chain.

REFERENCES

1. Lindström G, Haug LS, Nicolaysen T, Dybing E: Comparability of world-wide analytical data of PCDDs, PCDFs and non-ortho PCBs in samples of chicken, butter and salmon. *Chemosphere,* 47, 139-146, 2002.

2. Sasamoto T, Ushio F, Kikutani N, Saitoh Y, Yamaki Y, Hashimoto T, Horii S, Nakagawa J, Ibe A: Estimation of 1999-2004 dietary daily intake of PCDDs, PCDFs and dioxin-like PCBs by a total diet study in metropolitan Tokyo, Japan. *Chemosphere,* 64, 634-641, 2006.

3. Ferrario J, Byrne C: The concentration and distribution of 2,3,7,8-dibenzo-p-dioxins/-furans in chickens. *Chemosphere,* 40, 221-224, 2000.

4. Hallikainen A, Vartiainen T: Food control surveys of polychlorinated dibenzo-p-dioxins and dibenzofurans and intake estimates. *Food Addit Contam,* 14, 355-366, 1997.

5. Liem AKD, Fürst P, Rappe C: Exposure of populations to dioxins and related compounds. *Food Addit Contam,* 17, 241-259, 2000.

6. Birnbaum LS: Developmental toxicity of TCDD and related compounds: species sensitivities and differences. *Banbury Rep,* 35, 51-67, 1991.

7. Viluksela M, Stahl B, Birnbaum LS, Rozman KK: Subchronic/chronic toxicity of heptachlorodibenzop-dioxin (HpCDD) in rats. Part II: Biochemical effects. *Toxicol Appl Pharmacol,* 146, 217-226, 1997.

8. Pirard C, De Pauw E: Absorption, disposition and excretion of polybrominated diphenyl ethers (PBDEs) in chicken. *Chemosphere,* 66, 320-325, 2007.

9. USEPA: Test Method 8290 Polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) by high-resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS) Rev. Test Methods for Evaluating Solid Waste, SW-846 (NTIS PB88-239223). Environmental Protection Agency, Office of Solid Waste and Emergency Response, Washington, DC, September 1994.

10. Eppe G, Cofino WP, De Pauw E: Performances and limitations of the HRMS method for dioxins, furans and dioxin-like PCBs analysis if animal feedingstuffs Part I: Results of an inter-laboratory study, *Anal Chim Acta,* 519, 231-242, 2004.

11. WHO (World Health Organization): Executive Summary Report of Assessment of health risks of dioxins; re-evaluation of the Tolerable Daily Intake (TDI), 1998.

12. Turrio-Baldassarri L, Abate V, Alivernini S, Battistelli CL, Carasi S, Casella M, Iacovella N, Iamiceli AL, Indelicato A, Scarcella C, La Rocca C: A study on PCB, PCDD/PCDF industrial contamination in a mixed urban-agricultural area significantly affecting the food chain and the human exposure. Part I: Soil and feed. *Chemosphere,* 67, 1822-1830, 2007.

13. Bakoglu M, Karademir A, Durmusoglu E: Evaluation of PCDD/F levels in ambient air and soils and estimation of deposition rates in Kocaeli, Turkey. *Chemosphere,* 59, 1373-1385, 2005.

14. Mari M, Nadal M, Schuhmacher M, Domingo JL: Monitoring PCDD/Fs, PCBs and metals in the ambient air of an industrial area of Catalonia, Spain. *Chemosphere,* 73, 990-998, 2008.

15. König J, Theisen J, Günther WJ, Liebl KH, Büchen M: Ambient air levels of polychlorinated dibenzofurans and dibenzo(p)dioxins at different sites in Hessen. *Chemosphere,* 26, 851-861, 1993.

16. Schuler F, Schmid P, Schlatter C: Transfer of airborne polychlorinated dibenzo- p-dioxins and dibenzofurans into dairy milk. *J Agric Food Chem,* 45, 4162-4167, 1997.

17. Papadopoulos A, Vassiliadou I, Costopoulou D, Papanicolaou C, Leondiadis L: Levels of dioxins and dioxin-like PCBs in food samples on the Greek market. *Chemosphere,* 57, 413-419, 2004.

18. Guruge KS, Seike N, Yamanaka N, Miyazaki S: Polychlorinated dibenzo-p-dioxins,-dibenzofurans, and biphenyls in domestic animal food stuff and their fat. *Chemosphere,* 58, 883-889, 2005.

19. Kim M, Kim S, Yun SJ, Kim DG, Chung GS: Background levels and dietary intake of PCDD/Fs in domestic and imported meat in South Korea. *Chemosphere,* 69, 479-488, 2007.

20. European Commission: Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official J Europ Union,* L364, 5-24, 20.12.2006.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S13-S16, 2010
DOI:10.9775/kvfd.2009.793

RESEARCH ARTICLE

Determination of Aflatoxin Levels in Raw Milk, Cheese and Dehulled Hazelnut Samples Consumed in Samsun Province, Turkey [1]

Abdurrahman AKSOY * Oguzhan YAVUZ * Dilek GUVENC *
Yavuz Kursad DAS * Goknur TERZİ ** Suleyman CELİK ***

[1] *This study was supported by the Commission of Scientific Research Projects of Ondokuz Mayis University (Project No: VET-018) and the results of the study were presented at the 6th International Congress of Turkish Society of Toxicology in Antalya, Turkey on November 2-5, 2006.*

* Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ondokuz Mayis University, TR-55139 Samsun - TURKEY
** Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Ondokuz Mayis University, TR-55139 Samsun - TURKEY
*** Department of Pharmacology, Faculty of Medicine, Ondokuz Mayis University, TR-55139 Samsun - TURKEY

Makale Kodu (Article Code): KVFD-2009-793

Summary

Thirty six samples of raw cows' milk, 50 samples of milk products (25 samples each of fresh white cheese and Kashar cheese), and 50 samples of dehulled hazelnut were randomly collected from the Samsun province of Turkey. The dairy products and hazelnut samples were analyzed for the presence of aflatoxin M1 (AFM1) and aflatoxin B1 (AFB1), respectively, by microtiter-plate enzyme-linked immunosorbent assay (ELISA). The incidence of AFM1 contamination in the samples of raw cow milk, fresh white cheese and Kashar cheese were 61%, 12%, and 80%, respectively. AFB1 contamination was detected in 43 (86%) of the dehulled hazelnut samples (ranging from less than 1 to 11.3 μg kg^{-1}). The AFM1 levels determined in the samples of milk and dairy products were lower than the limit set by the Turkish Food Codex and European Union, whereas the AFB1 levels of two dehulled hazelnut samples exceeded the legal limit.

Keywords: *Aflatoxin, Milk, Dairy products, Dehulled hazelnut, ELISA, Samsun*

Samsun Çevresinde Tüketime Sunulan Çiğ Süt, Peynir ve Fındık Örneklerinde Aflatoksin Seviyelerinin Belirlenmesi

Özet

Otuz altı adet çiğ inek sütü, 50 adet süt ürünü (25'er adet taze beyaz peynir ve kaşar peyniri) ve 50 adet fındık örneği Samsun çevresinden rastgele toplandı. Süt ürünü ve fındık örnekleri sırasıyla aflatoksin M1 (AFM1) ve aflatoksin B1 (AFB1) yönünden ELISA testi kullanılarak analiz edildi. Çiğ inek sütü, taze beyaz peynir ve kaşar peyniri örneklerinde AFM1 ile bulaşma oranları sırasıyla %61, %12 ve %80 olarak belirlendi. Fındık örneklerinin 43 adedinde (%86) AFB1 tespit edildi (<1 ile 11.3 μg/kg^{-1} arasında). Süt ve süt ürünlerinde tespit edilen AFM1 miktarlarının Türk Gıda Kodeksi ve Avrupa Birliği tarafından belirlenen en yüksek tolere edilebilir limitlerin altında olduğu, bununla birlikte 2 fındık örneğinde AFB1 miktarının yasal limitlerden yüksek olduğu görüldü.

Anahtar sözcükler: *Aflatoksin, Süt, Süt ürünleri, Fındık, ELISA, Samsun*

INTRODUCTION

Mycotoxicosis, which can occur in both industrialized and developing countries, arises when environmental, social and economic conditions combine with weather conditions (humidity and temperature) that favor the growth of moulds [1]. The four main aflatoxins are B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2), and were

İletişim (Correspondence)
☎ +90 362 3121919/ 2816
✉ aaksoy@omu.edu.tr

named on the basis of their fluorescence (blue or green) under UV light and their relative chromatographic mobility during thin-layer chromatography [2]. The foremost food sources of aflatoxins include grains (particularly corn, sorghum and millet), peanuts, beans and tree nuts, including almonds and pistachios hazelnuts [3].

Exposure of animals to AFB1 occurs mainly by the ingestion of contaminated feeds. In the liver, ingested AFB1 is biotransformed by the hepatic microsomal cytochrome P450 into aflatoxin M1 (AFM1), which is then excreted into the milk of lactating animals [4,5]. In dairy cows the amount of AFM1 excreted into milk can be up to 3% of the AFB1 intake and is affected by milk yield [6]. The consumption of milk and milk products by human populations is quite high, particularly by infants and young children, thereby increasing the risk of exposure to AFM1 [7]. The European Commission (EC) and Turkish authorities have adopted 50 ng kg^{-1} as the maximum residue limit (MRL) for raw milk, heat-treated milk and milk used for the manufacture of milk-based products. However, the MRL is 25 ng kg^{-1} for infant milk [8,9].

Globally, the leading hazelnut producers are Turkey (73% of market share), followed by Italy (14%), the United States (4%), Spain (3%) and others (6%) [10]. In Turkey, hazelnuts are traditionally sun-dried and thus may be subject to mould growth and subsequent aflatoxin formation due to prolonged drying under humid or rainy conditions, similar to the process in other nuts [11]. The EC and Turkish governments have set the MRL for total aflatoxins in hazelnut, which is offered for direct human consumption, at 10 ng kg^{-1} [8,9].

The aim of this study was to determine the occurrence and levels of aflatoxins in dairy products and dehulled hazelnut samples consumed in Turkey.

MATERIAL and METHODS

Thirty six samples of raw cow's milk, 50 samples of milk products (25 samples each of fresh white cheese and Kashar cheese) and 50 dehulled hazelnut samples were randomly collected from the Samsun province in Turkey. The samples of dairy products and hazelnut were analyzed for the presence of aflatoxin AFM1 and AFB1, respectively, by ELISA.

The analysis was performed according to the procedures described by R-Biopharm GmbH [12]. Milk samples were centrifuged for 10 min at 3.500 rpm and 10°C for degreasing. After centrifuging, the upper layer of cream was removed completely by aspiration using a Pasteur pipette. Skimmed milk (defatted supernatant) was used directly in the test (100 µl per well). The limit of detection (LOD) for AFM1 in milk was <10 ng l^{-1}.

Samples of two grams of white or Kashar cheese and 40 ml of dichloromethane were used for extraction of AFM1. The suspension was filtered and a 10 ml aliquot was evaporated under a nitrogen stream. The extraction procedure was repeated with 0.5 ml of phosphate buffered saline (PBS), 0.5 ml of methanol and 1 ml of heptane. The extract obtained was subsequently centrifuged for 15 min at 2500 rpm and 15°C. The methanol layers were used for testing. The LOD for AFM1 in white and Kashar cheese was <100 ng kg^{-1}.

Two grams of powdered hazelnut samples were shaken for 10 min with 7 ml of methanol for AFB1 extraction. Then two milliliters of filtrate were transferred into a screw-top centrifuge vial, 2 ml of distilled water and 3 ml of dichloromethane were added and mixed for 5 min, and the solution was centrifuged for 5 min at 3250 rpm and 15°C. The upper aqueous layer was removed and the entire dichloromethane layer was used for the subsequent steps. Firstly, the dichloromethane layer was evaporated at 50-60°C. The extraction procedure was repeated with 0.4 ml PBS and 1.5 ml heptane, as described above. The upper heptane layer was removed, and the methanol layer was used for AFB1 testing. The LOD for AFB1 in hazelnut samples was 625 ng kg^{-1}.

ELISA was carried out on a Digital and Analog Systems microplate reader (Rome, Italy). RIDASCREEN Aflatoxin ELISA kits (R-Biopharm AG, Darmstadt, Germany) were used to determine the levels of AFM1 and AFB1. The basis of the test was the detection of specific antigens using the antigen-antibody reaction in the presence of an enzyme. The wells in the microtiter strips were coated with antibodies specific to AFM1 or AFB1. Measurements were carried out photometrically at 450 nm, and the absorption was inversely proportional to the concentration of aflatoxins present in the samples.

RESULTS

AFM1 contamination in the dairy products (raw fresh milk, white fresh cheese and Kashar cheese) is shown in *Table 1*. The AFM1 levels were lower than the maximum levels (cheese: 500 ng kg^{-1}; milk: 50 ng kg^{-1}) allowed by the Turkish Food Codex [9]. The AFB1 levels of dehulled hazelnut samples are shown in *Table 2*. They exceeded the legal limits of the EC (5 µg kg^{-1}) [8] in only two samples.

Table 1. *Aflatoxin M1 levels in samples of milk, white cheese, and Kashar cheese*
Tablo 1. *Süt, beyaz peynir ve kaşar peyniri örneklerinde aflatoksin M1 seviyeleri*

AFM1 (ng kg^{-1})	Milk		White Cheese		Kashar Cheese	
	n	%	n	%	n	%
Not detected	14	38.89	22	88.00	5	20.00
Detected	22	61.11	3	12.00	20	80.00
<1	7	19.44	-	-	-	-
1-10	15	41.67	1	4.00	3	12.00
10-50	-	-	2	8.00	13	52.00
50-100	-	-	-	-	3	12.00
100-250	-	-	-	-	1	4.00
>250	-	-	-	-	-	-
Total (n)	36	100.00	25	100.00	25	100.00
X±Sx	2.32±0.40		19.67±6.53		41.93±10.69	
Legal limit ng kg^{-1}	50 *		500 **		500 **	

***** *According to Turkish Food Codex and Commission Regulation of EC [8,9]*
****** *According to Turkish Food Codex [9]*

Table 2. *Aflatoxin B1 levels of dehulled hazelnut samples*
Tablo 2. *Fındık örneklerinde aflatoksin B1 seviyeleri*

AFB1 (µg kg^{-1})	n	%
Not detected	7	14.00
Detected	43	86.00
<1	26	52.00
1-5	15	30.00
5-10	1	2.00
>10	1	2.00
Total (n)	50	100.00
X±Sx	1.72±0.32	
Legal limit µg kg^{-1} *	5	

***** *According to Commission Regulation of EC [8]*

DISCUSSION

Monitoring programs for aflatoxin risk assessment in foodstuffs have been implemented in many countries, including Turkey, Italy, India and Spain [1,7,13-19].

Aycicek et al.[1] analyzed 223 samples of dairy products, 51 dehulled hazelnut samples and 40 cacao hazelnut creams in Turkey. Approximately 91.5% of white cheese samples were contaminated with AFM1, which is considerably higher than the result for our study (12%). However, 88.7% of the Kashar cheese samples from that study were positive for AFM1, similar to the result (80%) of the present study. Moreover, the AFB1 level of the dehulled hazelnut samples in that study (84.3%) was in parallel with our result (80%).

Tekinsen and Eken [13] investigated 100 ultra high temperature (UHT) milk and 132 Kashar cheese samples in Turkey for the presence of AFM1. It was determined that 67% of the UHT milk samples and 82.6% of the Kashar cheese samples were positive for AFM1. The results are similar to the results obtained from the samples of milk (61%) and Kashar cheese (80%) in the present study. However, in stark contrast, the AFM1 levels in 31 (31%) samples of UHT milk and 36 (27.3%) samples of Kashar cheese in the above study [13] exceeded the maximum tolerable limit of the EC and the Turkish Food Codex.

Gürses et al.[14] analyzed 77 samples of cheese in Turkey and found approximately 44% of samples to be positive for AFM1 residues. However, none of the levels was above the legal limit of the Turkish Food Codex [9].

Gürses [15] investigated 28 hazelnut, 24 walnut, 18 peanut, 13 almond and 11 roasted chickpea samples for aflatoxin contamination in Turkey. Nine of the 28 hazelnut samples were contaminated. The highest level of aflatoxin in those samples was 113 µg kg^{-1} and the mean concentration of aflatoxin was 33.4 µg kg^{-1}.

Bognanno et al.[16] examined 240 milk samples from dairy ewes in Italy. Eighty one percent of the samples were positive, which is higher than the result obtained in the present study (61%). Three samples with contamination above the legal limit (50 ng l^{-1}) were detected in that study.

Rastogi et al.[7] investigated AFM1 contamination in 87 samples of infant milk products and liquid milk in India. An 87.3% contamination rate was detected in the samples. It was also determined that the level of contamination by AFM1 (65-1012 ng l^{-1}) in infant milk products were higher than in liquid milk samples (28-164 ng l^{-1}). Almost 99% of the contaminated samples had contamination exceeding the EC limits (50 ng l^{-1}).

Nachtmann et al.[17] analyzed 316 milk samples in Italy and detected two samples (0.6%) with levels greater than the limits set out in the regulations. Five samples (1.6%) had a contamination level of 50 µg l^{-1}. Only two of the 316 samples were above the legal limit, whereas there were five samples at the threshold of the legal limit, which parallels the results of the present study.

Blesa et al.[18] examined 58 food samples for aflatoxin contamination in Spain. Three positive samples, which were hazelnut (0.42 and 0.52 µg kg^{-1} for AFB1 and AFG1, respectively), nut cocktail (0.29 and 0.47 µg kg^{-1} for AFB1 and AFG1, respectively), were detected. Except for two samples, the contamination levels detected in that study were below the limits of EU standards.

Bircan et al.[19] analyzed food samples for export from Turkey for total aflatoxin concentrations. Three hundred and thirteen of 2.643 dried fig samples, two of 80 hazelnut samples, 17 of 28 pistachio samples, five of 10 peanut samples, and 19 of 23 paprika samples were positive and within the ranges of 0.2-162.76, 5.46-6.55, 2.31-63.11, 0.75-26.36 and 1.79-6.55 µg kg^{-1}, respectively.

In the present study, the AFM1 levels detected in samples of milk and cheese products were lower than the maximum tolerable limit set by the Turkish Food Codex and European Union standards, whereas the AFB1 levels of two dehulled hazelnut samples exceeded the legal limit. However, the incidence of AFM1 in milk and Kashar cheese was high. In addition, AFB1 was also detected in most of the dehulled hazelnut samples. Although the contamination level was low, these residues would probably be harmful for humans. Clearly, any risk assessment study of a naturally occurring toxic compound must be based on information about its occurrence, exposure and toxicology, as indicated in the literature [1].

Therefore, it is concluded that the aflatoxin levels of dairy products and hazelnut samples should be monitored regularly and rigorously by the appropriate government agency and with mandatory public reporting. At the same time, it is imperative that producers, processors and consumers be educated about the hazards of these compounds and how to manage food products to minimise contamination. Consumer rights groups and consumers themselves should be vigilant and demand that producers, processors and government all work conscientiously to minimise the hazard of aflatoxin in animal and human foodstuffs.

Acknowledgements

The authors thank Gregory T. Sullivan of OYDEM at Ondokuz Mayis University in Samsun, Turkey for editing the English in this manuscript.

REFERENCES

1. Aycicek H, Aksoy A, Saygi S: Determination of aflatoxin levels in some dairy and food products which consumed in Ankara, Turkey. *Food Cont,* 16 (3): 263-266, 2005.

2. Bennett JW, Klich M: Mycotoxins. *Clin Microbio Rev,* 16 (3): 497-516, 2003.

3. Guzman-de-Pena D, Pena-Cabriales JJ: Regulatory considerations of aflatoxin contamination of food in Mexico. *Rev Latinoam Microbiol,* 47 (3-4): 160-164, 2005.

4. Battacone G, Nudda A, Palomba M, Pascale M, Nicolussi P, Pulina G: Transfer of aflatoxin B1 from feed to milk and from milk to curd and whey in dairy sheep fed artificially contaminated concentrates. *J Dairy Sci,* 88 (9): 3063-3069, 2005.

5. Gallo A, Moschini M, Masoero F: Aflatoxins absorption in the gastro-intestinal tract and in the vaginal mucosa in lactating dairy cows. *Ital J Anim Sci,* 7, 53-63, 2008.

6. Masoero F, Gallo A, Moschini M, Piva G, Diaz D: Carryover of aflatoxin from feed to milk in dairy cows with low or high somatic cell counts. *Animal,* 1 (9): 1344-1350, 2007.

7. Rastogi S, Dwivedi PD, Khanna SK, Das M: Detection of aflatoxin M1 contamination in milk and infant milk products from Indian markets by ELISA. *Food Cont,* 15 (4): 287-290, 2004.

8. European Commission: Commission Regulation (EC) No 1881/2006 of 19 December 2006. Setting maximum levels for certain contaminants in foodstuffs. *Off J Eur Commun,* L 364, 5-24, 2006.

9. Türk Gıda Kodeksi: Gıda Maddelerindeki Bulaşanların Maksimum Limitleri Hakkında Tebliğ. Resmi Gazete, 17 Mayıs 2008 - Sayı: 26879. Ankara Başbakanlık Basımevi, 2008.

10. Ozay G, Seyhan F, Yilmaz A: Sampling hazelnuts for aflatoxin: Uncertainty associated with sampling, sample preparation and analysis. *J AOAC Int,* 89 (4): 1004-1011, 2006.

11. Ozay G, Seyhan F, Pembeci C, Saklar S, Yilmaz A: Factors influencing fungal and aflatoxin levels in Turkish hazelnuts (*Corylus avellana* L.) during growth, harvest, drying and storage: A 3-year study. *Food Addit Contam,* 25 (2): 209-218, 2008.

12. R-Biopharm: Enzyme immunoassay for the quantitative analysis of aflatoxins. M1 Art. No: R-1101 and B1 Art. No: R-1201. R-Biopharm GmbH, Darmstadt, Germany, 1999.

13. Tekinsen KK, Eken HS: Aflatoxin M1 levels in UHT milk and kashar cheese consumed in Turkey. *Food Chem Toxicol,* 46, 3287-3289, 2008.

14. Gurses M, Erdoğan A, Cetin B: Occurrence of Aflatoxin M1 in some cheese types sold in Erzurum, Turkey. *Turk J Vet Anim Sci,* 28, 527-530, 2004.

15. Gurses M: Mycoflora and aflatoxin content of hazelnuts, walnuts, peanuts, almonds and roasted chickpeas (leblebi) sold in Turkey. *Int J Food Prop,* 9, 395-399, 2006.

16. Bognanno M, La Fauci L, Ritieni A, Tafuri A, De Lorenzo A, Micari P, Di Renzo L, Ciappellano S, Sarullo V, Galvano F: Survey of the occurrence of Aflatoxin M1 in ovine milk by HPLC and its confirmation by MS. *Mol Nutr Food Res,* 50 (3): 300-305, 2006.

17. Nachtmann C, Gallina S, Rastelli M, Ferro GL, Decastelli L: Regional monitoring plan regarding the presence of aflatoxin M1 in pasteurized and UHT milk in Italy. *Food Control,* 18 (6): 623-629, 2007.

18. Blesa J, Soriano JM, Molto JC, Manes J: Limited survey for the presence of aflatoxins in foods from local markets and supermarkets in Valencia, Spain. *Food Addit Contam,* 21 (2): 165-171, 2004.

19. Bircan C, Barringer SA, Ulken U, Pehlivan R: Aflatoxin levels in dried figs, nuts and paprika for export from Turkey. *Int J Food Sci Tech,* 43, 1492-1498, 2008.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S17-S22, 2010
DOI:10.9775/kvfd.2009.814

RESEARCH ARTICLE

Public's Knowledge, Opinions and Behaviors about Crimean-Congo Hemorrhagic Fever: An Example from Turkey

Nesrin ÇİLİNGİROĞLU * Fehminaz TEMEL * Hakan ALTINTAŞ *

* Hacettepe University, Faculty of Medicine, Department of Public Health, TR-06100 Ankara - TURKEY

Makale Kodu (Article Code): KVFD-2009-814

Summary

The aim of this study was to determine the knowledge, opinions and behaviors of the adults, regarding Crimean-Congo Hemorrhagic Fever (CCHF). This descriptive study data was collected from 405 adults who attended two health centers in Ankara city. The mean age of the adults was 38.2±14.5 years, 73.8% were female, and 63.0% had an educational level of secondary school or below. Of them, 91.6% were not involved with activities, which might have created risk in terms of CCHF. When the name of the disease or the tick was mentioned, 86.7% declared that they heard CCHF. The males (P<0.001), people with educational level of high school or more (P<0.001) and those who are currently working (P=0.004) heard CCHF more compared to others. Of the study population, 89.8% declared that disease was transmitted by ticks and caused fever (83.8%) and 65.0% knew there were ways of prevention from the disease. Mean knowledge score of CCHF was found to be 17.5±8.3 (min-max=0-30, median=20). Being younger than 50 years of age (P=0.003), being male (P=0.003), having an educational level of high school or higher (P<0.001), and working (P<0.001) increased the CCHF knowledge score. The study population did not have enough knowledge regarding CCHF. In order to increase the knowledge level, there should be cooperation among health authorities, health workers, veterinary public health service providers, educators, local authorities and the media.

Keywords: *Crimean-Congo Hemorrhagic Fever, Prevention, Tick, Knowledge, Behavior, Opinion*

Kırım Kongo Kanamalı Ateşinde Halkın Bilgi, Görüş ve Davranışları: Türkiye'den Bir Örnek

Özet

Çalışmanın amacı erişkinlerin Kırım-Kongo Kanamalı Ateşi (KKKA) ile ilgili bilgi, görüş ve davranışlarının saptanmasıdır. Bu tanımlayıcı çalışmada, veriler Ankara'da iki Sağlık Ocağı'na başvuran 405 erişkinden toplanmıştır. Erişkinlerin yaş ortalaması 38.2±14.5 yıl olup %73.8'i kadın ve %63'ü ortaokul ve altı eğitim düzeyine sahiptir. Yüzde 91.6'sı KKKA açısından risk taşıyabilecek işlerde çalışmamaktadır. Hastalığın adı belirtildiğinde veya kenelerin neden olduğu bir hastalık olarak hatırlatıldığında %86.7'si KKKA'ni duyduklarını söylemişlerdir. Erkek olmak (P<0.001), yüksek okul ve üstü düzeyde eğitimi olmak (P<0.001) ve halen bir işte çalışıyor olmak (P=0.004) KKKA'ni duymuş olmayı artıran faktörlerdir. Çalışmaya katılanların %89.8'si hastalığın kenelerle bulaştığını, %83.8'i ateşe neden olduğunu ve %65'i hastalıktan korunma yolları olduğunu bilmektedir. KKKA bilgi puanı ortalama 17.5±8.3 (En küçük-en büyük=0-30, ortanca=20) olarak bulunmuştur. Elli yaşından genç olmak (P=0.003), erkek olmak (P=0.003), yüksekokul ve üstü eğitim düzeyinde olmak (P<0.001) ve çalışıyor olmak (P<0.001) KKKA bilgi puanını artırmaktadır. Çalışmanın yapıldığı bireylerin KKKA hakkında yeterli bilgi düzeyine sahip olmadığı anlaşılmıştır. Toplumun bilgi düzeyinin artırılması için sağlık yöneticileri, sağlık çalışanları, toplum veterinerlik hizmetleri sunanlar, eğitimciler, yerel yönetimler ve medya arasında işbirliği yapılması gereklidir.

Anahtar sözcükler: *Kırım-Kongo Hemorajik Ateşi, Korunma, Kene, Bilgi, Davranış, Görüş*

INTRODUCTION

Crimean-Congo Hemorrhagic Fever (CCHF) is an acute illness affecting multiple organ systems and characterized by extensive ecchymosis, visceral bleeding, and hepatic dysfunction; and it has a case-fatality of 8% to 80%. This fatal viral infection was described in parts of Africa, Asia, Eastern Europe and the Middle East [1].

İletişim (Correspondence)
☎ +90 0312 3051599
✉ nesrinc@hacettepe.edu.tr

The virus belongs to the genus *Nairovirus* in the Bunyaviridae family, and causes severe disease in humans [2,3]. The disease was first characterized in the Crimea in 1944 and named as Crimean hemorrhagic fever. It was then recognized later in 1969 as the cause of disease in Congo, thus resulting in the current name of the disease. The CCHF virus may infect a wide range of domestic and wild animals. Animals become infected with CCHF, when infected ticks bite. Humans become infected through the bites of ticks, by contact with a patient who is in the acute phase of CCHF, or by contact with blood or tissues from veraemic livestock [4,5]. Changing social, economic and climatic conditions such as increased travelling, bio-terrorism and ecological disruption may increase the possibility of the introduction of Viral Hemorrhagic Fever (VHF)-associated viruses into new areas or increase the incidence in endemic areas [6]. Clinical features commonly show a dramatic progression characterized by haemorrhage, myalgia, and fever. Simple barrier precautions have been reported to be effective [7-17].

This infection is also an important public health issue in Turkey, because of its high case fatality rate [18]. *Hyalomma marginatum* is the main vector which transmits the virus to humans in Turkey [19-21]. The CCHF has not been officially reported in Turkey before 2002, although epidemics have been reported in neighboring countries before 2003 [22,23]. Cases infected with CCHF virus were first reported in Turkey in 2002 [24-26]. Between 2002-2008, 3135 cases of CCHF have been reported, and 155 (4.9%) of these cases died [27]. Since there is no effective treatment for this disease, means of prevention, and mainly public education on it is crucial [3].

The main objective of this study was to determine the knowledge, opinions and behaviors of the adults who admitted to two health centers for any kind of health related issue in Ankara, regarding CCHF.

MATERIAL and METHODS

In this descriptive study, 405 adults who attended two health centers in Ankara city, responded to a 27-item questionnaire, between 31 July-9 August 2006 (8 workdays included) since the seasonal peak was seen during this period. Although Ankara was not an endemic region for CCHF, many admissions due to tick bite were recorded in the city during the peak season. The first set of questions was related with the socio-demographic characteristics of the respondents. The second set of questions comprised knowledge and opinions on CCHF. Some of these questions included more than one alternative that could have been answered. Source of knowledge on CCHF, mode of transmission, acquisition from the animals, symptoms of the disease, knowledge on common preventive measures were asked. Behaviors of the participants were derived from both sets of questions. Data was collected by face to face interview technique. Each question about CCHF knowledge were scored as "1" (true) and "0" (false) and the knowledge level was evaluated according to the total score (Min=0, Max=30). In the further analysis, the cut-off point for the knowledge score was accepted as the median score for the study population. SPSS version 14 statistical software package (Chicago, IL) were used for data entry and chi-square test (χ^2) was used in statistical analysis for the study.

RESULTS

A total of 405 adults who accepted to participate in the study in Ankara city constituted the study group. Mean age of the adults was 38.2±14.5 years (min-max=18-80, median=36), 73.8% were female, 63.0% had a secondary school or lower level of education. Of the participants, 78.8% were not working and 85.7% were living in an apartment *(Table 1)*.

Table 1. *Some socio-demographic characteristics of participants*
Tablo 1 . *Katılımcıların bazı sosyo-demografik özellikleri*

Characteristics	n	%
Age		
29 and below	145	35.8
30-39	89	22.0
40-49	66	16.3
50 and above	105	25.9
Mean=38.2 years Min.=18 Max.=80 Sd=14.5		
Sex		
Male	106	26.2
Female	299	73.8
Education		
Illiterate	16	4.0
Secondary school or lower	239	59.0
High school or higher	150	37.0
Work Status		
Currently not working	319	78.8
Currently working	86	21.2
Health Insurance		
Insured	361	89.1
Not insured	44	10.9
House Type		
Apartment	347	85.7
Slum house	34	8.4
Detached house	24	5.9
Total	405	100.0

Of the adults, 8.4% were dealing with agriculture, livestock, and gardening activities, which had risk for CCHF *(Table 2)*.

Table 2. *Some job related risk characteristics of participants for CCHF*

Tablo 2. *KKKA açısından katılımcıların işleri ile ilgili bazı risk özellikleri*

Risk Ccharacteristics (n=405)	n	%
Involved in agriculture, livestock, and gardening		
Yes	34	8.4
No	371	91.6
Staying in rural areas during spring or summer		
Every year	91	22.5
Sometimes	100	24.7
Never	214	52.8

Of the participants, 86.7% declared that they heard this disease when the name of the disease or the tick was mentioned. There was statistically significant relationship between the sex of the adults and hearing the disease (P<0.001). Males heard the disease more than females. Adults who had an education level of high school and above declared that they heard the disease more when compared to the adults who had a lower level of education (P<0.001) *(Table 3)*.

Most of the adults knew that the disease was transmitted by ticks (89.8%) and caused fever (83.8%) *(Table 4)*, and more than half of them (65.0%) knew that there were precautionary measures against the disease.

Table 3. *The associations between some socio-demographic characteristics of participants and hearing the CCHF (%)*

Tablo 3. *Katılımcıların bazı sosyo-demografik özellikleri ile KKKA'ni duymuş olmaları arasındaki ilişkiler (%)*

Characteristics (n=405)	Ever Heard	Never Heard	Test Results
Sex			
Male	55.7	44.3	χ^2=17.265
Female	32.8	67.2	**P<0.001**
Age Group			
18-29	12.4	87.6	χ^2=0.962
30-39	5.6	94.4	**P=0.271**
40-49	7.6	92.4	
≥50	24.8	75.2	
Education			
Illiterate	18.8	81.2	χ^2=37.675
Primary/Secondary school	28.0	72.0	**P<0.001**
High school or more	58.0	42.0	
Work Status			
Currently working	52.3	47.7	χ^2=8.458
Currently not working	35.1	64.9	**P=0.004**
Total	38.8	61.2	

Regarding the information on vectors, majority of the responders mentioned tick (96.9%), dog (67.2%), livestock (69.2%), and false alternatives such as snake (25.1%). When knowledge on the symptoms of the disease was investigated, fever (83.8%) and lassitude (82.9%) were known as the most common symptoms, although false answers were also given such as hair loss (7.1%) *(Table 4)*. Ninety four percent of the adults stated that they learned the disease via television broadcasts. Only 6.4% stated that they learned the disease via the health centers, 7.4%, via the health care workers, and 4.0% via the internet.

Mean knowledge score was found to be 17.5±8.3 (min-max=0-30, median=20). The comparisons between the socio-demographic characteristics and knowledge score (≤19 and ≥20) were given in *Table 5*. It was found

Table 4. *The knowledge of participants regarding some characteristics of CCHF (%)**

Tablo 4. *Katılımcıların KKKA'nin bazı özellikleri hakkında bilgileri (%)**

Knowledge ** (n=405)	Yes	No	No Idea
Mode of Transmission			
Tick bite	89.8	2.8	7.4
Direct contact with patient's blood	60.4	17.1	22.5
Dirty water	30.8	41.6	27.6
Direct contact with infected animal's blood	72.1	9.4	18.5
Unwashed vegetables/fruits	37.6	46.4	16.0
Acquisition from the Animals			
Livestock	69.2	18.2	12.6
Fish	5.7	78.6	15.7
Tick	96.9	0.6	2.5
Snake	25.1	50.1	24.8
Bird	46.2	35.6	18.2
Dog	67.2	20.2	12.6
Symptoms of the Disease			
Fever	83.8	0.9	15.3
Infertility	3.7	45.3	51.0
Hair loss	7.1	45.0	47.9
Bleeding	43.0	24.5	32.5
Lassitude	82.9	2.3	14.8
Putting on weight	3.1	57.5	39.4
Headache	67.8	7.4	24.8
Precautions for the Disease			
Anti-tick treatment of animals (livestock)	52.8	2.5	44.7
The ticks should be killed by hands when seen	5.2	49.6	45.2
Insecticide use in the animal shelter	55.8	0.5	43.7
Animal shelters should be plastered and distempered	53.8	1.3	44.9
The sick people should be cured at home	4.7	51.1	44.2
No one should touch the blood and body fluids of sick animals or people without gloves	53.8	1.3	44.9

Correct answers are given in italics
** Row percentages, ** More than one correct answer*

that the knowledge score was significantly related to the participants' age (P=0.003), sex (P=0.003), education (P<0.001) and work status (P<0.001). Being younger than 50 years of age, being male, having an educational level of high school or higher, and working increased the CCHF knowledge score *(Table 5)*.

Table 5. *The distribution of associations between some characteristics of participants and knowledge scores (%)*

Tablo 5. *Katılımcıların bazı özellikleri ve bilgi puanları arasındaki ilişkilerin dağılımı (%)*

Characteristics (n=405)	Knowledge Score * (% **)		χ^2	P
	≤19	≥20		
Age Group				
≤ 29	44.1	55.9	13.723	**0.003**
30-39	47.2	52.8		
40-49	36.4	63.6		
≥50	62.9	37.1		
Sex				
Male	35.8	64.2	9.049	**0.003**
Female	52.8	47.2		
Educational Status				
Secondary school/lower	55.3	44.7	13.122	**0.000**
High school/higher	36.7	63.3		
Work Status				
Currently not working	53.9	46.1	18.352	**0.000**
Currently working	27.9	72.1		

** Scoring for knowledge: "1" (correct) and "0" (false),*
*** Row percentage*

DISCUSSION

In Turkey and other countries where the epidemic is common, the majority of cases have occurred in those who are involved in agriculture and animal husbandry. Slaughterhouse workers and veterinarians are also considered [8-15,26] as risk groups. In this respect, majority of our study group was not at risk as far as CCHF was concerned.

In the study, nearly eighty percent of the participants were females as most of the people who visited the health center were mainly housewives. They mostly brought their children for vaccination. Men were probably at work and often did not visit the health center. So, this limitation should be kept in mind while evaluating the results of the study.

Sex, education and employment status were found to be associated with knowledge regarding CCHF. Since most of the participants were housewives this can be a confounding factor in the analysis.

Most of the adults knew the transmission route of CCHF. This result was important as people could have protected themselves against the disease by knowing the mode of transmission. Most of the adults knew that CCHF caused headache, fever and lassitude. This knowledge might have been valuable in early diagnosis and treatment of CCHF, especially during the seasonal peak of the disease. Majority of the responders declared that there was a need for more information about the disease and its measures related to the prevention and control. There seemed to be some variations in terms of knowledge score depending on the socio-demographic characteristics of the study group, ie. young group, males, more educated and working people had high score probably due to being more aware on recent cases on CCHF.

As indicated by the responders, television broadcasts were the main source of information regarding CCHF. This result showed that television might have been an important tool to inform public on CCHF. Number of people who were informed by Internet was not high in this study. This result probably might have been due to the socio-economic status of the participants. Although there were educational tools, pamphlets, brochures as well as information on the website of Directorate of Health, they might have been ineffective due to distribution problems, lack of sufficient amounts, poor accessibility, level of literacy, or lack of Internet access.

Since most of the research on CCHF was concentrated on clinical and laboratory aspects of the disease in Turkey and neighboring countries, it was not possible to compare the study results with similar surveys. Two studies one from Iran and one from Turkey were conducted among healthcare workers regarding knowledge and attitudes of these personnel on CCHF and ticks. However, these two studies did not provide a comparable data as their target groups and items investigated were different [25,27].

Mean knowledge score was found to be 17.5±8.3 (min-max=0-30, median=20). There were statistically significant relationships between the age, (P=0.003), sex (P=0.003), education (P<0.001), work status (P<0.001) and the knowledge score. Being younger than 50 years of age, being male, having an educational level of high school or higher, and working increased the CCHF knowledge score. This statistical difference in age might have been due to the education level of the participants or communication difficulties with older people. As females were in majority in the study group, sex was also found to be associated with the knowledge.

In Turkey, primary health care was provided to the

majority of the population in health centers which were widely distributed throughout the country and these services were provided by 14956 physicians, and 31541 nurses/midwifes in health centers [28]. These figures indicated that majority of the population had the possibility to access primary care. This level of health care might contribute a lot in the struggle against CCHF by educating and providing information to the public, besides prompt and appropriate tick removal and early diagnosis of the disease. This study was just a pioneering and not a representative one. It provided some valuable clues regarding the general approach of the lay people on CCHF. Therefore, it might provide important information for the health care planners for future interventions such as implementing education for CCHF prevention. The general preventive measures may decrease the burden of this fatal disease. As it is not possible to eradicate it and there is no known treatment, prevention and tick-control remain as a vital mode of intervention. The questionnaire and results can form a baseline for the future studies.

It is known that people living in endemic areas should use personal protective measures, which include the avoidance of areas where tick vectors are abundant, particularly when the ticks are active; regular examination of clothing and skin for ticks, and their removal; and the use of repellents [8,29]. People who are exposed to potentially veraemic animal blood should take practical measures to protect themselves, including wearing gloves or other protective clothing to prevent skin contact with infected tissue or blood [8,9,16]. Informing public on precautions and practices is essential for preventing this disease. According to Scientific Advisory Board of CCHF in Turkey, preventive measures related to tick-control have to be taken by provincial veterinary bodies as well as related organs of the municipalities. A specific emphasis should be given to the people living in the rural area and people who go to picnic very often, since they are more vulnerable to tick bites. This will increase the chance of prevention from the tick bites and early diagnosis and treatment of the disease by increasing the awareness.

It was found that the study population did not have enough knowledge on CCHF. In Turkey where the epidemic is common, public's education on CCHF generally targeted the rural population. Therefore this can be one of the factors that affected this finding.

However, in the cities and in their suburbs, health centers can be the primary source of information or intervention since they are accessible for the middle and low-income groups who are more likely to go to the recreational places at weekends. To increase the knowledge there should be cooperation among health authorities, health workers, veterinary public health service providers, educators, local authorities and the media. Besides, most of the activities of the Provincial Directorate of Agriculture are planned and conducted in the rural areas. For the city centers, and the semi-urban areas the public information activities are insufficient [30]. Therefore, public authorities should provide support for developing projects to fight against various aspects of this public health problem.

REFERENCES

1. Hoogstraal H: The epidemiology of tick-borne Crimean-Congo hemorrhagic fever in Asia, Europe and Africa. A review. *J Med Entomol*, 15, 307-417, 1979.

2. Centers for Disease Control and Prevention: Viral hemorrhagic fever: Initial management of suspected and confirmed cases. *MMWR Morb Mortal Wkly Rep*, 32, 27-39, 1983.

3. Ergönül O: Crimean-Congo haemorrhagic fever. *Lancet Infect Dis,* 6, 203-214, 2006.

4. Whitehouse CA: Crimean-Congo hemorrhagic fever. *Antiviral Res,* 64, 145-160, 2004.

5. Vatansever Z: Vektör Kene Ekolojisi, *16. Ulusal Parazitoloji Kongresi Program ve Özet Kitabı,* s. 96-99, 1-7 Kasım 2009.

6. Crowcroft NS, Morgan D, Brown D: Viral haemorrhagic fevers in Europe-effective control requires a co-ordinated response. *Euro Surveill,* 7, 1-32, 2002.

7. Elaldı N: Kırım-Kongo Kanamalı Ateşi (KKKA): Klinik Özellikler ve Tedavi, *16. Ulusal Parazitoloji Kongresi Program ve Özet Kitabı,* s. 94-95, 1-7 Kasım 2009.

8. TC Sağlık Bakanlığı Temel Sağlık Hizmetleri Genel Müdürlüğü: Kırım-Kongo Kanamalı Ateşi Vaka ve Ölümlerinin Yıllara Göre Dağılımı, Türkiye, 2002-2008. http://www.kirim-kongo.saglik.gov.tr/, *Accessed:* 12.06.2009.

9. Vorou R, Pierroutsakos IN, Maltezou HC: Crimean-Congo hemorrhagic fever. *Curr Opin Infect Dis,* 20 (5): 495-500, 2007.

10. Peters CJ: Infections caused by Arthropod and rodent-borne viruses. **In,** Fauci AS, Braunwald E, Isselbacher KJ, Wilson JD, Martin JB, Kasper DL, Hauser SL, Longo DL, Harrison TR (Eds): Harrison's Principles of Internal Medicine. 14th ed. pp. 1136–1140, McGraw-Hill, New York, 1998.

11. World Health Organization: Crimean-Congo Hemorrhagic Fever. Geneva: WHO Fact Sheet No: 208; 2001.

12. Papa A, Bino S, Papadimitriou E, Velo E, Dhimolea M, Antoniadis A: Suspected Crimean Congo Haemorrhagic Fever cases in Albania. *Scand J Infect Dis,* 40, 978-980, 2008.

13. Papa A, Christova I, Papadimitriou E, Antoniadis A: Crimean-Congo hemorrhagic fever in Bulgaria. *Emerg Infect Dis,* 10, 1465-1467, 2004.

14. Onishchenko GG, Efremenko VI: Crimean-Congo haemorrhagic fever in southern Russia. *Zh Mikrobiol Epidemiol Immunobiol,* 4, 86-90, 2004.

15. Mandell GL, Bennet JE, Dolin R: Bunyaviridae-California

encephalitis, hantavirus pulmonary syndrome and Bunyavirid hemorrhagic fevers. **In,** Mandell GL (Ed): Principles and Practice of Infectious Diseases. 5th ed, pp. 1849-1853, Churchill Livingstone, New York, 2000.

16. Rahnavardi M, Rajaeinejad M, Pourmalek F, Mardani M, Holakouie-Naieni K, Dowlatshahi S: Knowledge and attitude toward Crimean-Congo haemorrhagic fever in occupationally at-risk Iranian healthcare workers. *J Hosp Infect,* 69, 77-85, 2008.

17. Athar MN, Khalid MA, Ahmad AM, Bashir N, Baqai HZ, Ahmad M, Balouch AH, Bashir K: Crimean-Congo hemorrhagic fever outbreak in Rawalpindi, Pakistan, February 2002: Contact tracing and risk assessment. *Am J Trop Med Hyg,* 72, 471-473, 2005.

18. Akkaya H: Entegre kene mücadelesi. *16. Ulusal Parazitoloji Kongresi Program ve Özet Kitabı,* s. 75-77, 1-7 Kasım 2009.

19. Estrada-Pena A, Vatansever Z, Gargili A, Aktas M, Uzun R, Ergonul O, Jongejan F: Modeling the Spatial Distribution of Crimean-Congo Hemorrhagic Fever Outbreaks in Turkey. *Vector Borne Zoonotic Dis,* 7, 667-678, 2007.

20. Vatansever Z, Midilli K, Ergin S, Aktaş M, Gargılı A: Kırım Kongo Kanamalı Ateşi açısından endemik bölgede aç kenelerde KKKA virüsünün yaygınlığı. *16. Ulusal Parazitoloji Kongresi Program ve Özet Kitabı,* 205, 1-7 Kasım 2009.

21. Vatansever Z, Uzun R, Estrada-Pena A, Ergonul O: Crimean-Congo Hemorrhagic Fever in Turkey, **In,** Ergonul O, Whitehouse CA (Eds): Crimean-Congo Hemorrhagic Fever: A Global Perspective. pp. 59-74, 2007.

22. Avsic-Zupanc T: Epidemiology of Crimean-Congo Hemorrhagic Fever in the Balkans. **In,** Ergonul OWCA (Ed): Crimean-Congo Hemorrhagic Fever: A Global Perspective. pp. 75-88, Dordrecht, Springer; 2007.

23. Avsic-Zupanc T, Ahmeti S, Petrovec M, Rossi CA: Retrospective analysis of an outbreak of Crieman-Congo hemorrhagic fever in the Kosovo during 1991-1992. *Am J Trop Med Hyg,* 61, 318-319. 1999.

24. Ergonul O, Celikbas A, Dokuzoguz B, Eren S, Baykam N, Esener H: The characteristics of Crimean-Congo Hemorrhagic Fever in a recent outbreak in Turkey and the impact of oral ribavirin therapy. *Clin Infect Dis,* 39, 285-289, 2004.

25. Karti SS, Odabasi Z, Korten V, Yilmaz M, Sonmez M, Caylan R, Akdogan E, Eren N, Koksal I, Ovali E, Erickson BR, Vincent MJ, Nichol ST, Comer JA, Rollin PE, Ksiazek TG: Crimean-Congo Hemorrhagic Fever in Turkey. *Emerg Infect Dis,* 19, 1379-1384, 2004.

26. Bakir M, Ugurlu M, Dokuzoguz B, Bodur H, Tasyaran MA, Vahaboglu H: Crimean-Congo Hemorrhagic Fever outbreak in Middle Anatolia: A multicenter study of clinical features and outcome measures. *J Med Microbiol,* 54, 1-5, 2005.

27. İnceboz T, Över L, Şimşek H: Sağlık çalışanlarının kene ve kene vektörlüğüne ilişkin bilgi düzeyleri. *16. Ulusal Parazitoloji Kongresi Program ve Özet Kitabı, s.* 301-302, 1-7 Kasım 2009.

28. Hamzaoğlu O, Özcan U: Türkiye Sağlık İstatistikleri 2006. Birinci Baskı, s. 67, Türk Tabipleri Birliği Yayını, Ankara, Aralık 2005.

29. Aydın L: Kenelerde korunma ve kontrol. *16. Ulusal Parazitoloji Kongresi Program ve Özet Kitabı, s.* 102-103, 1-7 Kasım 2009.

30. Sümer A: Kene ısırığı nedeniyle Kaş Devlet Hastanesi acil servisine başvuran hastaların değerlendirilmesi. *Kafkas Univ Vet Fak Derg,* 16 (1): 49-53, 2010.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S23-S29, 2010
DOI:10.9775/kvfd.2009.1147

RESEARCH ARTICLE

Effect of Oregano Essential Oil on Biofilms Formed By Staphylococci and *Escherichia coli*

Nebahat BİLGE ORAL * Leyla VATANSEVER * Berna DUMAN AYDIN *
Çiğdem SEZER * Abamüslüm GÜVEN * Murat GÜLMEZ *
Kemal Hüsnü Can BAŞER ** Mine KÜRKÇÜOĞLU **

* University of Kafkas, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, TR-36100 Kars - TÜRKİYE
** Anadolu University, Farmacy Faculty, Department of Pharmacognosy, TR-26470 Eskişehir - TÜRKİYE

Makale Kodu (Article Code): KVFD-2009-1147

Summary

In the present study, it was aimed to investigate the effect of oregano *(Origanum onites)* essential oil (EO) on biofilm formation and established biofilm. *Staphylococcus aureus* (n=6), *Staphylococcus lugdunensis* (n=1), *Staphylococcus haemolyticus* (n=1), *Staphylococcus sciuri* (n=1) and *Escherichia coli* (n=1) were used as the test organisms. The crystal violet assay was used for assessing the growth of the biofilm on 96-well polystyrene microtitre plates. The minimum inhibitory concentration (MIC) was determined by broth dilution method as 0.05% (v/v) for staphylococci with the exception of *Staph. sciuri* (0.8%, v/v) and 0.1% (v/v) for *E. coli.* Oregano EO inhibited biofilm formation and eradicated established biofilm at MIC level. Subinhibitory concentrations of the EO reduced the level of biofilm formation of the test strains. Further investigations should be examined whether these observations extend to biofilms formed on other surfaces, particularly those found in food processing plants. The ability of biofilm-embedded microorganisms to resist clearance by antimicrobial agents points to the importance of a continuous search for novel agents. The use of oregano EO as a natural antimicrobial agent can be an effective alternative or a supplement for the control of microorganisms.

Keywords: *Biofilm, Staphylococci, Escherichia coli, Oregano, Essential oil*

Kekik Uçucu Yağının Stafilokoklar ve *Escherichia coli* Tarafından Oluşturulan Biyofilmler Üzerine Etkisi

Özet

Bu çalışmada, kekik *(Origanum onites)* uçucu yağının, biyofilm oluşumu ve oluşmuş biyofilm üzerine etkisini belirlemek amaçlanmıştır. Test mikroorganizmaları olarak *Staphylococcus aureus* (n=6), *Staphylococcus lugdunensis* (n=1), *Staphylococcus haemolyticus* (n=1), *Staphylococcus sciuri* (n=1) ve *Escherichia coli* (n=1) suşlarından yararlanılmıştır. Biyofilm gelişiminin 96 kuyucuklu polistiren plaklarda görüntülenebilmesi için, Kristal Viyole deneyi uygulanmıştır. Broth Dilusyon Yöntemi kullanılarak değerlendirilen minimum inhibisyon konsantrasyonları (MİK), *Staph. sciuri* (%0.8 v/v) dışındaki stafilokok suşları için %0.05 (v/v), *E. coli* için ise %0.1 (v/v) olarak belirlenmiştir. Kekik uçucu yağı, MİK düzeyinde kullanıldığında biyofilm oluşumunu inhibe etmiş, oluşmuş biyofilmi eradike etmiştir. İnhibitörik düzeyin altındaki konsantrasyonlarda ise test suşları tarafından biyofilm oluşturma düzeyini düşürmüştür. Başta gıda işleme yerlerinde bulunanlar olmak üzere, diğer yüzeylerde de benzer etkilerin meydana gelip gelmeyeceğinin belirlenmesi amacıyla, yeni çalışmaların yapılmasının yerinde olacağı kanaatine varılmıştır. Biyofilm içinde yer alan mikroorganizmaların, antimikrobiyel ajanlarla temizliğe direnç göstermesi, yeni ajanların bulunması yönündeki çalışmaların önemine işaret etmektedir. Bu anlamda kekik uçucu yağının, doğal bir antimikrobiyel madde olarak, mikroorganizmaların kontrol altına alınmasında etkili bir alternatif olabileceği ya da bu doğrultuda katkıda bulunabileceği görülmektedir. .

Anahtar sözcükler: *Biyofilm, Stafilokok, Escherichia coli, Kekik, Uçucu yağ*

İletişim (Correspondence)
☎ +90 474 2426807/1178
✉ nebahatbilgeoral@hotmail.com

INTRODUCTION

A biofilm is a multicellular layer of adherent bacteria surrounded by a matrix of extracellular polysaccharides [1]. Typically, anywhere that there is a flow of water, organisms and a solid surface, a biofilm can be formed. The solid surfaces that can harbour biofilms in food plants include stainless steel, aluminium, glass, nylon materials, Buna-N and Teflon seals. Surfaces that are pitted, scratched or cracked provide an excellent opportunity to trap food particles and bacteria, which begins the formation of a biofilm. Corrosion patches and dead ends are also areas where biofilms can grow [2].

In nature, biofilms or adhesion of microorganisms may be composed of a single species or represent a consortium of numerous species [3]. They can cause significant problems in many areas, both in medical settings (e.g. persistent and recurrent infections, device-related infections) and in non-medical (industrial) settings (e.g. biofouling in drinking water distribution systems and food processing environments [4,5]. The attachment of bacteria with subsequent development of biofilms in food processing environments is a potential source of contamination that may lead to food spoilage or transmission of disease [6].

It is well established that bacteria contained within biofilms exhibit increased resistance to antimicrobial treatments compared to individual cells grown in suspension. As the biofilm matures, resistance against various disinfectants is greater than with younger (less than 24 h) biofilms [2]. To control these problems, it has been recognized that a greater understanding of the interaction between microorganisms and food processing surfaces is required [3]. The best way of controlling biofilms is to prevent their development [2].

The control of food borne pathogens such as Staphylococci and *Escherichia coli* has received a great deal of attention because these organisms can form resilient biofilms on a range of surfaces [7,8]. Several genes and mechanisms are involved in biofilm production, but the regulatory mechanisms are poorly understood. The finding that biofilm formation may be promoted at conditions in the food industry indicates that the food producers should be aware that controlling biofilm formation by *S. aureus* may be of importance [7]. *E. coli* can behave as a commensal, intestinal diarrhoeagenic, and extraintestinal pathogenic microorganism. It has been shown that *E coli* strains causing prostatitis produce biofilms *in vitro* more frequently than those causing urinary tract infections, and that they are more likely to be haemolysin producers. In addition, biofilm-forming strains show significantly greater haemolysin and type 1 fimbriae expression [9].

Eradication usually requires the use of alkaline or acidic detergents and/or iodophores. Though efficacious, issues such as corrosion, product contamination, and toxicity limit the use of these compounds [10]. The ability of biofilm-embedded microorganisms to resist clearance by antimicrobial agents points to the importance of a continuous search for novel agents that are effective against bacteria in this mode of growth or work in synergy with the currently available myriad of anti-microbials [11]. The use of natural antimicrobial agents can be an effective alternative or supplement for the control of microorganisms [10]. One approach may be the use of essential oils that have been shown to be potential agents in the treatment of infections, and are safe in terms of human and animal health. In this context, oregano oil and its major phenolic components, carvacrol and thymol are known for their wide spectrum of antimicrobial activity, which has been the subject of several investigations *in vitro* and *in vivo* [12]. The objective of this study was to evaluate the activity of oregano oil *(Origanum onites)* on biofilm-grown Staphylococci and *E. coli* strains, as well as the effect of oil on biofilm formation.

MATERIAL and METHODS

Essential Oil

The oregano essential oil *(Origanum onites)* was provided by Türer Tarım Ltd. Şti. (Türer Tarım ve Orman Ürünleri İthalat İhracat Sanayii ve Ticaret Limited Şirketi, Kavaklıdere Köyü, Bornova, İzmir, Türkiye). The oil was analyzed by capillary GC and GC/MS using an Agilent GC-MSD system. The GC/MS analysis was carried out with an Agilent 5975 GC-MSD system. Innowax FSC column (60 m x 0.25 mm, 0.25 μm film thickness) was used with helium as carrier gas (0.8 ml/min). GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, and kept constant at 220°C for 10 min and then programmed to 240°C at a rate of 1°C/min. Split ratio was adjusted 40:1. The injector temperature was at 250°C. MS were taken at 70 eV. Mass range was from m/z 35 to 450. The GC analysis was carried out using an Agilent 6890N GC system. In order to obtain same elution order with GC/MS, simultaneous injection was done by using same column and appropriate operational conditions. FID temperature was 300°C. The components of essential oils were identified by comparison of their mass spectra with those in the Baser Library of Essential Oil Constituents, Wiley GC/MS Library, Adams Library, Mass

Finder Library and confirmed by comparison of their retention indices. Alkanes were used as reference points in the calculation of relative retention indices (RRI). Relative percentage amounts of the separated compounds were calculated from FID chromatograms. The results of analysis are shown in *Table 1*.

Bacterial Strains

The bacteria used were *Staphylococcus aureus* (n=5) (Sa1, Sa2, Sa3, Sa4, Sa5), *S. lugdunensis* (n=1) (SL), *S. haemolyticus* (n=1) (Sh), *S. sciuri* (n=1) (Ss), isolated from restaurant workers and milk samples, belonging to our private collection, and the reference strains *S. aureus* NCTC 8325 (n=1) (Sa), *Escherichia coli* (Strain no: 97010) (n=1) (Ec) provided by Refik Saydam Hygiene Center Presidency National Type Refik Saydam Culture Collection Laboratory, Ankara, Turkey. Each isolate was characterized for biofilm related properties as reported previously [13]. The isolates were capable of forming biofilms with an OD_{570} ranging from 0.305 to 0.521.

Efficacy of Oregano Oil on Planktonic Cells

The minimum inhibitory concentrations (MIC) of oregano essential oil (EO) on planktonic cells were determined in Tryptic Soy Broth (TSB, Difco 211822) using the broth dilution method according to Nostro et al.[14]. An overnight bacterial culture was inoculated to TSB with EO at the level of 1%. The final concentrations of EO in the medium ranged from 0.25% to 3%. Tween 80 was used at concentration of 0.1% to enhance EO solubility in medium. The growth of test strains in TSB including EO was evaluated by plating on Tryptic Soy Agar (TSA, Difco 236950) after incubation at 37°C, 24 h. The MIC was defined as the lowest concentration of the oregano oil inhibiting the growth of each strain. All determinations were performed in duplicate and two growth controls consisting of TSB medium and TSB with 0.1% (v/v) Tween 80 included.

Effect on Biofilm Formation

The effect of different concentrations of oil on biofilm forming ability was tested on polystyrene flat-bottomed microtitre plates as described Hammer et al.[13] and Nostro et al.[12] with some modifications. As a treatment solution, the concentrations of oregano oil were prepared in TSB with 0.25% glucose and 0.1% Tween 80 (TSBG) at the level of 0.25 MIC, 0.50 MIC and MIC. Then, cultures were grown overnight in TSBG and 10 µl were dispensed into each well of microtitre plate containing 90 µl of treatment solutions. For the negative controls, 10 µl of TSBG were dispensed into each well consisting 90 µl of treatment solutions. The positive control group was also obtained by inoculating 10 µl of cultures to 90 µl of TSBG. After incubation at 37°C for 24 h, the contents of each well were removed and the wells were washed three times with sterile physiological saline (0.85% NaCl). Trays were shaken vigorously to remove non-adherent bacteria. Adherent bacteria were fixed by adding 99% methanol to wells and leaving for 15 min at room temperature. The wells were then emptied and left to dry. Biofilm was stained by adding 200 µl of 2% crystal violet stain for 5 min. The trays were then rinsed with water. After drying, stain was resolubilised by adding 160 µl of 33% glacial acetic acid to each well and agitating gently, and then OD_{570} was measured by spectrophotometer using an ELISA reader. Each assay was performed in triplicate. As a measure of efficacy, OD_{570} of negative control was subtracted from corresponding absorbance reading and compared to that of positive control.

Effect on Established Biofilms

The effect on established biofilms was verified as described by Nostro et al.[12] with some modifications. All isolates were grown as biofilms in wells of polystyrene flat-bottomed microtitre plates for treatment and positive control groups. Five wells for each isolate were not inoculated (Negative controls). After 24 h of incubation at 37°C, the planktonic-phase cells were gently removed and the wells were washed three times with physiological saline and filled with 200 µl twofold dilutions of the EO, ranging from MIC to 8 MIC. For the negative controls, not inoculated wells were also filled with those EO dilutions. The positive control group was also obtained by adding 200 µl of TSBG. The plates were incubated for 24 h at 37°C. The OD_{570} was measured at time 0 and after incubation for 24 h. The biofilm inhibitory concentration (BIC) was determined as the lowest concentration where no growth occurred in the supernatant fluid, confirmed by no increase in optical density compared with the initial reading. Samples of biofilms from the bottom of these wells were scarified by a metal loop, spread over the surface of TSA and incubated for 72 h at 37°C. The biofilm eradication concentration (BEC) was determined as the lowest concentration at which no bacterial growth occurred on the TSA plates. Data from four replicates were evaluated.

Statistical Analysis

The data were initially tested for normal distribution by one-sample Kolmogrov-Smirnov test. Following the confirmation of normal distribution ($P>0.001$), differences for individual parameters between control and treated groups were tested by paired-sample t-test using SPSS Version 9.05 for Windows. Differences were considered significant if the P value was less than 0.001.

RESULTS

Composition of Oregano Oil

GC/MS results indicated that the two phenols, carvacrol and thymol were the major components of oregano essential oil *(Table 1)*. Some of the researchers reported that compounds are mainly responsible for its antimicrobial activity [15,16].

Table 1. *The composition of Origanum onites oil*
Tablo 1. *Origanum onites yağının bileşimi*

RRI	Main Compounds	%
1280	*p*-cymene	7.0
1553	Linalool	3.6
1611	Terpinen-4-ol	1.7
1719	Borneol	1.2
1741	β-bisabolene	1.9
2198	Thymol	7.4
2239	Carvacrol	70.2
	Others	7

Table 2. *Minimum inhibitory concentration (MIC) of oregano EO on test strains*
Tablo 2. *Kekik uçucu yağının test suşlarına karşı minimum inhibisyon kansantrasyonu*

MIC (%, v/v, EO Concentration in Distilled Water)	Strain (Number)
0.05 %	*S. aureus* (n=6)
0.05 %	*S. lugdunensis* (n=1)
0.05 %	*S. haemolyticus* (n=1)
0.8 %	*S. sciuri* (n=1)
0.1 %	*E. coli* (n=1)

Efficacy of Oregano Oil on Planktonic Cells

A total of 10 isolates was tested for their susceptibility to oregano EO. The MICs of oregano EO for each organism are given in *Table 2*. The values ranged from 0.1% to 0.8%.

Efficacy of Oregano Oil on Biofilm Formation

Despite a different inhibitory effect among the strains, a reduced level of biofilm formation in the presence of subinhibitory concentrations of oregano EO was observed *(Table 3)*. Doses of MIC and .05 MIC showed a greater influence than that of 0.25 MIC.

Efficacy of Oregano Oil on Established Biofilm

A statistically significant reduction was noted in biofilms that were treated with oregano EO even at MIC level. The findings indicating biofilm eradication effect of EO are given in *Table 4.*

Table 3. *Effect of oregano oil on biofilm formation (means± standard deviation)*
Tablo 3. *Kekik yağının biyofilm oluşumu üzerine etkisi (ortalama±standart sapma)*

Strain	Biofilm Formation			
	MIC	0.5 MIC	0.25 MIC	Positive Control
Sa1	0.14±0.020 ^{a}	0.19±0.010 ^{a}	0.40±0.050 ^{b}	0.50±0.010 ^{b}
Sa2	0.07±0.050 ^{a}	0.23±0.020 ^{b}	0.36±0.020 ^{b,c}	0.46±0.010 ^{c}
Sa3	0.10±0.006 ^{a}	0.23±0.030 ^{b}	0.37±0.010 ^{c}	0.49±0.006 ^{d}
Sa4	0.08±0.007 ^{a}	0.13±0.010 ^{a,b}	0.24±0.030 ^{b,c}	0.32±0.010 ^{c}
Sa5	0.10±0.002 ^{a}	0.20±0.010 ^{b}	0.30±0.006 ^{c}	0.39±0.010 ^{d}
SL	0.08±0.004 ^{a}	0.18±0.010 ^{a}	0.34±0.030 ^{b}	0.47±0.010 ^{c}
Sh	0.09±0.004 ^{a}	0.14±0.003 ^{b}	0.22±0.003 ^{c}	0.40±0.006 ^{d}
Ss	0.10±0.008 ^{a}	0.23±0.020 ^{b}	0.36±0.020 ^{c}	0.46±0.010 ^{c}
Sa	0.10±0.003 ^{a}	0.24±0.020 ^{b}	0.38±0.010 ^{c}	0.50±0.010 ^{d}
Ec	0.08±0.005 ^{a}	0.13±0.004 ^{b}	0.23±0.010 ^{c}	0.41±0.010 ^{d}

Means with different letters (a, b) in the same row for each EO concentration are significantly different (P≤0.001)

DISCUSSION

Bacteria in biofilm are known to be much more resistant to antimicrobial agents than free-living cells and may act as continuous sources of spoilage and pathogenic bacteria that contaminate food. This increased resistance is not often considered during disinfection, and many studies dealing with the effect of disinfectant are carried out in broth cultures. The current interest in natural antimicrobial compounds has increased due to changes in consumer attitudes toward the use of synthetic preservative agents in food, surface detergents and disinfectants that have a negative impact on the environment [17]. Selected natural products that originate in plants can influence microbial biofilm formation through different mechanisms. Many plant essential oils, which are mixtures of numerous organic chemicals, contain compounds that inhibit microbial growth [18]. In our study, oregano essential oil showed antibacterial activity against all the test organisms. Moreover, it also had effect on biofilm formation and established biofilm even at MIC level. Nostro et al.[12] also reported that *Origanum vulgare* L. essential oil inhibited growth of preformed biofilm and interfered with biofilm formation during planktonic growth. It was documented in their article that carvacrol and thymol which are the principal phenolic components of oregano oil may be responsible for the effects observed on biofilm formation. They could diffuse through the polysaccharide matrix of the biofilm and destabilize it due to their strong intrinsic antimicrobial properties, the researchers continued. In this study, carvacrol (70.2%), thymol

Table 4. *Effect of oregano oil on established biofilm formation (means±standard deviation)*
Tablo 4. *Kekik yağının oluşmuş biyofilm üzerine etkisi (ortalama±standart sapma)*

Strain	Biofilm Formation				
	MIC	2MIC	4MIC	8MIC	Control
Sa1 (BI)	0.081±0.002	0.098±0.008	0.099±0.002	0.093±0.002	0.097±0.003 ^{k}
Sa1 (AI)	0.096±0.003 ^{a}	0.097±0.002 ^{a}	0.100±0.002 ^{a}	0.097±0.002 ^{a}	0.559±0.076 ^{b,l}
Sa2 (BI)	0.081±0.006	0.100±0.002	0.090±0.001	0.090±0.002	0.116±0.009 ^{k}
Sa2 (AI)	0.096±0.003 ^{a}	0.096±0.004 ^{a}	0.097±0.002 ^{a}	0.098±0.001 ^{a}	0.397±0.013 ^{b,l}
Sa3 (BI)	0.100±0.004	0.102±0.003	0.097±0.001	0.100±0.004	0.100±0.013 ^{k}
Sa3 (AI)	0.095±0.004 ^{a}	0.102±0.004 ^{a}	0.100±0.004 ^{a}	0.101±0.005 ^{a}	0.617±0.017 ^{b,l}
Sa4 (BI)	0.083±0.007 ^{a}	0.089±0.003 ^{a,b}	0.094±0.003 ^{a,b}	0.097±0.002 ^{b}	0.091±0.003 ^{a,b,k}
Sa4 (AI)	0.088±0.007 ^{a}	0.100±0.002 ^{a}	0.100±0.002 ^{a}	0.100±0.002 ^{a}	0.664±0.005 ^{b,l}
Sa5 (BI)	0.081±0.006 ^{a}	0.086±0.007 ^{a,b}	0.096±0.003 ^{a,b}	0.098±0.003 ^{b}	0.100±0.004 ^{b,k}
Sa5 (AI)	0.088±0.044 ^{a}	0.093±0.003 ^{a}	0.096±0.002 ^{a}	0.098±0.002 ^{a}	0.609±0.014 ^{b,l}
SL (BI)	0.101±0.003	0.098±0.001	0.100±0.003	0.093±0.006	0.099±0.003 ^{k}
SL (AI)	0.103±0.005 ^{a}	0.104±0.003 ^{a}	0.103±0.003 ^{a}	0.105±0.004 ^{a}	0.568±0.061 ^{b,l}
Sh (BI)	0.079±0.003	0.073±0.005	0.076±0.002	0.079±0.003	0.083±0.005 ^{k}
Sh (AI)	0.080±0.002 ^{a}	0.075±0.005 ^{a}	0.074±0.002 ^{a}	0.078±0.003 ^{a}	0.581±0.015 ^{b,l}
Ss (BI)	0.068±0.002	0.070±0.002	0.075±0.003	0.077±0.004	0.069±0.002 ^{k}
Ss (AI)	0.068±0.002 ^{a}	0.069±0.002 ^{a}	0.072±0.002 ^{a}	0.074±0.002 ^{a}	0.695±0.059 ^{b,l}
Sa (BI)	0.067±0.003	0.070±0.002	0.075±0.004	0.076±0.003	0.069±0.002 ^{k}
Sa (AI)	0.068±0.002 ^{a}	0.070±0.002 ^{a}	0.074±0.002 ^{a}	0.076±0.003 ^{a}	0.511±0.019 ^{b,l}
Ec (BI)	0.080±0.003 ^{a}	0.093±0.003 ^{a,b}	0.094±0.004 ^{a,b}	0.090±0.003 ^{a,b}	0.116±0.009 ^{c,k}
Ec (AI)	0.096±0.002 ^{a}	0.096±0.00 ^{a}	0.097±0.003 ^{a}	0.098±0.003 ^{a}	0.418±0.012 ^{b,l}

BI: *Before incubation,* ***AI:*** *After incubation*
Means with different letters (a, b) in the same row for each EO concentration are significantly different (P≤0.001)
Means with different letters (k, l) in the same column for each strain are significantly different (P≤0.001)

(7.4%) and *p*-cymene (7%) were the main components of oregano essential oil that we used.

Microbial cell surface interaction in leakage of intracellular constituents has been suggested as the biocidal mechanism for carvacrol-mediated antimicrobial activity [19]. Exposure of *S. aureus* to carvacrol during the early stages of biofilm development led to potent inhibition of matrix formation, with shedding of proteinaceous mass after each antimicrobial pulse. Rapid killing of *S. aureus* by carvacrol also led to disruption of the proteinaceous matrix of the film. However, the shedding of such proteinaceous mass did not coincide with viability reductions of staphylococci in the biofilm, possibly due to continuous exfoliation of the matrix. Lysostaphin is an agent with a mode of action similar to that of carvacrol [10] and in a study conducted by Wu et al.[20]; the researchers demonstrated that, *in vitro,* lysostaphin disrupted *S. aureus* biofilms on polystyrene, polycarbonate, and glass surfaces. Their findings also showed this antimicrobial eradicated both sessile *S. aureus* cells of the biofilm and the extracellular matrices.

Thymol is the one of the main compounds of oregano EO. Lebert et al. [17] reported that thymol did not kill any bacteria including *E. coli* and *S. aureus* in biofilm, while Satureja thymbra oil, containing thymol (41%), γ-terpinene (22.2%) and p-cymene (11.8%) as previously informed by Chorianopoulos et al.[21], reduced the population of *S. aureus* and *E. coli* grown in biofilm. Burt [16] suggested that the differences observed between the effects of pure thymol and the essential oil may be due to a combined effect of thymol and other molecules, such as terpinene and cymene, which can have a synergic or additive effect. In this study, oregano oil showed strong effect against *E. coli* and *Staphylococci strains* including *S. aureus.* This data supports the suggestion of Burt [16]. Similar results were reached by Quave et al.[22] for methicillin-resistant *S. aureus* biofilm using *Lonicera alpigena, Castanea sativa, Juglans regia, Ballota nigra, Rosmarinus officinalis, Leopoldia comosa, Malva sylvestris, Cyclamen hederifolium, Rosa canina var. canina* and *Rubus ulmifolius* extracts and also by Kuźma et al.[23] for antibiotic resistant staphylococci biofilm using *Salvia sclarea* L. extract.

According to our findings, oregano EO was effective on biofilm formed by test strains. It was reached that results using Crystal violet (CV) staining technique and it

was observed that CV assay suitable for determining the effect of essential oil on biofilm formation. In a study conducted by Niu and Gilbert [18], the researchers reported that Cinnamomum cassia essential oil reduced the extent of biofilm formation by *E. coli*. They also used this assay and informed CV staining has been widely adopted by microbiologists to investigate mutants with respect to adhesion or biofilm formation, attachment to diverse surfaces, and to compare biofilm development in different pathogens. Its greatest features are that it is inexpensive, relatively quick, and adaptable for use high-throughput screening with microtitre plates.

In this study, a reduced level of biofilm formation by Staphylococci and *E. coli* in the presence of subinhibitory concentrations of oregano EO was observed. Doses of MIC and 0.5 MIC showed a greater influence than that of 0.25 MIC. A statistically significant reduction was observed in biofilms that were treated with oregano EO even at MIC level. The MIC was 0.05% for *S. aureus* strains and 0.1% for *E. coli*. In contrast to our results Özkan et al.[24] noted higher concentrations (0.2-1%) of marjoram *(Origanum majorana),* oregano *(Origanum vulgare L.),* black thyme *(Thymbra spicata L.)* and thyme *(Thymbra sintenesi)* essential oils which have similar composition with *Origanum onites* EO to inhibit the growth of *S. aureus* and *E. coli*. This difference may be attributed to the paper disc diffusion method they used to detect the antibacterial activity of essential oils. Diffusion assays, in which the agent is applied to a well or paper disc in the centre of an agar plate seeded with the test microorganism, are unsuited to essential oil testing because the oil components are partitioned through the agar according to their affinity with water [25]. Broth and agar dilution methods are widely used to determine MIC of essential oils. In addition, when testing non-water-soluble antimicrobials such as essential oils, it is necessary to incorporate an emulsifier or solvent into the test medium to ensure contact between the test organism and the agent for the duration of the experiment. Tween 80 (polysorbate 80) is one of the most commonly used agents [26]. In this study, we used broth dilution method in TSB containing Tween 80 at the level of 0.1% (v/v).

In summary, we have shown that oregano EO exhibited antibacterial action on planktonic *S. aureus, S. lugdunensis, S. haemolyticus, S. sciuri, E. coli* and was able to prevent or at least interfere with biofilm formation on polystyrene surfaces. It also eradicated established biofilm even at MIC level. Further investigations should be examined whether these observations extend to biofilms formed on other surfaces, particularly those found in food processing plants.

REFERENCES

1. Costerson JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin Scott HM: Microbial biofilms. *Annu Rev Microbiol,* 49, 711-745, 1995.

2. Deibel V: Biofilms. *Int J Food Safety,* 1, 6-7, 2003.

3. Hood SK, Zottola EA: Biofilms in food processing. *Food Cont,* 6, 9-18, 1995.

4. Kumar CG, Anand SK: Significance of microbial biofilms in food industry: A review. *Int J Food Microbiol,* 42, 9-27, 1998.

5. Flemming HC: Biofouling in water systems - cases, causes and countermeasures. *Appl Microbiol Biotechnol,* 59, 629-640, 2002.

6. Oulahal N, Brice W, Martial A, Degraeve P: Quantitative analysis of survival of *Staphylococcus aureus* or *Listeria innocua* on two types of surfaces: Polypropylene and stainless steel in contact with three different dairy products. *Food Cont,* 19, 178-185, 2008.

7. Rode TM, Langsrud S, Holck A, Mørettø T: Different patterns of biofilm formation in *Staphylococcus aureus* under food-related stress conditions. *Int J Food Microbiol,* 116, 372-383, 2007.

8. Naves P, del Prado G, Huelves L, Gracia M, Ruiz V, Blanco J, Dahbi G, Blanco M, Ponte MC, Soriano F: Correlation between virulence factors and *in vitro* biofilm formation by *Escherichia coli* strains. *Microb Pathog,* 45, 86-91, 2008.

9. Soto SM, Smithson A, Martinez JA, Horcajada JP, Mensa J, Vila J: Biofilm formation in uropathogenic *Escherichia coli* strains: Relationship with prostatitis, urovirulence factors and antimicrobial resistance. *J Urol,* 177, 365-368, 2007.

10. Knowles JR, Roller S, Murray DB, Naidu AS: Antimicrobial action of carvacrol at different stages of dual-species biofilm development by *Staphylococcus aureus* and *Salmonella enterica* Serovar Typhimurium. *Appl Environ Microbiol,* 71, 797-803, 2005.

11. Jabra-Rizk MA, Meiller TF, James CE, Shirtliff ME: Effect of Farnesol on *Staphylococcus aureus* biofilm formation and antimicrobial susceptibility. *Antimicrob Agents CH,* 50, 1463-1469, 2006.

12. Nostro A, Roccaro AS, Bisignano G, Marino A, Cannatelli MA, Pizzimenti FC, Cioni PL, Procopio F, Blanco AR: Effects of oregano, carvacrol and thymol on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *J Med Microbiol,* 56, 519-523, 2007.

13. Hammer KA, Carson CF, Riley TV: Effects of tea tree oil on *Staphylococcus aureus* virulence factors. A report for the rural industries research and development corporation. RIRDC Publication No: 05/115, 2005.

14. Nostro A, Bisignano G, Cannatelli MA, Crisafi G, Germano MP, Alonzo V: Effects of *Helichrysum italicum* extract on growth and enzymatic activity of *Staphylococcus aureus. Int J Antimicrob Agents,* 17, 517-520, 2001.

15. Adam K, Sivropoulou A, Kokkini S, Lanaras T, Arsenakis M: Antifungal activities of *Origanum vulgare* subsp. hirtum, *Mentha spicata, Lavandula angustifolia* and *Salvia fruticosa* essential oils against human pathogenic fungi. *J Agric Food Chem,* 46, 1739-1745, 1998.

16. Burt S: Essential oils: Their antibacterial properties and potential applications in foods: A review. *Int J Food Microbiol,* 94, 223-253, 2004.

17. Lebert I, Leroy S, Talon R: Effect of industrial and natural biocides on spoilage pathogenic and technological strains grown in biofilm. *Food Microbiol,* 24, 281-287, 2007.

18. Niu C, Gilbert ES: Colorimetric method for identifying plant essential oil components that affect biofilm formation and structure. *Appl Environ Microbiol,* 70, 6951-6956, 2004.

19. Ultee A, Kets EPW, Smid EJ: Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus. Appl Environ Microbiol,* 65, 4606-4610, 1999.

20. Wu JA, Kusuma C, Mond JJ, Kokai-Kun JF: Lysostaphin disrupts *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms on artificial surfaces. *Antimicrob Agents CH,* 47, 3407-3414, 2003.

21. Chorianopoulos N, Kalpoutzakis E, Aligiannis N, Mitaku S, Nychas GJ, Haroutounian SA: Essential oils of Satureja, Origanum, and Thymus species: Chemical composition and antibacterial activities against foodborne pathogens. *J Agric Food Chem,* 52, 8261-8267, 2004.

22. Quave CL, Plano LRW, Pantuso T, Bennett BC: Effects of extracts from Italian medicinal plants on planktonic growth, biofilm formation and adherence of methicillin-resistant *Staphylococcus aureus. J Ethnopharmacol,* 118, 418-428, 2008.

23. Kuźma L, Różaski M, Walencka E, Różaska B, Wysokińska H: Antimicrobial activity of diterpenoids from hairy roots of *Salvia sclarea* L.: Salvipisone as a potential anti-biofilm agent active against antibiotic resistant Staphylococci. *Phytomedicine,* 14, 31-35, 2007.

24. Özkan G, Sağdıç O, Özcan M: Note: Inhibition of pathogenic bacteria by essential oils at different concentrations. *Food Sci Technol Int,* 9, 85-88, 2003.

25. Southwell IA, Hayes A, Markham J, Leach DN: The search for optimally bioactive Australian tea tree oil. *Acta Hortic,* 334, 256-265, 1993.

26. Mann CM, Markham JL: A new method for determining the minimum inhibitory concentration of essential oils. *J Appl Microbiol,* 84, 538-544, 1998.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S31-S35, 2010
DOI:10.9775/kvfd.2009.1174

RESEARCH ARTICLE

Determination of Antibiotic Residues in Milk Samples [1]

Seyda ERGIN KAYA * Ayhan FILAZI **

[1] *This work was summarised from same named PhD thesis*
* Vilsan Company, Product Manager, Yenice Koyu Mevkii, TR-06760 Esenboğa, Ankara - TURKEY
** Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ankara University, TR-06110 Diskapi, Ankara - TURKEY

Makale Kodu (Article Code): KVFD-2009-1174

Summary

In this study, antibiotic residues in raw milk and pasteurized milk products sold in Ankara- Turkey investigated. For this purpose; a total of 240 milk samples, which contains 10 raw and 10 pasteurized milk samples per month collected from various markets in Ankara between April 2003 and March 2004 were analyzed in terms of penicillin G, oxytetracycline, gentamicin, streptomycin and neomycin by using TLC (Thin Layer Chromatography)/Bioautographic method, in which *Bacillus subtilis* ATCC 6633 was used as test microorganism. The minimum detectable concentrations for penicillin G, oxytetracycline, gentamicin, streptomycin and neomycin, as μg/L were 4, 100, 200, 100 and 1000, respectively and recovery rate as percentage were 75.6, 79.7, 80.9, 84.7 and 73.5, respectively. The concentrations found among pasteurized samples were 150.4 μg/L oxytetracycline and 33.5 g/L penicillin G and 7688.4 μg/L of neomycin among raw samples, which are higher than the maximum residue limits in milks accepted in Turkey and European Countries. According to the total number of samples analysed, the ratio of contamination with antibiotics was detected as 1.25%.

Keywords: *Milk, Antibiotic, Residue, TLC/Bioautographic method*

Sütlerde Antibiyotik Kalıntılarının Belirlenmesi

Özet

Bu çalışmada Ankara'da satılan çiğ süt ve pastörize süt ürünlerinin antibiyotik kalıntılarının araştırılması amaçlandı. Nisan 2003 ile Mart 2004 tarihleri arasında, çeşitli satış yerlerinden her ay 10 çiğ süt ve 10 pastörize süt olmak üzere toplam 240 adet süt örneği toplanarak penisilin G, oksitetrasiklin, gentamisin, streptomisin ve neomisin yönünden analiz edildi. Bunun için test mikroorganizması olarak *Bacillus subtilis* ATCC 6633'ün kullanıldığı İnce Tabaka Kromatografisi /Biyootografi yönteminden yararlanıldı. Buna göre penisilin G, oksitetrasiklin, streptomisin, gentamisin ve neomisin'in en küçük belirlenme miktarları sırasıyla μg/L olarak 4, 100, 200, 100 ve 1000, geri alınma oranları ise yüzde olarak yine sırasıyla 75.6, 79.7, 80.9, 84.7 ve 73.5 olarak belirlendi. Pastörize süt örneklerinin birinde 150.4 μg/L oksitetrasiklin, birinde 33.5 μg/L penisilin G ve bir adet çiğ süt örneğinde 7688.4 μg/L neomisin tespit edilmiş ve bu konsantrasyonların Türkiye ve Avrupa Birliği ülkelerinde sütlerde bulunmasına izin verilen maksimum kalıntı limitlerinin üstünde olduğu belirlendi. Toplam analiz örneği sayısına göre antibiyotikle kirlenme sıklığı %1.25 olarak bulundu.

Anahtar sözcükler: *Süt, Antibiyotik, Kalıntı, İnce Tabaka Kromatografisi/Biyootografi*

INTRODUCTION

The first use of antimicrobials for treatment of infections in veterinary medicine was in the late 1940s, shortly after their development [1]. In the treatment bovine mastitis, antibiotics are widely used and improper application can lead to the contamination of milk at farm level. Nowadays, beta lactam (penicillin G etc), aminoglycoside (streptomycin, neomycin, etc) and tetracycline (oxytetracycline, etc) antibiotics are the most frequently used antimicrobials for treatment of mastitis in dairy cows and consequently, the most

İletişim (Correspondence)
☎ +90 312 3170315
✉ filazi@veterinary.ankara.edu.tr

commonly found type residues in milk [2]. Residues are of concern due to their possible adverse effects on people allergic to antibiotics, potential buildup of antibiotic-resistant organism in humans and inhibition of starter cultures used to produce cultured milk products such as yogurt and cheeses [3,4]. These reasons make it important to effectively control antibiotic residues in milk and therefore, regulatory authorities have enacted maximum residue limits (MRLs) for a number antiinfective agent in milk. National monitoring programs to control the veterinary drug residues in various animal origined foods, including milk, are compulsory in all EC countries and Turkey [5-7]. Detectable concentrations of antibiotic residues in milk supplies higher than the MRLs are illegal.

Running effective monitoring program requires specific, sensitive and reliable analytical methods that can detect all drug residues below regulated levels. The overall objective is to develop and validate multiresidue methods in order to support the implementation of both existing as well as future regulations in the area of food control [8].

Various analytical methods have been described to determine antibiotic residues in milk, such as microbiological, chromatographic, immunochemical, receptor and enzyme-based tests. Microbiological tests are commonly applied in dairy and in survey studies [9,10]. For the detection of antimicrobial residues in milk; microbiological screening tests are used that utilize bacterial test strains such as *Bacillus stearothermophilus* var. *calidolactis, Streptococcus thermophilus* and *Bacillus subtilis* ATCC 6633 [11,12].

The coupling of thin layer chromatography with microbiological detection (TLC/bioautography) has been used for the identification and quantification of several antibiotics. It is considered a simple, cheap and quite sensitive and specific method [13,14]. The application of bioautography combined with TLC in antibiotic residues detection in milk and animal tissue has been demonstrated previously by Choma et al.[15] and Neidert et al.[16].

The purpose of this study was to analyse residues of streptomycin, penicillin G, oxytetracycline, gentamicin, and neomycin by the using TLC/bioautography; that are frequently seen in raw and pasteurized milk products sold in Ankara-Turkey.

MATERIAL and METHODS

Apparatus and Reagents

Antimicrobial standards penicillin G potassium, oxytetracycline, streptomycin sulphate, gentamicin and neomycin sulphate were purchased from Sigma-Aldrich Ltd. HPLC grade acetone, glycerine, chloroform, potassium hydrogen phthalate, n-propanol, Whatman cellulose TLC plates (200X200 mm) (Merck), Standard Plate Count Agar and Tryptose Soy Agar (Oxoid) were purchased. Bioplates (sterilized glass plates-245x245 mm) prepared privately.

Impregnation liquid: Mix 0.1 N phthalate buffer pH 3.75 and glycerine (19+1).

Stock solutions of standards were prepared by dissolving each compound in milli-Q water at a concentration of 1 mg/ml and stored in amber vials in a freezer (-20°C). Fresh stock solutions were made every month. Working standard solutions (different concentrations) were made by serially diluting the stock solutions with milli-Q water every time just before use and stored at 4°C in amber glass vials. Method validation samples were prepared at levels of ½, 1 and 2 or 4 times MRL values. All spiked samples (standards and method validation samples) were assayed 6 times in duplicate spread over three working days. The variation coefficient of the within-laboratory reproducibility for method validation samples was calculated.

Sample Collection

As part of the study, between 03.04.2003 and 30.03.2004 (for one year), a total of 240 milk samples, 10 of which were raw milk and 10 were pasteurized milk every month were collected from different points of sales in Ankara-Turkey. Attention was paid to ensure that the milk samples analysed were of different brands and were collected from different locations. Milk samples were kept in the refrigerator (4°C) until analysis and were analysed within two days at most.

Assay organism: *Bacillus subtilis* ATCC 6633 Difco spore solutions were obtained from The Ministry of Agricultural and Rural Affairs, Central Veterinary Control and Research Institute. It cultured on Tryptone Soya Agar (European Pharmacopeia).

TLC solvent system: Acetone-chloroform-n-propanol-impregnation liquid (16 + 20 + 27 + 16)

Preparation of Milk Samples

For extraction of the antibiotic residues of milk samples, methods specific to liquid chromatography developed by Tyczkowska et al.[17] were used. Accordingly, it was extracted 1 ml from each milk sample and placed in centrifuge tubes. In order to precipitate the proteins in milk 1 ml of acetonitrile - methanol, deionized water

(40:20:20) mixture was added on it. After stirring with hand thoroughly, it was centrifuged at 3000 rpm for 10 min and the portion remaining on supernatant after proteins were precipitated was used for analysis.

Thin Layer Chromatography

Cellulose plates were divided into 10 equal channels. And then the 10 µl extracts were applied to the channels. During application, air current was applied on the plate to prevent the sample from decomposing. The plate was left to rest for 5 min in room temperature to dry them. Afterwards, the plates were placed in the development tanks prepared at least one hour in advance, in order to help the ambiance reach saturation Then it is waited for the solution to rise up to 15 cm on the plate. Plate was removed from the tank and dried. The dried plate was then applied to the newly prepared bio-plate.

Bioautography

Seventeen and a half grams of Standard Plate Count Agar was measured and 1 L distilled water was added and was melted in 100°C water for 2 h and was sterilized by keeping for 16 min under 1 atmosphere pressure at 121°C. And by adjusting its pH to 7.2, its temperature was decreased by helping it contact water occasionally up to the temperature (38-40°C) required for *B. subtilis* to reproduce. For every 100 ml agar, 400 µl from *Bacillus subtilis* ATCC 6633 spore solution was added and as a result, 4 ml was added to 1 L agar and it was shaken for 15 sec. to help it diffuse homogenously. 300 ml agar was added to every bio-plate sterilized in advance for one h at 121°C under 1 atmospheric pressure. After waiting for agar to become solid (for around 20 min), the TLC plates that were developed by applying sample extracts or antibiotics standards and dried, were placed on the food-lot surface and was allowed to contact agar for 20 min. At the end of contact period, plates were removed and bio-plates were left for incubation for 16 h at 37°C. The inhibition zones diameters that developed at the end of the period were measured using calliper [16].

RESULTS

The R_f means obtained by preparing and using the standards, at certain concentrations, for detecting the streptomycin, penicillin G, oxytetracycline, gentamicin and neomycin residues using the TLC/bioautography method as well as the their low impact densities, their recovery rates and their minimum detectable concentrations are given in the *Table 1*.

As a result of the analysis in one of the pasteurized milk samples collected in April 2003, 150.4 µg/l oxytetracycline and 33.5 µg/l penicillin in another company's sample was detected, whereas in the raw milk sample obtained from Ankara-Ayaş Gökyayla village, 7688.4 g/l neomycin was detected. Based on the total number of analysis samples, the ratio of contamination with antibiotics was detected as 1.25%.

DISCUSSION

TLC/bioautography method developed by Neidert et al.[16] for detecting antibiotic residues in animal tissue was used. The method developed by Tyckowska et al.[17] for extraction and detection of penicillin G from cow milk by HPLC was used to extract the antibiotic in milk. As a result of the recovery procedure carried out using these methods, 75.6% for penicillin G, 79.7% for oxytetracycline, 80.9% for streptomycin, 84.7% for gentamicin, and 73.5% for neomycin were calculated as recovery rates. In the recovery study in meat conducted by Neidert et al.[16], these values were as follows; 95% for penicillin G, 95-98% for oxytetracycline, 80% for streptomycin, 100% for gentamicin, and neomycin. Accordingly, recovery levels in this study were lower for antibiotics other than streptomycin. The difference is believed to be due to differences between samples. Besides the fact that, TLC/ bioautography is not a costly method compared to the other analysis methods, which can be adapted to laboratory conditions and applied easily; it has also certain advantages such as enabling distinction between

Table 1. *R_f mean, minimum detectable concentration, recovery and MRL of antibiotics measured by TLC/bioautography*

Tablo 1. *TLC/Biyootografi ile ölçülen antibiyotiklerin R_f düzeyi, belirlenebilen en düşük konsantrasyonu, geriye kazanç yüzdeleri ve maksimum kalıntı düzeyleri*

Antibiotics	Tested Concentrations (µg/L)	Minimum Detectable Concentrations (µg/L)	Recovery Rates (%)	Rf Values	MRLs [a] (µg/L)
Penicillin G	2, 4, 8, 16	4	75.6	0.84	4
Oxytetracycline	50, 100, 200, 400	100	79.7	0.34	100
Streptomycin	100, 200, 400, 800	200	80.9	0.50	200
Gentamicin	50, 100, 200, 400	100	84.7	0.24	100
Neomycin	750, 1500, 3000, 6000	1000	73.5	0.07	1500

a: Maximum Residues Limits (EC directive and Turkish Food Codex)

antibiotics when appropriate plate and development systems are selected, leading more frequent use.

The degree of contamination of milk and dairy products with anti-bacterial additive residues differs, depending on the level of education, legislations and effectiveness of food inspection in different countries [18]. According to the results of the technical report prepared for milk hygiene, by World Health Organization and Joint Expert Committee on Food Additives (JECFA), the rate of contamination of milk and dairy products with anti-bacterial additives in developed countries such as USA, Australia, UK and Scotchland was 7-10% until 1969, after that year, the rate of contamination of the same products decreased to 0.5% in USA, 2.1% in Australia, 1.5% in UK and 3.4% in Scotland due to the precautions taken after the given date. The same report indicates that, in underdeveloped and developing countries, which fall behind in terms of increasing the level of awareness of stock breeders, improvement of hygienic conditions and in terms of inspection effectiveness, the rate of contamination for milk and dairy products might be higher [19].

In Turkey, there are only a limited number of anti-bacterial residue studies in milk [20-27]. The most comprehensive is the one related to detection of veterinary medicine residues in foods carried by 5 Veterinary Control and Research Institutes in various cities in Turkey and by the 9 Provincial Control Laboratories, under the leadership of the Ministry of Agriculture and Rural Affairs. In this study, 3084 milk sample were analysed using intertest method for the presence of penicillin, tetracycline and chloramphenicol in which 377 of the samples (12%) were found as positive [21].

In another study by Ceyhan and Bozkurt [22] from a total of 200 milk samples (100 raw milk, 50 pasteurized milk and 50 UHT sterilized milk) collected around Ankara region, 11 of them were reported as penicillin positive (5.5%).

In a study carried out by Aydın et al.[23] in 307 milk samples (204 raw milk and 103 pasteurized milk samples) using intertest and agar diffusion tests with the goal of determining antibiotic and other inhibitor substances, the following results were obtained; with the intertest method, raw milk and pasteurized milk, 44% and 29% positive, 1.5% and 15.5% suspicious and 49.5% and 53% negative respectively; with the agar diffusion method, 15.7% and 14.5% positive, 4.4% and 7.8% suspicious and 80% and 77.7% negative respectively.

In another study carried out by Şanlı et al.[19], in 89 milk samples chloramphenicol contamination was found as 6.9% with the residue level of 1.15 ppm.

In a study carried out by Demet et al.[24], where they tried to determine the level of penicillin G, ampicilline and penicillin V residues, using HPLC method in 50 milk samples collected from various dairy farms and creameries, 6 of the milk samples demonstrated penicillin G-potassium but none of the samples demonstrated penicillin V and ampicilline. Again in a study carried out by Demet et al.[25] in 61 milk samples collected from various dairy farms in Konya, in order to detect the level of chloramphenicol using HPLC method, 28 milk samples demonstrated chloramphenicol residues.

Dokuzlu and Tayyar [26] reported that, using the intertest method, they have detected chloramphenicol residue in 2 of the 150 raw milk samples collected in Bursa Province.

As a result of the study conducted by Önal et al.[27], for the detection of chloramphenicol residues on 444 raw and pasteurized milk samples collected from public and private sector establishments in Ankara and the surrounding region, the following rates were obtained; with the Intertest method, 78 positive (17.56%), 65 suspicious (14.63%), 301 negative (67.79%); with the triple plate method, carried out using *B. subtilis,* 24 (5.40%) positive, 1 suspicious (0.22%), 419 (94.36%) negative. It is indicated that this difference is due to the method used, sampling time and the establishments.

Chloramphenicol is an additive that was banned for use in livestock used for milk production and breeding, with the circular dated 19 April 1993 and numbered 419, issued by the Ministry of Agriculture and Rural Affairs due to its certain unwanted affects [28]. However it is noteworthy and frustrating that it was detected in milk in various residue detection studies conducted after that date.

Even though the contamination rates discovered in milk samples as a result of residue detection studies carried in Turkey in previous years are so high, the contamination level detected in this study was only 1.25%. This can be explained by the increased public awareness about food safety and healthy nutrition and efforts of producers to market high quality products after the media started emphasizing the issue.

As a result the recovery values of the residues of penicillin G, oxytetracycline, gentamicin, streptomycin and neomycin detected using the TLC/Bioautography method in milk were found to be satisfactory. Based on the results of this study and previous studies, it was determined that the probability of detecting antibiotic

in milk during spring and autumn is higher than the probability of detecting it in other months. The values detected are higher than the MRLs accepted in European Countries and Turkey. However, the fact that there is less antibiotic contamination in the milk produced in Turkey, compared with the residue rates obtained in previous studies, is considered to be a positive sign in terms of food safety.

REFERENCES

1. Mitchell JM, Griffiths MW, McEwen SA, McNab WB, Yee AJ: Antimicrobial drug residues in milk and meat: Causes, concerns, prevalance, regulations, tests and test performance. *J Food Prot,* 61, 742-756, 1998.

2. Gustavsson E, Degelaen J, Bjurling P, Sternesjö A: Determination of beta lactams in milk using a surface plasmon resonance-based biosensor. *J Agric Food Chem,* 52 (10): 2791-2796, 2004.

3. Jones GM, Seymour EH: Cowside antibiotic residue testing. *J Dairy Sci,* 71 (6): 1691, 1988.

4. Seymour EH, Jones GM, McGilliard ML: Persistence of residues in milk following antibiotic treatment of diary cattle. *J Dairy Sci,* 71 (8): 2292-2296, 1988.

5. EC Council Directive: 96/23/EC of April 1996 on measures to monitor certain substances and residues thereof in live animals and animal product. *Off J Eur Comm,* L125, 10-32, 1996.

6. EEC Council Regulation: No. 2377/90 of 26 june 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *Of J Eur Comm,* L224, 1-8, 1990,

7. Turkish Food Codex: Türk Gıda Kodeksi Hayvansal Kökenli Gıdalarda Veteriner İlaçları Maksimum Kalıntı Limitleri Tebliğinde Değişiklik Yapılması Hakkında Tebliğ (No: 2007/17). 9 Mart 2007 tarih ve 26457 sayılı Resmi Gazete.

8. Samanidou V, Nisyriou S: Multi-residue methods for confirmatory determination of antibiotics in milk. *J Sep Sci,* 31 (11): 2068-2090, 2008.

9. Kang JH, Jin JH, Kondo F: False-positive outcome and drug residue in milk samples over withdrawal times. *J Dairy Sci,* 88 (3): 908-913, 2005.

10. Knecht BG, Strasser A, Dietrich R, Martlbauer E, Niessner R, Weller MG: Automated microarray system for the simultaneous detection of antibiotics in milk. *Anal Chem,* 76 (3): 646-654, 2004.

11. Nouws JFM, Loeffen G, Schouten J, Van Egmond H, Keukens H, Stegeman H: Testing of raw milk for tetracycline residues. *J Dairy Sci,* 81 (9): 2341-2345, 1998.

12. Nouws J, Van Egmond H, Smulders I, Loeffen G, Schouten J, Stegeman H: A microbiological assay system for assessment of raw milk exceeding EU maximum residue levels. *Int Dairy J,* 9 (2): 85-90, 1999.

13. Petz M, Solly R, Lymburn M, Clear MH: Thin-layer chromatographic determination of erytromicin and other macrolide antibiotics in livestock products. *J Assoc Off Anal Chem,* 70 (4): 691-697, 1987.

14. Ramirez A, Gutierrez R, Diaz G, Gonzalez C, Perez N, Vega S, Noa M: High-performance thin-layer chromatography -bioautography for multiple antibiotic residues in cow's milk. *J Chromotogr B Analyt Technol Biomed Life Sci,* 784 (2): 315-322, 2003.

15. Chooma IM, Choma A, Staszczuck K: Determination of flumequine in milk by thin - layer chromatography-bio-autography. *J Liq Chrom Rel Technol,* 25 (10): 1579-1587, 2002.

16. Neidert E, Saschenbrecker PW, Tittiger F: Thin-layer chromotograpic/bioautographic method for identification of antibiotic residues in animal tissues. *J Assoc Off Anal Chem,* 70 (2): 197-200, 1987.

17. Tyczkowska K, Voyksner RD, Aronson AL: Development of analytical method for penicillin G in bovine milk by liquid chromatography with ultraviolet-visiable detection and confirmation by mass spectrometric detection. *J Chromatogr,* 490 (1): 101-113, 1989.

18. Allison JRD: Antibiotic residues in milk. *Br Vet J,* 141 (1): 9-16, 1985.

19. Şanlı Y, Kaya S, Aydın N, Yavuz H, Akar F, Doğan A: Süt örneklerinde kloramfenikol kalıntıları. *Ankara Univ Vet Fak Derg,* 38 (3): 402-416, 1991.

20. Temamogullari F, Kaya S: Ankara piyasasında satılan sütlerde bazı antibiyotik kalıntılarının ince tabaka kromatografisi ve biyootografik yöntemle saptanması. *Kafkas Univ Vet Fak Derg,* 16 (2): 187-191, 2010.

21. Gıdalarda Katkı- Kalıntı ve Bulaşanların İzlenmesi: Gıdalarda veteriner ilaç ve anabolizan maddelerin kalıntı düzeylerinin tespiti. Uludağ Üniversitesi Basımevi, Bursa, ISBN 925-7657-97-2. 1997.

22. Ceyhan I, Bozkurt M: Ankara piyasasında satılan sütlerde penisilin araştırması. *Türk Hij Den Biyol Derg,* 44, 1-5, 1987.

23. Aydın N, Cambazoğlu M, Ayhan H: İntertest yöntemi ile sütteki antibiyotiklerin ve diğer inhibitör maddelerin saptanması üzerine çalışmalar. *Etlik Vet Mikrobiyol Derg,* 6, 11-22, 1989.

24. Demet Ö, Acet A, Traş B, Baş L, Eğilmez İ: Konya'da faaliyet gösteren çeşitli mandıralardan toplanan süt örneklerinde penisilin G, ampisilin ve penisilin V kalıntılarının araştırılması. *Selçuk Univ Vet Fak Derg,* 8, 33-35, 1992.

25. Demet Ö, Acet A, Traş B: Konya'da faaliyet gösteren çeşitli mandıralardan toplanan süt örneklerinde kloromfenikol ilaç kalıntılarının araştırılması. *Selçuk Univ Vet Fak Derg,* 8, 35-37, 1992.

26. Dokuzlu C, Tayar M: Bursa ve çevresinde çiğ sütlerde antibiyotik varlığının belirlenmesi. *Vet Bil Derg,* 17 (1): 153-157, 2001.

27. Önal A, Aydın N, Ayaz Y, İşcan D, Savaş N: Süt ve etlerde bulunan bazı antibiyotiklerin çeşitli yöntemlerle saptanması. *Etlik Vet Mikrobiyol Derg,* 7, 48-51, 1993.

28. Kaya S: Antibiyotikler. **In,** Kaya S, Pirinçci İ, Bilgili A (Eds): Veteriner Uygulamalı Farmakoloji. Cilt 2. Baskı 2, s. 247-408, Medisan Yayınevi. Ankara, 2000.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S37-S40, 2010
DOI:10.9775/kvfd.2009.1208

RESEARCH ARTICLE

Fatty Acid Composition and Conjugated Linoleic Acid (CLA) Content of Some Commercial Milk in Turkey [1]

Gokalp Ozmen GULER * Yavuz Selim CAKMAK ** Gokhan ZENGIN **
Abdurrahman AKTUMSEK ** Kazım AKYILDIZ **

[1] *This study was financed by Selcuk University Scientific Research Foundation (BAP). The authors wish to acknowledge their support of this Project*

* Department of Biological Education, Ahmet Kelesoglu Education Faculty, Selcuk University, TR-42090 Konya - TURKEY
** Department of Biology, Science Faculty, Selcuk University, TR-42075 Konya - TURKEY

Makale Kodu (Article Code): KVFD-2009-1208

Summary

In this study, conjugated linoleic acid (CLA) and *trans* fatty acid content and fatty acid composition of some commercial milk in Turkey were determined. Total 41 milk samples belonging to 23 different national milk brands were analyzed. These samples categorized as whole milk, light milk and fruity milk. Generally, C 16:0, palmitic acid, was major fatty acid in all samples. Other predominant fatty acids were C 18:1, oleic acid, and C 18:0, stearic acid. The average of total CLA was ranged between 0.961% and 1.020%.

Keywords: *Commercial milk, Conjugated linoleic acid, Fatty acid composition, Turkey*

Türkiye'deki Bazı Ticari Sütlerin Yağ Asidi Bileşimi ve Konjuge Linoleik Asit (CLA) İçeriği

Özet

Bu çalışmada, Türkiye'deki bazı ticari sütlerin konjuge linoleik asit (CLA) ile *trans* yağ asidi içeriği ve yağ asidi bileşimi belirlenmiştir. 23 farklı ulusal markaya ait 41 süt numunesi analizlenmiştir. Bu süt numuneleri, yağlı süt, yarım yağlı süt ve meyveli süt olarak gruplandırılmıştır. Genel olarak C 16:0, palmitik asit, majör yağ asididir. Diğer en yüksek yüzdeye sahip yağ asitleri de C 18:1, oleik asit ve C 18:0, stearik asit, olarak belirlenmiştir. Toplam CLA ortalama 0.961% ile 1.020% arasında bulunmuştur.

Anahtar sözcükler: *Ticari süt, Konjuge linoleik asit, Yağ asidi bileşimi, Türkiye*

INTRODUCTION

Milk fatty acid composition is important for milk processing and human health [1]. Fatty acid composition of milk fat is highly saturated [2] which have been implicated as a factor for increased risk of heart disease [3]. n-3 fatty acids which may help prevent coronary heart disease [4] account for less than 1% of total fatty acids in milk fat [5].

CLA is a term to describe a mixture of positional and geometrical isomers of linoleic acid (*cis*-9, *cis*-12 octadecadienoic acid) with conjugated double bonds. A portion of CLA in milk fat derives from the uncompleted biohydrogenation of linoleic acid in the rumen and another portion comes from the action of Δ^9 desaturase enzyme on vaccenic acid (C18:1 *trans*-11) to form rumenic acid within the tissues of the mammary gland [6,7]. Two CLA isomers, *cis*-9, *trans*-11 (rumenic acid) and *trans*-10, *cis*-12, have been reported as the most biologically

İletişim (Correspondence)
☎ +90 332 2231866
✉ aktumsek@selcuk.edu.tr

active [8]. The main isomer present in both milk and meat fat is the *c*-9, *t*-11 CLA that accounts for 80 to 90% of the total CLA present [9].

A number of health benefits are associated with dietary conjugated linoleic acid (CLA) including anti-carcinogenic and antiatherogenic activities [10,11].

CLA occurs naturally in many foods however; main dietary sources are dairy products and foods derived from ruminants [12]. Diet is the most important factor influencing milk CLA concentrations [13].

There is some research about the fatty acid composition and CLA content of dairy products various countries [14,15]. However, there are only a few reports about fatty acid composition and CLA content in dairy products in Turkey [16-18]. Therefore, the objective of this study was to determine fatty acid composition and conjugated linoleic acid content of commercial milk belonging to some brands in Turkey.

MATERIAL and METHODS

In this study, 41 commercial milk samples belonging to 23 different national milk brands were analyzed in 2007. Commercial milk was sampled in local dairy markets and stores in Konya, Turkey. These samples categorized as whole milk (n=21), light milk (n=10) and fruity milk (n=10). All of these samples were UHT milk.

Milk samples were frozen and stored at -27°C for fatty acid composition analysis. Milk fat was extracted by the method of Erickson and Dunkley [19] with hexane as the extraction solvent. The fatty acids in the total lipid were esterified into methyl esters by saponification with 0.5 N methanolic NaOH and transesterified with 14% BF_3 (w/v) in methanol [20].

The fatty acid methyl esters were analyzed on a HP (Hewlett Packard) Agilent 6890N model gas chromatograph (GC), equipped with a flame ionization detector (FID) and fitted with a HP-88 capillary column (100 m, 0.25 mm ID and 0.2 µm). Chromatographic conditions were performed according to Ledoux et al.[21] method modified as follows: Injector and detector temperatures were 250°C and 280°C, respectively. The oven was programmed at 60°C initial temperature and 1 min initial time. Thereafter the temperature increased 20°C/min to 190°C held for 60 min then increased at 1°C/min to 220°C and held for 10 min at 220°C. Total run time was 107.5 min. Carrier gas was helium (1 ml/min).

Identification of fatty acids and *trans* isomers were carried out by comparing sample FAME peak relative retention times with those obtained for Alltech standards. Linoleic acid conjugated methyl ester (mixture of *cis*- and *trans*-9,11- and -10,12-octadecadienoic acid methyl esters, catalog number O5632) was purchased from Sigma-Aldrich (St Louis, MO, USA). Results were expressed as FID response area relative percentages. Each reported result is the average value of three GC analyses. The results are offered as mean±SD.

RESULTS

Results of analysis are presented in *Table 1.* Thirty seven fatty acids in milk samples were identified and evaluated. Palmitic acid (30.849-32.200%), oleic acid (27.399-29.179%), stearic acid (13.791-14.788%) and myristic acid (8.131-9.419%) were of high percentages in milk samples. Generally, palmitic acid was the major fatty acid in all samples *(Table 1).*

Saturated fatty acids (SFAs) were higher than mono-unsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) and varied from 59.387 to 61.505%. MUFAs ranged from 31.828% to 33.345 and oleic acid was the major MUFA in all samples. Oleic acid was found to be 29.179%, 28.043% and 27.399% of total fatty acids in whole milk, light milk and fruity milk, respectively. Other predominant MUFA was palmitoleic acid (2.204-2.257%) in milk samples. PUFAs content varied from 3.142% to 3.199% and linoleic acid was major PUFA in all samples. Linoleic and linolenic acid ranged from 2.427 to 2.656% and 0.210 to 0.413%, respectively.

The average total CLA content of milk samples was 1.020%, 0.965% and 0.961% of total fatty acids in whole milk, light milk and fruity milk, respectively. *c*9, *t*11 CLA is major CLA isomers in all samples.

Trans vaccenic acid was found to be the highest *trans* fatty acid (1.585-2.013%) in all samples and C 14:1*t*9, C 16:1*t*9, C 18:2 *t*9*t*12 and C 18:2 *t*9c12 were other *trans* fatty acids in all samples.

DISCUSSION

In our study, palmitic acid was the most abundant fatty acid in milk samples. These results agree with Prandini et al.[14] who reported that palmitic acid (31.01 g/100 g) was major fatty acid in fermented milk. In the present study, the other predominant fatty acids were oleic, stearic and myristic acid in milk samples. Prandini et al.[14] found that SFA was higher than MUFA and PUFA in fermented milk. Similarly, in the present study, SFAs were found to be higher than MUFAs and PUFAs in all samples and ranged from 59.387% to 61.505%.

Table 1: *Fatty acid composition (as methyl esters) of milk samples (%)*

Tablo 1: *Süt numunelerinin yağ asidi bileşimi (metil esteri olarak) (%)*

Fatty Acids	Whole Milk (n=21)	Light Milk (n=10)	Fruity Milk (n=10)
C 4:0	0.134±0.18	0.194±0.26	0.307±0.25
C 6:0	0.160±0.41	0.036±0.03	0.575±0.59
C 8:0	0.152±0.16	0.183±0.17	0.539±0.45
C 10:0	0.825±0.44	1.050±0.67	1.516±0.93
C 11:0	0.106±0.12	0.070±0.06	0.149±0.11
C 12:0	1.641±0.44	1.958±0.66	2.198±0.77
C 13:0	0.156±0.07	0.174±0.07	0.120±0.03
C 14:0	8.131±1.13	8.834±0.66	9.419±1.17
C 15:0	1.057±0.11	1.102±0.12	1.138±0.09
C 16:0	31.205±2.04	32.200±1.44	30.849±2.17
C 17:0	0.744±0.10	0.707±0.10	0.682±0.12
C 18:0	14.788±1.56	14.329±1.11	13.791±1.59
C 19:0	0.040±0.06	0.026±0.03	0.015±0.01
C 20:0	0.089±0.06	0.067±0.04	0.035±0.02
C 21:0	0.043±0.03	0.043±0.02	0.058±0.04
C 22:0	0.109±0.03	0.104±0.03	0.107±0.02
C 24:0	0.007±0.01	0.016±0.02	0.007±0.01
Σ SFA [a]	**59.387±2.32**	**61.093±2.15**	**61.505±2.38**
C 14:1 n5	1.099±0.22	1.220±0.11	1.350±0.19
C 15:1 n5	0.384±0.06	0.394±0.08	0.361±0.05
C 16:1 n7	2.226±0.22	2.204±0.31	2.257±0.19
C 17:1 n8	0.438±0.05	0.436±0.05	0.439±0.05
C 18:1 n9	29.179±1.91	28.043±1.57	27.399±2.49
C 20:1 n9	0.019±0.01	0.022±0.02	0.022±0.01
Σ MUFA [a]	**33.345±1.83**	**32.319±1.79**	**31.828±2.41**
C 18:2 n6	2.656±0.38	2.578±0.30	2.427±0.20
C 18:3 n6	0.031±0.03	0.033±0.01	0.065±0.03
C 18:3 n3	0.280±0.06	0.210±0.06	0.413±0.10
C 20:2 n6	0.014±0.01	0.026±0.02	0.029±0.02
C 20:4 n6	0.173±0.04	0.207±0.06	0.175±0.03
C 20:5 n3	0.018±0.03	0.044±0.05	0.068±0.06
C 22:6 n3	0.015±0.02	0.044±0.06	0.022±0.03
Σ PUFA [a]	**3.187±0.41**	**3.142±0.38**	**3.199±0.26**
C 14:1 *t*9	0.252±0.05	0.255±0.06	0.269±0.04
C 16:1 *t*9	0.550±0.12	0.455±0.10	0.464±0.12
C 18:1 *t*11	2.013±0.52	1.585±0.56	1.699±0.53
C 18:2 *t*9, *t*12	0.147±0.20	0.124±0.14	0.063±0.02
C 18:2 *t*9, *c*12	0.100±0.10	0.070±0.04	0.025±0.01
Σ TFA [a]	**3.062±0.68**	**2.489±0.71**	**2.520±0.60**
CLA *c*9*t*11	0.937±0.21	0.859±0.18	0.891±0.12
CLA *t*10*c*12	0.083±0.03	0.106±0.06	0.070±0.02
Σ CLA [a]	**1.020±0.22**	**0.965±0.18**	**0.961±0.13**

[a] ***SFA:*** *Saturated fatty acid,* ***MUFA:*** *Monounsaturated fatty acid,* ***PUFA:*** *Polyunsaturated fatty acid,* ***TFA:*** *Trans fatty acid,* ***CLA:*** *Conjugated linoleic acid*

Seckin et al.[17] determined that oleic acid was predominant MUFA and ranged from 23.12 to 32.78 g/ 100 g total fatty acid in some dairy products in Turkey. We have also found that oleic acid was the highest MUFA in all samples. Prandini et al.[14] found that MUFA content were 21.61% and 20.63% in fermented milk and fermented milk mountain pasture. Similarly, they determined that oleic acid was predominant MUFA in the fermented milk and fermented milk mountain pasture.

PUFA was reported to range from 0.34 to 2.58 g/ 100 g total fatty acid in some Turkish dairy products [17]. Prandini et al.[14] found that PUFA was 2.23 g/ 100 g of total fatty acids in fermented milk. The value of PUFA (3.142-3.199%) in our experiment is considerably higher than those reported by Prandini et al.[14] and Seckin et al.[17].

Trans vaccenic acid which is the predominant *trans* isomer in milk fat [22] is formed as an intermediate during the biohydrogenation of dietary linoleic acid to stearic acid [23,24]. Other TFA isomers such as 14:1 *t*9, 16:1 *t*9, 18:1 positional isomers, 18:2 *t* and 18:3 *t* have lower amounts in dairy products [25]. Similarly, in our study, the *trans* vaccenic acid was found to be the most abundant *trans* fatty acid in all samples. Mendis et al.[26] analyzed the fatty acid profile of popular brands of Canadian dairy products including milk (n=8). In their study, the average total trans fatty acids of milk was 5.8% of total fatty acids and vaccenic acid was the major trans-octadecenoic acid isomer accounting for 31.0% of total trans-18:1. In our study, total *trans* fatty acid contents of milk (2.489-3.062% in total fatty acid) was relatively lower than Canadian milk. Total *trans* fatty acids in whole milk (3.062%) were found to be higher than light milk and fruity milk because of high percentages of *trans* vaccenic acid. Σ CLA and trans vaccenic acid were higher in whole milk than other samples. CLA content of processed compounded products will reflect the type and amount of the component fats [27].

Fritsche and Steinhart [28] found that CLA (C 18:2 *c*9*t*11) in German foods was 1.16, 0.98, 0.80 and 0.63% in raw milk, pasteurized milk, UHT milk and condensed milk, respectively. Chin et al.[12] reported total CLA to be 0.55 g/100 g fat in homogenized milk and 0.7 g/100 g fat in condensed milk. In our study, the milk samples which were categorized into various groups have varying contents of CLA. The differences in CLA content may also be explained by differences in origin and initial CLA content of raw milks, and production technologies [17]. Prandini et al.[14] stated that animal diet, specific characteristics of milk used in manufacturing, with special reference to the species and CLA content of the milk, processing and production methods play and important role in setting the CLA levels in dairy products.

Rodriguez-Alcala and Fontecha [15] analyzed the total fatty acid content and CLA isomer composition of 6 commercially available CLA-fortified dairy products during processing and refrigerated storage in Spain. They stated that the CLA isomers C18:2 *cis*-9, *trans*-11 and C18:2 *trans*-10, *cis*-12 were the predominant fatty acids at a ratio ranging from 0.97 to 1.05. Rumenic acid was the major CLA isomer of dairy products in Canada [26]. Park et al.[29] stated that based on the distribution ratios

of CLA isomers, the c-9, t-11 isomer appeared to be the major CLA isomer in low-fat milk (89.87%). In our study, the *cis*-9, *trans*-11 isomer was the predominant CLA isomer present in milk samples (0.859-0.937%), with lesser amounts (0.07-0.106%) of *trans*-10, *cis*-12 CLA.

In conclusion, conjugated linoleic acid, *trans* fatty acid and fatty acid composition of some commercial milk in Turkey were determined. Whole milk had highest CLA and *trans* vaccenic acid which is the most common *trans* fatty acid in milk fat from ruminants. More research is needed about CLA content and *trans* fatty acids in Turkish dairy products with regard to food processing and manufacturing because of its health effect.

REFERENCES

1. Kay JK, Weber WJ, Moore CE, Bauman DE, Hansen LB, Chester-Jones H, Crooker BA, Baumgard LH: Effects of week of lactation and genetic selection for milk yield on milk fatty acid composition in Holstein cows. *J Dairy Sci,* 88, 3886-3893, 2005.

2. Loor JJ, Herbein JH, Jenkins TC: Nutrient digestion, biohydrogenation, and fatty acid profiles in blood plasma and milk fat from lactating Holstein cows fed canola oil or canolamide. *Anim Feed Sci Tech,* 97, 65-82, 2002.

3. Ney DM: Potential for enhancing the nutritional properties of milk fat. *J Dairy Sci,* 74, 4002–4012, 1991.

4. Daviglus ML, Stamler J, Orencia AJ, Dyer AR, Liu K, Greenland P, Walsh MK, Morris D, Shekelle RB: Fish consumption and the 30-year risk of fatal myocardial infarction. *New Engl J Med,* 336,1046-1053, 1997.

5. Chilliard Y, Ferlay A, Doreau M: Effect of different types of forages, animal fat or marine oils in cow's diet on milk fat secretion and composition, especially conjugated linoleic acid (CLA) and polyunsaturated fatty acids. *Livest Prod Sci,* 70, 31-48, 2001.

6. Griinari JM, Bauman DE: Biosynthesis of conjugated linoleic acid and its incorporation into meat and milk in ruminants. **In,** Yurawecz MP, Mossoba MM, Kramer JKG, Pariza MW, Nelson GJ (Eds): Advances in Conjugated Linoleic Acid Research. Vol. I, pp. 180-200. AOCS Press, Champaign, IL, 1999.

7. Griinari JM, Corl BA, Lacy SH, Chouinard PY, Nurmela KVV, Bauman DE: Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by Δ^9-desaturase. *J Nutr,* 130, 2285-2291, 2000.

8.Bauman DE, Baumgard LH, Corl BA, Griinari JM: Biosynthesis of conjugated linoleic acid in ruminants. Proc Am Soc Anim Sci Online. 1999. Available http://www.asas.org/jas/symposia/proceedings/0937.pdf. *Accessed:* 1.11.2003.

9. Chin SF, Strokson JM, Pariza MW: Conjugated dienoic derivatives of linoleic acid: A new class of-food derived anticarcinogens. *ACS Symposium Series,* 528, 262-271, 1993.

10. Ip C, Chin F, Scimeca JA, Pariza MW: Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. *Cancer Res,* 51, 6118-6124, 1991.

11. Lee KN, Kritchevsky D, Pariza MW: Conjugated linoleic acid and atherosclerosis in rabbits. *Atherosclerosis,* 108, 19-25, 1994.

12. Chin SF, Liu W, Storkson JM, Ha YL, Pariza MW: Dietary sources of conjugated dienoic isomers of linoleic acid, a newly recognized class of anticarcinogens. *J Food Compos Anal,* 5, 185-197, 1992.

13. Collomb M, Butikofer U, Sieber R, Jeangros B, Bosset JO: Composition of fatty acids in cow's milk fat produced in the lowlands, mountains and highlands of Switzerland using high-resolution gas chromatography. *Int Dairy J,* 12 (8): 649-659, 2002.

14. Prandini A, Sigolo S, Tansini G, Brogna N, Piva G: Different level of conjugated linoleic acid (CLA) in dairy products from Italy. *J Food Compos Anal,* 20, 472-479, 2007.

15. Rodriguez-Alcala LM, Fontecha J: Hot topic: Fatty acid and conjugated linoleic acid (CLA) isomer composition of commercial CLA-Fortified dairy products: Evalution after processing and storage. *J Dairy Sci,* 90, 2083-2090, 2007.

16. Donmez M, Seckin AK, Sagdic O, Simsek B: Chemical characteristics, fatty acid compositions, conjugated linoleic acid contents and cholesterol levels of some traditional Turkish cheeses. *Int J Food Sci Nutr,* 56, 157-163, 2005.

17. Seckin AK, Gursoy O, Kinik O, Akbulut N: Conjugated linoleic acid (CLA) concentration, fatty acid composition and cholesterol content of some Turkish dairy products. *LWT-Food Sci Technol,* 38, 909-915, 2005.

18. Guzel-Seydim ZB, Seydim AC, Greene AK, Tas T: Determination of antimutagenic properties of acetone extracted fermented milks and changes in their total fatty acid profiles including conjugated linoleic acids. *Int J Dairy Technol,* 59, 209-215, 2006.

19. Erickson DR, Dunkley WL: Spectrophotometric determination of tocopherol in milk and milk lipids. *Anal Chem,* 36, 1055-1058, 1964.

20. Paquot C: IUPAC Standard Methods for the Analysis of Oils, Fats and Derivatives. sixth ed., Pergamon Press, Oxford, 1979.

21. Ledoux M, Chargdigny JM, Darbois M, Soustre Y, Sebedio JL, Laloux L: Fatty acid composition of French butters, with special emphasis on conjugated linoleic acid (CLA) isomers. *J Food Compos Anal,* 18, 409-425, 2005.

22. Parodi PW: Distribution of isomeric octadecenoic fatty acids in milk fats. *J Dairy Sci,* 59, 1870-1873, 1976.

23. Jiang J, Bjoerck L, Fonden R, Emanuelson M: Occurence of conjugated *cis*-9, *trans* 11-octadecadieonic acid in bovine milk: Effects of feed and dietary regimen. *J Dairy Sci,* 79, 438-445, 1996.

24. Kepler CR, Tove SB: Biohydrogenation of unsaturated fatty acids. *J Biol Chem,* 242, 5686-5692, 1967.

25. Fritsche J, Steinhart H: Contents of *trans* fatty acids (TFA) in German foods and estimation of daily intake. *Fett/Lipid,* 99, 314-318, 1997.

26. Mendis S, Cruz-Hernandez C, Ratnayake WMN: Fatty acid profile of Canadian dairy products with special attention to the *trans*-octadecenoic acid and conjugated linoleic acid isomers. *JAOAC Int,* 91, 811-819, 2008.

27. Parodi PW: Conjugated linoleic acid in food. **In,** Sébédio JL, Christie WW, Adlof RO (Eds): Advances in Conjugated Linoleic Acid Research. Vol. 2, pp. 101-122, AOCS Press, Champaign, IL, 2003.

28. Fritsche J, Steinhart H: Amounts of conjugated linoleic acid (CLA) in German foods and evaluation of daily intake. *Z Lebensm Unters Forsch A,* 206, 77-82, 1998.

29. Park SY, Aw JE, Kim GB, Jung MY, Lee BH: Distribution and content of geometric isomers of conjugated linoleic acid in dairy foods from the Quebec province of Canada. *Food Sci Biotechnol,* 17, 31-37, 2008.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S41-S46, 2010
DOI:10.9775/kvfd.2009.1280

RESEARCH ARTICLE

The Effects of Organic Acid and *Origanum onites* Supplementations on Some Physical and Microbial Characteristics of Broiler Meat Obtained from Broilers Kept Under Seasonal Heat Stres

Ergün Ömer GÖKSOY * Mustafa AKŞİT ** Şükrü KIRKAN ***

* Adnan Menderes University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Batı Kampusu, TR-09016, Isikli, Aydin - TURKEY
** Adnan Menderes University, Faculty of Agriculture, Department of Animal Science, Guney Kampusu, TR-09100, Cakmar, Aydin - TURKEY
*** Adnan Menderes University, Faculty of Veterinary Medicine, Department of Microbiology, Batı Kampusu, TR-09016, Isikli, Aydin - TURKEY

Makale Kodu (Article Code): KVFD-2009-1280

Summary

This study was conducted to determine the effects of dietary organic acid combination or *Origanum onites* supplementations on the microbiological quality and some meat quality parameters of broiler meat obtained from heat stressed broilers. 540 broiler chicks (Ross 308) were divided into three groups as control, organic acid (OA) supplemented group and *Origanum onites* supplemented group (OO). After the period of 0-3 weeks, chickens were raised under seasonal heat stress. On the 42nd day, 10 birds were randomly chosen from each group and slaughtered then some meat quality parameters, colour, drip and cooking loss were determined on the breast muscles. The microbiological quality and some meat quality parameters of breast muscles and skins were analysed at the 0, 3rd, 8th and 15th days of storage period at 4°C. Coliforms in faces samples taken from gastrointestinal tract were counted. Control group had the lowest pH ultimate value, whereas the values of drip loss (18.28±0.99%) and cooking loss (31.15±0.99%) of this group were the highest (P<0.05). Chickens in control group had the highest L* value (62.23±1.53) whereas the OO supplemented group had the lowest L* value (54.93±1.53) (P<0.05). OA supplementation affected broiler meat microbiological quality significantly (P<0.05). OA supplemented group had significantly lower coliform count (P<0.05). OO supplementation improved the meat quality parameters, such as drip loss, cooking loss and lightness, in broilers under seasonal heat stress. Organic acid supplementations improved microbiological quality of chicken meat during storage.

Keywords: *Broiler, Origanum onites, Organic acid, Microbiologic quality, Meat quality*

Mevsimsel Sıcaklık Stresinde Yetiştirilen Broilerlerin Rasyonlarına İlave Edilen Organik Asit veya *Origanum onites*'in Bazı Fiziksel ve Mikrobiyolojik Et Kalitesi Özellikleri Üzerine Olan Etkileri

Özet

Bu çalışma mevsimsel sıcak stresine maruz kalıp organic asit veya esansiyel yağ asidi ile beslenilmiş olan broilerlerden elde edilen kanatlı etinin bazı et kalitesi özellikleri ve raf ömrünün belirlenmesi amacıyla yapılmıştır. 540 broiler tavuk (Ross 308) kontrol, organik asit ilave edilmiş (OA) veya *Origaum onites* ilave edilmiş (OO) grup olmak üzere 3 ayrı gruba ayrıldı. İlk 3 haftanın takibinde broilerler mevsimsel sıcak stresine maruz bırakıldılar. 42. günde kesilen broilelerden her bir gruptan 10'ar tane rastgele seçilerek göğüs kaslarında renk, pişme ve soğutma kaybı gibi bazı et kalitesi parametreleri incelendi. Göğüs etlerinin raf ömürleri özellikleri soğuk muhafazanın 0, 3., 8. ve 15. günlerinde mikrobiyolojik yöntemlerle incelendi ve bazı et kalitesi özellikleri kontrol edildi. Broilerlerin barsak sisteminden alınan dışkı örnekleri de 0. günde coliform sayısı açısından incelendi. Kontrol grubu en düşük son pH'a ulaşırken fire (%18.28±0.99) ve pişirme kaybı (%31.15±0.99) en yüksek olan gruptu. Kontrol grubunda L*değeri en yüksek bulunurken (62.23±1.53), OO ilave edilen grupta en düşüktü (54.93±1.53). OA ilavesi mikrobiyolojik kaliteyi istatistiksel olarak belirgin bir şekilde (P<0.05) etkiledi. OA ilavesi ile dışkıda coliform sayısında belirgin bir azalma görüldü (P<0.05). Yemlere OO ilavesi ile Lightness, drip ve pişirme kaybı gibi et kalitesi parametrelerinde iyileşme görülürken organic asit ilavesinin raf ömrü süresince mikrobiyel kaliteyi karşılaştırılan diğer gruplara göre arttırdığı gözlemlendi.

Anahtar sözcükler: *Broiler, Origanum onites, Organik asit, Mikrobiyolojik kalite, Et kalitesi*

İletişim (Correspondence)
☎ +90 256 2470700
✉ egoksoy@hotmail.com

INTRODUCTION

Broiler meat quality depends on the inherent characteristics of the animal, long- and short-term environmental influences on the animal and processing variables. Most of these factors are likely to alter muscle post-mortem metabolism which largely determines the processing ability of meat. Environmental conditions existing even shortly before the animal is slaughtered have been known to be stressful for broilers and alter some of their meat characteristics [1].

To the consumer, appearance is the major criterion for purchase selection and initial evaluation of meat quality. Other quality attributes, such as tenderness, juiciness, drip-loss, cook-loss, and shelf-life are important to the consumer after purchasing the product, as well as to the processor when producing value-added meat products [2].

A selective exclusion of the gut pathogens and a consequent promotion of the favourable microbes like the lactobacilli may be the plausible mechanism of action of the organic acids [3]. In addition the beneficial effects of organic acid supplementation in livestock have been attributed to a lowering of pH, mainly in the stomach, and intestines which controls the growth of pathogenic bacteria [4].

Herbs and species have been used for centuries to provide favourable flavours. They also have anti-microbial activity [5,6]. Antibacterial activity of herbs and species is mainly caused by essential oil components such as carvacrol, α-terpineol, terpinen-4-ol, eugenol, (±)-linalool, (-)-thujone, (cis+trans) citral, nerol, geraniol, menthone [7]. The essential oils are hydraphobic and their primary site of toxicity is the membrane. They accumulate in the lipid bilayer according to a partition coefficient that is specific for the compound applied, leading to disruption of the membrane structure and function.

The aim of this study is to determine the effects of organic acid or *Origanum onites* supplementation on some meat quality parameters and microbiological quality of broiler meat of which obtained from of broilers kept under seasonal heat stress.

MATERIAL and METHODS

This study was conducted in summer, during July and August, 2007. 540 broiler chicks (Ross 308), mixed sex kept in deep litter. The temperature was 32°C in housing on day 1 and was decreased weekly by 2.5°C until wk 3. From 3 to 6 wk, groups were kept at ambient temperature which was reaching to 43°C during the day time. From 3 to 6 wk, the average temperature and relative humidity from 10:00 to 18:00 and from 18:00 to 10:00 were 34.2°C, 46% and 26.6°C, 57%, respectively. For each group, broiler chicks were kept in different pens (1.75x2.5 m) based on the diet regimes for 42 days. Birds were randomly divided into 3 groups (180 birds for each group; 60 birds x 3 pens): Control group (basal diet), *Origanum onites* group (the basal diet supplemented with *Origanum onites*: *Origanum onites,* 15 g/kg) and organic acid group (the basal diet supplemented organic acid combination; Lactic acid 200 g/kg; Formic acid 250 g/kg, and Propionic acid, 80 g/kg). Diets were fed ad libitum and birds had free access to drinking water. Ingredients of the diets were shown in *Table 1*.

Birds were slaughtered on 42nd day of age. Slaughtering was carried out under commercial conditions in a pilot plant (Poultry Processing plant of ADU, Faculty of Agriculture). The equipment and plant were disinfected after slaughtering for each group. Samples were taken immediately after air chilling.

Table 1. *Composition of experimental basal diets and calculated nutrient content*

Tablo 1. *Deneysel bazal diyet kompozisyonu ve hesaplanmış besin içeriği*

	Basal Diets	
Ingredients	**%**	
Corn	52.00	60.00
Soybean Meal	34.50	29.50
Fish Meal	5.00	1.50
Vegetable Oil	5.00	6.00
Di Calcium Phosphate	1.50	1.30
Calcium Carbonate	1.05	0.94
Salt	0.30	0.30
Trace Vitamin Premix [1]	0.25	0.25
Trace Mineral Premix [1]	0.10	0.10
DL-Methionin	0.10	0.10
L-Lysine	0.05	0
Choline	0.05	0.01
Coccidiostat (Cygro) [2]	0.10	0.10
Origanum onites [3]	0	0
Organic Acid Combination [4]	0	0
Calculated chemical analyses, %		
Crude protein	23.11	19.35
Calcium	1.18	0.92
Total Phosphor	0.79	0.66
Methionin	0.51	0.35
Lysine	1.47	1.06

[1]: For each kg of the diet; vitamin A 12.000 IU; vitamin E 35.0 mg; vitamin K_3 5.0 mg; vitamin B_1 3.0 mg; vitamin B_2 7.0 mg; vitamin B_6 5.0 mg; vitamin B_{12} 0.015 mg; Calcium D-Pentotenat 10.0 mg; Folic acid 1.0 mg; D-Biotin 0.045 mg; Choline chloride 125.0 mg; vitamin C 50.0 mg; Mn 80 mg; Fe 60.0 mg; Cu 5 mg; Co 0.2 mg; Se 0.15 mg,

[2]: For each kg of cygro; maduramycin Amonium, 5.000 ppm

[3]: Origanum onites, 15 g/kg

[4]: Lactic acid 200 g/kg; Formic acid 250 g/kg, and Propionic acid, 80 g/kg

Determination of Meat Quality Parameters
pH and Color Measurements

The pH values were determined after 15 min post slaughter (initial pH, pH_{15}) and after chilling for 24 h at 4°C in sealed plastic bags (ultimate pH, pHu), on the left breast muscles of carcasses by using pH meter (Hanna Instrument Model 211) with a penetration electrode (FC-200). Color (L*, a*, and b*) was measured on the left breast muscle at 24 h using a Minolta chromameter (Minolta Corp., Ramsey, NJ).

Drip Loss and Cooking Loss

Drip loss (DL) of the Pectoralis major muscle at 24 h post-mortem was measured as described by Remington et al [8]. The left Pectoralis major muscle was excised and weighed, then placed in a plastic bag and stored at 4°C. The muscle was removed from the bag 24 h post slaughter, wiped, and weighed to evaluate DL which was expressed as a percentage of the initial muscle weight. To obtain cooking loss, samples were cooked individually in heat-and-seal bags immersed in 75°C water to internal temperature of 70°C. Temperature was measured with a penetrative probe (Hanna Instrument 8521) in a meat sample in a bag. Samples were weighed before and after cooking to determine cooking loss. Weight loss was expressed as a percentage of initial weight for drip and cooking losses.

Microbiological Analyses

Ten birds from each group (30 broiler chickens in total) were slaughtered under commercial conditions. After chilling, the left breasts were removed and put in sterile bags. The intestinal truct of the birds were also collected separately and put into individual sterile bags. Breasts (skin on), and intestinal tracts were brought to the laboratory in two cold boxes (4°C). The shelf life and organoleptic features of breast muscles and skins were analysed at the 0, 3rd, 8th and 15th days of storage period at 4°C. 10 gram of skin and flesh samples from each breast were put in a sterile bag and homogenised in 90 ml of pepton water (Merck 1.07228) then serial dilutions were carried out. Plating out was done on PCA (Plate count Agar, Oxoid CM 325) from appropriate serial dilutions and the plates were incubated at 30°C for 3 days. The results were found as log cfu/g of the meat and skin samples analysed.

Organoleptic Analyses

The samples were analyzed by 3 referees on the sampling days based on their odors and appearances. The referees recorded the sensory evaluation results as good and spoiled. The spoiled evaluation was carried out by smelling uncommon flesh odors and observing sticky exudates on the skin and flesh of meat.

Analysis of Faeces

Ten gram of faces samples taken from gastro-intestinal truct were homogenised in 90 ml of pepton water then serial dilutions were carried out. Dilutions were plated out on Violet Bile Lactose Agar (VRBA: Lab M, Lab 31) and plates were incubated at 37°C for 1 day then colonies were counted. The results were found as log cfu/g of the material analysed.

Statistics

All data were subjected to Univariate using the General Linear Models procedure of SPSS. Means differing significantly were separated using the Duncan's test option of SPSS ($P<0.05$).

RESULTS

Meat Quality Parameters

Meat quality parameters of broiler chickens for different groups are given at *(Table 2)*. Although no statistical differences were observed at the initial pH values measured at 15 min postmortem, *Origanum onites* (OO) supplemented group had the highest pH ultimate value (6.10±0.49) then the others at the pH measurement conducted 24 h post mortem. The drip loss and cooking loss parameters were statistically different between groups. The highest losses for these parameters were in control group. The lowest drip loss (7.40±0.99%) and cooking loss (19.69±0.99%) were observed in the group supplemented with OO *(Table 2)*.

The colour measurements showed that there were significant differences between groups. Chickens kept at

Table 2. *Effect of diets on breast meat quality parameters of broiler chickens under seasonal heat stress (X±Sx)*

Tablo 2. *Mevsimsel sıcaklık stresine maruz kalan broilerlerin et kalitesi üzerine verilen diyetlerin etkisi (X±Sx)*

Parameters	Control	Organic Acid	*Origanum onites*
pH 15	6.01±0.60 ^{a}	6.13±0.60 ^{a}	6.19±0.60 ^{a}
pHu 24	5.84±0.49 ^{a}	5.96±0.49 ^{ab}	6.10±0. 49 ^{b}
DL %	18.28±0.99 ^{a}	11.86±0.99 ^{b}	7.40±0.99 ^{c}
CL %	31.15±0.99 ^{a}	27.26±0.99 ^{b}	19.69±0.99 ^{c}
L*	62.23±1.53 ^{a}	58.63±1.53 ^{ab}	54.93±1.53 ^{b}
a *	3.41±0.41 ^{a}	2.46±0.41 ^{a}	2.32±0.41 ^{a}
b*	5.60±0.54 ^{a}	2.26±0.54 ^{b}	3.16±0.54 ^{ab}

a-c *Means in the same column with no common superscript differ significantly,* ***DL:*** *Drip loss;* ***CL:*** *cooking loss;* ***L**** *= lightness;* ***a**** *= redness;* ***b**** *= yellowness*

control group had the highest lightness (L*) values whereas the lowest was the OO group. Although redness (a*) values did not show any significance, mean yellowness (b*) values obtained from the control birds was significantly higher than OA supplemented group *(Table 2)*.

Microbiological Quality

Examinations carried out during the sampling days showed that OA supplementation affected broiler breast meat microbiological quality *(Table 3)*. On day 0, breast muscles from OA group had significantly lower (P<0.05) microbial load. On 3rd day of storage, OA group had lowest microbial load (4.98±0.09 log cfu/g) and there was a statistically significant difference (P<0.05) between OA supplemented group and the others. No statistical difference was observed between control group and OO group (P>0.05). No spoilage signs were observed in any of these groups. The mean microbiological load on the breast muscles from OA supplemented group was 5.51±0.09 log cfu/g and no spoilage signs were observed on the 8th day of storage. However, muscle samples from the other groups showed spoilage signs such as a heavy odour and slight sticky exudates on their surfaces. On the 15th day of trial, all samples were spoiled. Intestinal pH was not affected (P>0.05) from the different diets *(Table 4)*. However, OA group had significantly lower coliform count than the other groups.

Table 3. *Numbers of total bacteria grown during the storage on the breast meat of broiler carcasses (log cfu g^{-1}) fed by various feed supplementations under seasonal heat stress*

Tablo 3. *Mevsimsel sıcaklık stresine maruz kalıp çeşitli yem ilaveleriyle beslenilen broilerlerin göğüs etlerinde soğuk muhafaza sırasında gelişen toplam mezofilik bakteri sayıları (log kob g^{-1})*

Storage Time (Day)	Control	Organic Acid Combination	*Origanum onites*
0	4.67±0.10 ^{b}	4.45±0.10 ^{b}	5.30±0.10 ^{a}
3	5.87±0.09 ^{a}	4.98±0.09 ^{b}	5.74±0.09 ^{a}
8	6.40±0.09 ^{a}	5.51±0.09 ^{b}	6.32±0.09 ^{a}
15	8.49±0.15 ^{a}	7.76±0.15 ^{b}	7.88±0.15 ^{b}

$^{a-b}$ Means in the same row with no common superscript differ significantly

Table 4. *Coliform counts in the faecal material taken from broilers (log cfu g^{-1}) and intestinal pH (X±Sx)*

Tabo 4. *Broilerlerden alınan dışkı materyalinde coliform sayısı (log kob g^{-1}) ve barsak içeriğinin pH'ı (X±Sx)*

Characteristic	Control	Organic Acid	*Origanum onites*
Intestinal pH	6.01±0.12 ^{a}	5.82±0.12 ^{a}	5.83±0.12 ^{a}
Coliforms in faeces	6.76±0.58 ^{a}	5.54±0.46 ^{b}	6.67±0.27 ^{a}

$^{a-b}$ Means in the same column with no common superscript differ significantly

DISCUSSION

Although repeated increases in environmental temperature or seasonal temperature changes do not affect broilers [9], acute heat stress does not allow broilers to adopt their physiological and metabolical conditions to increased temperatures causing quality defects and deaths. Especially in the Western and Southern seashore parts of Turkey acute heat stress causes massive deaths and meat quality defects between July and August months in the summer season [10].

The study reported here conducted during the summer season in order to determine the effects of organic acid or *Origanum onites* supplementations on some meat quality parameters and microbiological quality of broiler meat. It was reported that there was a significant correlation between muscle pH and extremes in color variation [11,12]. Cornforth [13] also stated that meat with a high pH has a higher water-holding capacity, hence making it appear darker. Poultry meat with low pH has been associated with low water-holding capacity (WHC), which results in increased cook-loss and drip loss [14,15]. Low pH has also been reported to decrease tenderness [14,16] and increase shelf-life [2]. Heat stress prior to slaughter was reported to cause PSE-like meat in turkeys [17] and broilers [15]. Lee et al [18], who found that birds held at a control temperature of 20°C (68°F), exhibited significantly higher terminal pH values than heat-stressed birds [16]. Mckee and Sams [19] were also showed that lighter meat colour, lower ultimate pH values and higher drip and cooking loss could be observed in turkeys having heat stress prior to slaughter. That is because higher drip losses observed in the control group and organic acid group L* values were higher than *Origanum onites* supplemented group. Based on the information given above meat with high ultimate pH (24 h) with low drip loss had the lowest L* value whereas meat with the low ultimate pH and high drip loss showed high L* value due to the reflectance effect of exudation. From these results, might be predicted that there were some positive effects of OO supplementation on the stress compensation of broilers.

In the food animal industry, organic acids were added to feeds to as fungistats, but in the past 30 yr, formic and propionic acids and various combinations have also been examined for potential bactericidal activity in feeds and feed ingredients contaminated with food-borne pathogens, particularly *Salmonella spp* [20-22]. The supplementation of OA or OO might be considered as beneficial in improving microbiological quality of broiler chickens during storage [23]. Organic acid supplementation

of feed or water has also been reported a promising non-antibiotic alternative [24,25] for poultry. *In vitro* studies revealed that the organic acid combinations have anti-microbial activities [25]. At low pH, organic acids are found predominantly in the undissociated (protonated) form. Undissociated organic acids are lipophilic and can diffuse across bacterial cell membranes. Once inside the bacterial cell, the organic acids will dissociate and decrease the cytoplasmic pH, disrupting enzymatic reactions, cellular growth and/or inducing cell death in a variety of bacterial species [4,24-26]. Certain microbes are more or less tolerant to decreases in intracellular pH levels. For example, some enterobacterial species such as *Escherichia coli* are generally more susceptible to organic acid induced toxicity whereas, other species (ie lactobacilli and streptococci) allow their intracellular pH levels to drop, and are often less susceptible [24,26]. In the present study, the total numbers of coliforms in OA supplemented group found in the faeces were significantly lower than the other groups examined, which might also affect the contamination rate of breast skin. As it was predicted, TVC obtained from the breast samples in OA supplemented group was lower than TVC obtained from the other groups. The inhibitory effects of OA combination continued during storage period and significantly lower numbers of total bacteria were observed on 0, 3rd and 8th sampling days when compared with the other groups. The control and OO supplemented groups both had the signs of the spoilage but the OA supplemented group did not show any of the spoilage signs 8th day of cold storage. Therefore, it could be predicted that approximately two days of shelf-life extension could be provided when OA combination supplementation was added to the ration of broilers.

Based on the data gathered from the study presented here it could be concluded that *Origanum onites* supplementation may have some beneficial effects on meat quality parameters of broilers, such as drip loss, cooking loss and meat colour. Organic acid combination supplemented to broiler feed may improve micro-biolgical quality of poultry carcasses during storage.

REFERENCES

1. Berri C, Debut M, Sante´-Lhoutellier C, Arnould B, Boutten B, Sellier N, Bae´Za E, Jehl N, Je´Go Y, Duclos MJ, Le Bihan Duval E: Variations in chicken breast meat quality: A strong implication of struggle and muscle glycogen level at death. *Br Poult Sci,* 46, 572-579, 2005.

2. Allen CD, Russell SM, Fletcher DL: The relationship of broiler breast meat color and pH to shelf-life and odor development. *Poult Sci,* 76, 1042-1046, 1997.

3. Overland M, Granil T, Kjos NP, Fjetland O, Steien SH, Stokstad M: Effect of dietary formats on growth performance, carcass traits, sensory quality, intestinal microflora, and stomach alterations in growing-finishing pigs. *J Anim Sci,* 78, 1875-1884, 2000.

4. Partanen KH: Organic acids their efficacy and modes of action in pigs. **In,** Piva A, Bach Knudsen KE, Lindberg JE (Eds): Gut Environment of Pigs. pp. 201-218, University Press, Notingham, UK, 2001.

5. Tassou CC, Drosinos EH, Nychas, GJE: Effects of essential oil from mint *(Mentha piperita)* on *Salmonella enteriditis* and *Listeria monocytogenes* in food model systems at 4°C and 10°C. *J Appl Bacteriol,* 78, 593-600, 1995.

6. Lambert RJW, Skandamis PN, Coote PJ, Nychas G-JE: A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J Appl Microbiol,* 91, 453-462, 2001.

7. Dorman, HJD Deans, SG: Antimicrobial agents from plants: Antibacterial activity of plant volatile oils. *J Appl Microbiol,* 88, 308-316, 2000.

8. Remignon H, Desrosier V, Marche G: Influence of increasing breast meat yield on muscle histology and meat quality in the chicken. *Reprod Nutr Dev,* 36, 523-530, 1996.

9. Yahav S, Hurwitz S: Induction of thermotolerance in male broiler chickens by temperature conditioning at an early age. *Poult Sci,* 75, 402-406, 1996.

10. Altan A, Bayraktar H, Önenç A: The effects of heat stress on colour and ph of broiler meat. *J Anim Prod,* 42 (2): 1-8, 2001.

11. Livingston DJ, Brown WD: The chemistry of myoglobin and its reactions. *Food Technol,* 35, 244-252, 1981.

12. Yang CC, Chen TC: Effects of refrigerated storage, pH adjustment, and marinade on color of raw and microwave cooked chicken meat. *Poult Sci,* 72, 355-362, 1993.

13. Cornforth, D P: Color and its importance. **In,** Pearson AM, Dutson TR (Eds): Quality attributes and their measurement in meat, poultry, and fish products. pp. 34-78, Chapman and Hall, London, UK, 1994.

14. Froning GW, Babji AS, Mather FB: The effect of preslaughter temperature, stress, struggle and anesthetization on color and textural characteristic of turkey muscle. *Poult Sci,* 57, 630-633, 1978.

15. Northcutt JK, Foegeding EA, Edens FW: Water-holding properties of thermally preconditioned chicken breast and leg meat. *Poult Sci,* 73, 308-316, 1994.

16. Holm CGP, Fletcher DL: Antemortem holding temperatures and broiler breast meat quality. *J Appl Poult Res,* 6, 180-184, 1997.

17. Babji AS, Froning GW, Nkoga DA: The effects of preslaughter environmental temperature in the presence of electrolyte treatment on turkey meat quality. *Poult Sci,* 61, 2385-2389, 1982.

18. Lee YB, Hqus GL, Hageberg EC, Forsylhe RH: Effect of antemortem environmental temperatures on postmortem colysi and tenderness in excised broiler breast muscle. *J Food Sci,* 41, 1466-1469, 1976.

19. Mckee SR, Sams AR: The effect of seasonal heat stress on rigor development and the incidence of pale, exudative turkey meat. *Poult Sci,* 76, 1616-1620, 1997

20. Dixon RC, Hamilton PB: Effect of feed ingredients on the antifungal activity of propionic acid. *Poult Sci,* 60, 2407-

2411, 1981.

21. Mchan F, Shotts EB: Effect of feeding selected short chain fatty acids on the *in vivo* attachment of *Salmonella typhimurium* in chick ceca. *Avian Dis,* 36, 139-142, 1992.

22. Hinton M, Linton AH: Control of salmonella infections in broiler chickens by the acid treatment of their feed. *Vet Rec,* 123, 416-421, 1988.

23. Akşit M, Göksoy EO Kök F, Özdemir D, Özdoğan M: The impacts of organic acid and essential oil supplements on the microbiological quality of chicken carcasses. *Arch Geflügelkd,* 70 (4): 168-173, 2006.

24. Van Immerseel F, Russell JB, Flythe MD, Gantois I, Timbermont L, Pasmans F, Haesebrouck F, Ducatelle R: The use of organic acids to combat salmonella in poultry: A mechanistic explanation of the efficacy. *Avian Pathol,* 35 (3): 182-188, 2006.

25. Dibner JJ, Buttin P: Use of organic acids as a model to study the impact of gut microflora on nutrition and metabolism. *J Appl Poult Res,* 11, 453-463, 2002.

26. Russell, JB, Diez-Gonzalez, F: The effects of fermentation acids on bacterial growth. *Adv Microb Physiol,* 39, 205-234, 1998.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S47-S52, 2010
DOI:10.9775/kvfd.2009.1284

RESEARCH ARTICLE

Detection of *Brucella* Antibody and DNA in Cow Milk by ELISA and PCR Methods

Göknur TERZİ * Özlem BÜYÜKTANIR ** Oktay GENÇ **
Ali GÜCÜKOĞLU * Nevzat YURDUSEV **

* Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, TR-55139 Kurupelit, Samsun - TURKEY
** Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Microbiology, TR-55139 Kurupelit, Samsun - TURKEY

Makale Kodu (Article Code): KVFD-2009-1284

Summary

The aim of the present study was to determine comparatively the presence of anti-*Brucella* antibody and *Brucella* DNA in cow milk. Anti-*Brucella* antibody was detected by ELISA based on the lipopolysaccharide (LPS) as diagnostic antigen. Besides, the presence of *Brucella* DNA in milk samples was screened by *ery*CD gene-targeted PCR and *B. abortus* DNA was determined by amplification of *alk*B genes. For this purpose, 70 raw cow milk samples collected from open markets were used. Among these samples, 15 samples (21.4%) were found positive for anti-*Brucella* LPS antibody in ELISA. In contrast, only 5 milk samples (7.1%) were determined as positive by *ery*CD gene-targeted PCR. All of the *ery*CD positive samples giving an amplicon of 904 bp indicated the presence of wild-type Brucella DNA but not *B. abortus* S19 vaccine strain allowing amplification of only an amplicon of 202 bp. In addition, amplification of the *alk*B gene demonstrated the presence of *B. abortus* DNA in 5 *ery*CD positive samples. No statistical agreement was observed between ELISA and PCR results with 95% confidence interval. These results strongly suggest that use of both ELISA and PCR methods could lead to more reliable diagnosis of brucellosis from bovine milk samples.

Keywords: *Brucella, Bovine, Milk, ELISA, PCR*

İnek Sütünde *Brucella* Antikoru ve DNA'sının ELISA ve PCR Yöntemleri ile Tespiti

Özet

Bu çalışmada inek sütünde anti-*Brucella* antikorları ve *Brucella* DNA'sının karşılaştırmalı yöntemlerle belirlenmesi amaçlanmıştır. Anti-*Brucella* antikorların tespitinde spesifik tanı antijeni olarak lipopolisakkaride (LPS) dayalı ELISA kullanıldı. Ayrıca, süt örneklerinde *Brucella* DNA varlığı *ery* genine dayalı PCR tekniği ile araştırıldı ve *B. abortus* DNA varlığı ise *alk*B geni amplifikasyonu ile belirlendi. Bu amaçla, 70 adet çiğ inek sütü örneği yerel pazarlardan toplandı. Bu örneklerin 15'i (% 21.4) anti-*Brucella* LPS antikoru yönünden ELISA'da pozitif bulundu. Buna karşın, 5 süt örneğinin (% 7.1) eryCD genine dayalı PCR'da pozitif olduğu belirlendi. Tüm *ery*CD pozitif örneklerin 904 bp boyutunda amplikon vermesi, *Brucella* DNA'sının saha suşuna ait olduğunu ancak yalnızca 202 bp büyüklüğünde amplikon çoğaltılabilenn *B. abortus* S19 aşı suşuna ait olmadığını gösterdi. Ayrıca, *alk*B geninin PCR'da çoğaltılması ile *ery*CD pozitif bulunan 5 örnekte de *B. abortus* DNA varlığı belirlendi. %95 güven aralığında, PCR ve ELISA sonuçları arasında istatistiksel uyumluluk gözlenmedi. Bu bulgular, inek süt örneklerinde brusellozis tanısının ELISA ve PCR yöntemlerinin birlikte kullanımı ile daha güvenilir olabileceğine kuvvetle işaret etmektedir.

Anahtar sözcükler: *Brucella, Sığır, Süt, ELISA, PCR*

INTRODUCTION

Brucellosis is a widespread zoonotic disease causing considerable economic losses in ruminants and transmission of the pathogenic *Brucella* strains to humans occurs as a result of consuming contaminated milk and milk products and direct contact with the infected animals [1-3]. Thus, the determination of the infected

İletişim (Correspondence)
☎ +90 362 3121919/2807
✉ yurdusev@omu.edu.tr

animals is of paramount importance for public and animal health. In dairy cattle, bacteriological, serological and molecular methods have been carried out for the diagnosis of brucellosis [4-6]. Although isolation of the bacteria leads to the definitive diagnosis of the disease, bovine brucellosis diagnosis is essentially based on the serological methods using serum or milk samples [4-6]. As milk particularly reflects IgG based antibody response of the animal [7,8] and is a non-invasive sampling method, its use instead of blood in serological detection represents an important advantage in lactating animals.

Milk Ring Test (MRT) has been used as a screening test for bovine brucellosis, and is an adaptation of the agglutination test performed with the milk [9]. MRT has relatively low sensitivity and leads to wrong interpretations with colostrums, milk at the end of lactation period and milk from cow with mastitis [6]. Its specificity, however, is doubtful when *Brucella* prevalence is low [10]. Milk ELISA was found more sensitive and specific than MRT in detecting anti-*Brucella* antibodies in milk [4,11].

In recent years, detection of *Brucella* DNA by PCR-based methods in milk samples have been developed [12-14]. These methods are rapid and accurate and allow testing a great number of samples to detect the presence of the pathogens [15]. A number of nucleic acid sequences have been targeted for the development of *Brucella* genus-specific PCR assays, including 16S rRNA, 16S-23S intergenic spacer region, *omp*2, *bcsp*31 [13,16]. In addition, *IS711* element downstream of the *alk*B gene allows the detection of *B. abortus* and also *ery*CD gene regions facilitate the discrimination of *B. abortus* field strains from the S19 vaccine strain [17-20].

The aim of the present study was to detect comparatively the presence of anti-*Brucella* antibody and *Brucella* DNA in cow milk. For these purposes, our previously described LPS-based ELISA method was adapted to ELISA to detect anti-*Brucella* antibody in milk [21]. Furthermore, eryCD and *alk*B gene-based PCR assays using milk DNA as template were developed and used for determination of the presence of *Brucella spp*. and *B.abortus* and also discrimination of *B. abortus* field strains from the vaccine strain S19.

MATERIAL and METHODS

Milk Samples and Milk Whey Preparation

A total of 70 milk samples from cows with unknown brucellosis status were collected from 8 different open markets in Samsun (Turkey) between January-March 2008. Seven milk samples from *B. abortus* isolated animals and 16 samples from *B. abortus* S19 vaccinated cattle were collected. Forty five milk samples from non-vaccinated cows evaluated as negative with MRT and whey agglutination tests as described in a previous study [22] were used as negative controls. Milk whey was prepared by the addition of 200 µl of commercial liquid rennet (strength 1: 10000; 0.3%, w/v) to 10 ml of each milk sample and incubation at 37°C for 30 min until the coagulation occurred [23]. Following removal of the casein by filtration and centrifugation, clear milk whey was collected and stored at -20°C until use.

B. abortus Strain, Culture, Enumeration and LPS Preparation

Brucella abortus S19 vaccine strain supplied from Veterinary Research and Control Institute (Pendik, İstanbul) was grown at 37°C for 3 days on blood agar base (Merck, Germany). A single smooth colony selected by morphology and acriflavine agglutination was isolated and cultured in Brain Heart Infusion Broth (BHI, Oxoid, Cambridge, UK). Colony count was determined by inoculation of 0.1 ml suspensions from ten-fold serial dilutions onto BHI agar plates and incubation at 37°C for 24 h.

To prepare *B. abortus* LPS as an immunodominant antigen, fresh *Brucella* culture was inactivated by formaldehyde (0.5%) and stored at 4°C for 2 days. Inactivated cells were harvested by centrifugation, washed three times in physiological saline solution and LPS was extracted by hot phenol-water method as previously described by Caroff et al.[24].

ELISA Procedure

Indirect ELISA method previously described by Genç et al.[21] was adapted to test milk whey. Briefly, micro-plates were coated with 100 µl of 5 µg/ml of *B. abortus* crude LPS prepared in carbonate buffer (0.1 M, pH 9.6) and kept overnight at +4°C. The microwells blocked using 200 µl/well of 1% fish gelatine in phosphate buffered saline (PBS, Sigma Aldrich, St-Louis, USA) containing 0.1% Tween 20 (FG-PBST) were incubated at 37°C for 1 h. One hundred microliters of milk whey samples diluted 1:5 in FG-PBST were added to microwells. Alkaline phosphatase conjugated rabbit anti-bovine IgG (Sigma-Aldrich, St-Louis, USA) diluted 1:30 000 were added and incubated for 1 h at 37°C. After addition of 100 µl of pNPP (p-Nitrophenyl Phosphate, Sigma-Aldrich) as substrate, and incubation for 1 h at 37°C, the absorbance was read at 405 nm in ELISA reader (Digital Analog Systems, DAS RS 232, Rome, Italy). Each assay was carried out in duplicate.

DNA Extraction

Total milk DNA was obtained with phenol-

chloroform-isoamyl alcohol extraction method as described by Leal-Klevazas et al.[25]. Briefly, 400 µl of lysis solution (2% Triton X-100, 1% sodium dodecyl sulphate, 100 mM NaCl, 10 mM Tris-HCl [pH 8.0]) and 10 µl of proteinase K (10 mg/ml) were added to 400 µl of samples taken from the cream layer of each milk. Following centrifugation, the pellet was rinsed with 1 ml of 70% ethanol, dried and resuspended in 20 µl of TE buffer (10 mM Tris-HCl [pH 8.0], 1mM disodium EDTA). Concentration and purity of DNA were measured by absorbance at 260 and 280 nm wavelengths using a UV spectrophotometer (Helios Gamma, Thermo Spectronic, Cambridge, UK) and stored at -20°C until use.

PCR Assays

Primer sequences of *ery*CD and *alk*B genes and PCR assay conditions were given in *Table 1.* PCR was carried out in a total volume of 50 µl, using 1xPCR buffer, 200 ng of purified genomic DNA, 20 pmol of each oligo-nucleotide primer, 2 mM $MgCl_2$, 200 mM of each dNTP and 2.5 U of Taq DNA polymerase (MBI Fermentas, Germany). To assess the detection limit of the PCR assay, ten-fold dilutions of *B. abortus* S19 vaccine strain in PBS ranging from 1.0 x 10^9 to 1.0 x 10^1 CFU ml^{-1} was inoculated into *Brucella* negative milk samples. Total DNA extraction and determination of its concentration were carried out and PCR assays were performed as described above. The reactions were performed with a DNA thermocycler (Biometra-Tpersonal, Göttingen, Germany). PCR products were separated by electrophoresis on 1.5% (w/v) agarose gel in 1xTBE buffer, pH 8.0 and visualized under ultraviolet light after staining with ethidium bromide. A Gene Ruler™ 100 bp DNA Ladder Plus (MBI Fermentas) was used as a DNA size marker.

Statistical Analysis

All statistical analyses were performed at 95% confidence interval (CI) by using Win Episcope version 2.0 programme. The cut-off point, sensitivity and specificity of ELISA were determined using receiver-operating characteristic (ROC) analysis. ELISA and PCR results were compared by test agreement analysis and evaluated on the basis of kappa (κ) value.

RESULTS

Detection of Anti-Brucella Antibody in Milk by ELISA

The cut off value of LPS-based ELISA was determined as 0.500 at OD405 by ROC analysis on the basis of the results obtained from 23 positive and 45 negative pre-evaluated milk whey samples described in details in material and methods. The sensitivity and specificity of the test at 95% confidence interval was found 87% and 100%, respectively. Positive and negative predictive values (PPV and NPV) were detected as 100% and 93.75%, respectively. Area under curve (AUC) being found 92.42% by ROC analysis demonstrates high diagnostic performance of ELISA, particularly for its specificity. When 70 cow milk whey samples were tested with ELISA, 15 samples (21.4%) were found positive and 55 samples (78.6%) were negative *(Table 2).*

PCR Analysis for Detecting Brucella eryCD and alkB Genes

PCR amplification of *ery*CD gene region was performed and only an amplification of 904 bp fragment was detected from 5 (7.1%) out of 70 milk samples *(Fig. 1).* These *ery*CD amplicon positive samples were further analyzed by *alk*B gene-targeted PCR to determine *Brucella* species and all of these samples were confirmed as *B. abortus* by amplification of a 136 bp DNA fragment *(Fig. 2).* All positive milk samples contained only wild-type *B. abortus* DNA but not *B. abortus* S19 vaccine strain DNA, giving only a 202 bp-amplicon *(Fig. 1 lane 3).*

Determination of Detection Limit of PCR

PCR detection limit was determined as 1.0 x 10^3 CFU ml^{-1} *Brucella* DNA from artificially contaminated negative milk samples by *ery*CD-targeted PCR.

Table 1. *PCR conditions and primers for eryCD and alkB genes*
Tablo 1. *eryCD ve alkB genleri için PCR koşulları ve primerler*

Cyling Parameters	*eryCD gene*		*alkB gene*	
	Temperature	**Duration**	**Temperature**	**Duration**
Initial denaturation	95°C	10 min	95°C	10 min
Denaturation	94°C	30 sec	94°C	15 sec
Annealing	60°C	30 sec	57°C	1 min
Extention	72°C	1 min and 40 sec	72°C	1 min and 40 sec
Final extention	72°C	10 min	72°C	10 min
	40 cycles		45 cycles	
Primer (F)	5′-GATCGCCATCGACTGCTGGG-3′		5′-GCGGCTTTTCTATCACGGTATTC-3′	
Primer (R)	5′-GGTCATCGGCATCGGCCATGGC -3′		5′-CATGCGCTATGATCTGGTTACG-3′	

Table 2. *Comparative analysis between ELISA and PCR results*
Tablo 2. *ELISA ve PCR sonuçlarının karşılaştırmalı analizi*

PCR Results	ELISA Results		Test Agreement
	Positive (n=15)	Negative (n=55)	
Positive (n=5)	1	4	κ= -0.008
Negative (n=65)	14	51	(-0.202/+0.186)

(*) Values in parenthesis indicate lower and upper kappa values

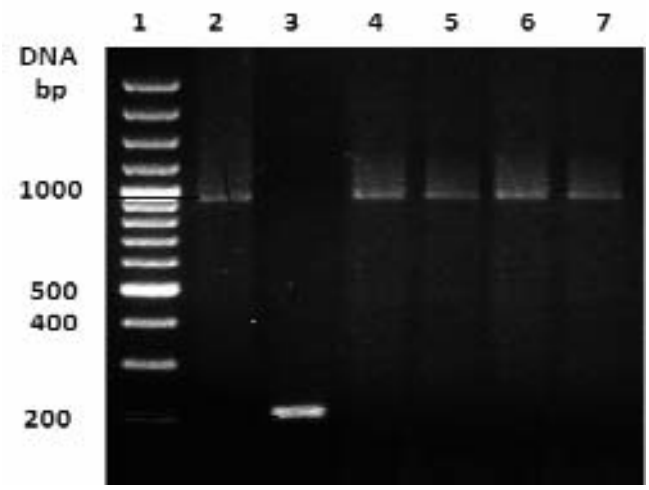

Fig 1. Gel electrophoresis of *Brucella ery*CD genes region amplicon from total DNA of cow milk. **Lane 1:** 100 bp DNA ladder; **Lane 2:** *B. abortus* field strain (904 bp); **Lane 3:** *B. abortus* S19 vaccine strain (202 bp); **Lane 4-7:** positive milk samples

Şekil 1. İnek sütü total DNA'sından çoğaltılan *Brucella ery*CD gen bölgesi jel elektroforezi. **Sütun 1:** 100 bp DNA belirteci; **Sütun 2:** *B. abortus* saha suşu (904 bp); **Sütun 3:** *B. abortus* S19 aşı suşu (202 bp); **Sütun 4-7:** pozitif süt örnekleri

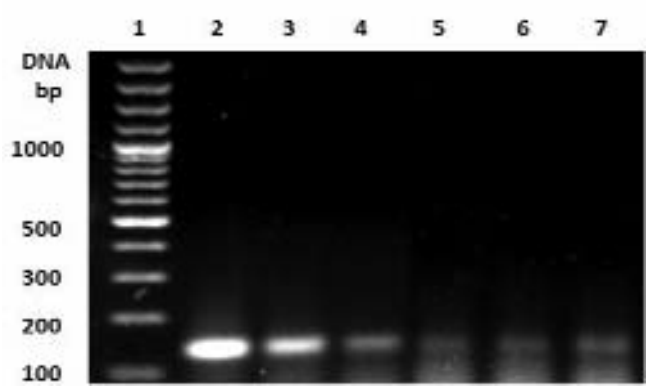

Fig 2. Gel electrophoresis of *B. abortus alk*B gene amplicon from total DNA of cow milk. **Lane 1:** 100 bp DNA ladder; **Lane 2:** *B. abortus* field strain (136 bp); **Lane 3-7:** positive milk samples

Şekil 2. İnek sütü total DNA'sından çoğaltılan *B. abortus alk*B geni jel elektroforezi. **Sütun 1:** 100 bp DNA belirteci; **Sütun 2:** *B. abortus* saha suşu (136 bp); **Sütun 3-7:** pozitif süt örnekleri

DISCUSSION

Serological tests such as MRT and ELISA are widely used for the detection of anti-*Brucella* antibody in milk [7,26,27]. However, MRT often causes wrong results [5,6] and its sensitivity and specificity have been found lower than ELISA methods [7,26,28]. Because of accuracy and less cross-reactions in determining *Brucella* antibody in milk and serum, LPS-based ELISA is preferentially used [4,27]. We adapted a previously described LPS-based ELISA [21] to milk antibody detection and determined its cut-off value as 0.500 at OD_{405} by ROC analysis in order to obtain a specificity of 100%. Although its sensitivity decreased to 87%, percentage of *Brucella* antibody positivity of the milk samples tested in this study was found 21% and considered as high for Samsun region, where vaccination against brucellosis is rarely applied.

Based on these results, PCR amplification targeting the genus and species-specific genes, *ery*CD and *alk*B, was performed to determine and confirm the presence of *Brucella* DNA in milk samples. *Brucella ery*CD gene-targeted PCR was carried out not only for detecting *Brucella spp.* DNA in milk samples but also for discriminating DNA of wild-type *B. abortus*, from S19 vaccine strain DNA as described in earlier studies [12,13,18]. In this study, amplification of a 904 bp fragment by *ery*CD gene-targeted PCR demonstrated the presence of the wild-type *Brucella* DNA in 5 milk samples. Detection of an amplicon of 136 bp by *alk*B gene-targeted PCR from the same samples allowed, however, identifying the presence of *B. abortus* DNA. Furthermore, no amplification of a 202 bp fragment by *ery*CD gene-targeted PCR confirmed only the presence of *B. abortus* wild-type strain in the same samples but not the S19 vaccine strain containing a deletion of 702 bp. From these results, it can be proposed that *ery*CD in follows *alk*B genes-targeted PCRs would be used as screening and differentiating molecular diagnostic tests for investigation of brucellosis status of the animal from milk.

When PCR results were compared with ELISA results, 51 milk samples were detected as negative with both tests *(Table 2).* While 15 samples were found positive in ELISA, only one sample of them was detected as positive by PCR. In the same manner, among 5 PCR positive samples only one sample was detected as positive in ELISA. As seen in *Table 2,* statistical analysis showed no agreement between both ELISA and PCR test results because the kappa value was significantly lower than 1. Our findings were similar to that of Romero et al.[12] reporting that 7 PCR negative samples were found positive in ELISA, and only one ELISA negative sample was detected as positive by PCR. This difference can be

due to i) long term persistence of *anti-Brucella* antibody without presence of the disease agent in milk or ii) relatively low detection limit of PCR. Because detection limit of eryCD gene-targeted PCR was determined as 1.0 x 10^3 CFU ml^{-1}, it is possible that some milk samples containing bacteria less than the detection limit failed to be found as positive. On the other hand, no detection of *Brucella* DNA by PCR in majority of the ELISA positive samples could be explained by having samples from animals in their chronic phase of the disease.

The consumption of contaminated milk and milk products is one of the main transmission ways of pathogenic *Brucella* strains to humans. For that reason, fast and accurate evaluation of brucellosis status of the milk and its products is paramount for public health. In conclusion, as significantly different results were obtained by the detection of anti-*Brucella* antibody and *Brucella* DNA in cow milk, the present study suggests that accurate evaluation of brucellosis status of cow milk and discrimination of *B. abortus* field strains from the vaccine strain S19 would be assured by simultaneous use of both ELISA and PCR assays.

REFERENCES

1. Young EJ: An overview of human Brucellosis. *Clin Infect Dis,* 21, 283-290, 1995.

2. Blood DC, Radostits OM: Diseases caused by *Brucella spp.* Veterinary medicine, 7th ed. Bailliere, Tindall, London, pp. 677-690, 1989.

3. Nielsen K: Brucellosis: Development and success using ELISAs for diagnosis. **In,** *Proceeding of the International Symposium on Diagnosis and Control of Livestok Diseases Using Nuclear and Related Tecniques,* Vienna, Austria, 7-11 April, 1997.

4. Neilsen K, Smith P, Gall D, Perez B, Cosma C, Muller P, Trottier J, Cote G, Boag L, Bosse J: Development and validation of an indirect enzyme immunoassay for detection of antibody to *Brucela abortus* in milk. *Vet Microbiol,* 52, 165-173, 1996.

5. Nielsen K: Diagnosis of brucellosis by serology. *Vet Microbiol,* 90, 447-459, 2002.

6. OIE: Bovine Brucellosis. **In,** Manual of standard for diagnostic test. List B disease OIE Terrestrial Manual, Chapter 2.4.3, 2009.

7. Nielsen KH, Kelly L, Gall D, Nicoletti P, Kelly W: Improved competitive enzyme immunoassay for the diagnosis of bovine Brucellosis. *Vet Immunol Immunopathol,* 46, 285-291, 1995.

8. Chand P, Rajpurohit BS, Malhotra AK, Poonia JS: Comparison of milk-ELISA and serum-ELISA for the diagnosis of *Brucella melitensis* infection in sheep. *Vet Microbiol,* 108, 305-311, 2005.

9. Hunter D, Allan J: An evaluation of milk and blood tests used to diagnose brucellosis. *Vet Rec,* 91, 310-312, 1972.

10. Rolfe DC, Sykes WE: Monitoring of dairy herds for *Brucella abortus* infection when prevalence is low. *Aust Vet J,* 64, 97-100, 1987.

11. Kerkhofs P, Botton Y, Thiange P, Dekeyser P, Limet JN: Diagnosis of bovine Brucellosis by Enzyme immunoassay of milk. *Vet Microbiol,* 24, 73-80, 1990.

12. Romero C, Pardo M, Grillo MJ, Diaz R, Blasco JM, Lopez-Goni I: Evaluation of PCR and indirect enzyme linked immunosorbent assay on milk samples for diagnosis of brucellosis in dairy cattle. *J Clin Microbiol,* 33, 3198-3200, 1995.

13. Rijpens NP, Jannes G, Asbroeck MV, Rossau R, Herman LMF: Direct detection of *Brucella spp.* in raw milk by PCR and reverse hybridization with 16S-23S rRNA spacer probes. *Appl Environ Microbiol,* 62, 1683-1688, 1996.

14. Hamdy MER, Amin AS: Detection of *Brucella* species in the milk of infected cattle, sheep, goats and camels by PCR. *Vet J,* 163, 299-305, 2002.

15. Romero C, Lopez-Goni I: Improved method for purification of bacterial DNA from bovine milk for detection of *Brucella spp.* by PCR. *Appl Environ Microbiol,* 65, 3735-3737, 1999.

16. Gupta VK, Verma DK, Rout PK, Singh SV, Vihan VS: Polymerase chain reaction (PCR) for detection of *Brucella melitensis* in goat milk. *Small Rumin Res,* 65, 79-84, 2006.

17. Sperry JF, Robertson DC: Inhibition of growth by erythritol catabolism in *Brucella abortus. J Bacteriol,* 124, 391-397, 1975.

18. Sangari FJ and Agüero J: Identification of *Brucella abortus* B19 vaccine strain by the detection of DNA polymorphism at the ery locus. *Vaccine,* 12, 337-342, 1994.

19. Sangari FJ, Jesus A, Garcia-Lobo JM: The genes for erythritol catabolism are organized as an inducible operon in *Brucella abortus. Microbiol,* 146, 487-495, 2000.

20. Probert WS, Schrader KN, Khuong NY, Bystrom SL, Graves MH: Real-time multiplex PCR assay for detection of *Brucella spp., B. abortus,* and *B. melitensis. J Clin Microbiol,* 42, 1290-1293, 2004.

21. Genç O, Büyüktanır Ö, Yurdusev N: Development of qualitative and quantitative ELISA models for bovine *Brucellosis* diagnosis. *Kafkas Univ Vet Fak Derg,* 16, 287-291, 2010.

22. Terzi G: Samsun bölgesinden toplanan sütlerde milk ring test ve aglütinasyon testi ile *Brucella* antikorunun araştırılması. *TAF Prev Med Bull,* 5, 196-203, 2006.

23. Lopez-Fandino R, Ramos M, Olano A: Rennet coagulation of milk subjected to high pressures. *J Agric Food Chem,* 45, 3233-3237, 1997.

24. Caroff M, Bundle DR, Perry MB, Cherwonogrodzky JW, Duncan JR: Antigenic S-type lipopolysaccharide of *Brucella abortus* 1119-3. Infect Immun, 46, 384-388, 1984.

25. Leal-Klevezas DS, Martinez-Vazquez IO, Lopez-Merino A, Martinez-Soriano JP: Single-step PCR for detection of *Brucella spp.* from blood and milk of infected animals. *J Clin Microbiol,* 33, 3087-3090, 1995.

26. Sutherland SS, Evans RJ, Bathgate J: Application of an enzyme linked immunosorbent assay in the final stages of a bovine Brucellosis eradication program. *Aust Vet J,* 63, 412-415, 1986.

27. Vanzini VR, Aguirre N, LugaresiI CI, De Echaide ST, De Canavesio VG, Guglielmone AA, Marchesino MD, Nielsen K: Evaluation of an indirect ELISA for the diagnosis of bovine brucellosis in milk and serum samples in dairy cattle in Argentina. *Prev Vet Med,* 36, 211-217, 1998.

28. Vanzini VR, Aguirre NP, Valentini BS, Torioni de Echaide S, Lugaresi CI, Marchesino MD, Nielsen K: Comparison of an indirect ELISA with the *Brucella* milk ring test for detection of antibodies to *Brucella abortus* in bulk milk samples. *Vet Microbiol,* 82, 55-60, 2001.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S53-S58, 2010
DOI:10.9775/kvfd.2009.1342

RESEARCH ARTICLE

The Examination of the Microbiologic Quality in Örgü Cheese (Braided Cheese) Samples

Aydın VURAL * Mehmet Emin ERKAN * Hüsnü Şahan GÜRAN *

* Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, University of Dicle, TR-21280, Diyarbakır - TURKEY

Makale Kodu (Article Code): KVFD-2009-1342

Summary

In this study, totally 105 Örgü Cheese samples were analyzed in terms of total mesophilic aerob bacteria, coliform bacteria, *Escherichia coli, Escherichia coli 0157, Stapyhlococcus-Micrococcus spp., Lactobacillus spp., Lactic streptococcus,* mould, yeast, *Salmonella spp.* and *Listeria spp.* The contamination rate of coliform bacteria, *E. coli, E. coli 0157, Staphylococcus-micrococcus spp.,* mould, yeast, and *Listeria spp.* were found 80.00%, 65.71%, 7.62%, 84.76%, 32.38%, 93.33%, and 2.86%, respectively. *Salmonella spp.* could not be determined in any of samples. The resistance to antibiotics of *E. coli* and *E. coli 0157* were also examined. It was concluded that Örgü Cheese may be contaminated with some microorganisms during production or selling. These contaminations also may cause important public health risks.

Keywords: *Örgü cheese, Microbiological properties, Quality, Antibiotic resistance*

Örgü Peyniri Örneklerinde Mikrobiyolojik Kalitenin İncelenmesi

Özet

Bu çalışmada toplam 105 adet örgü peyniri örneği toplam mezofilik aerob bakteri, koliform bakteri, *Escherichia coli, Escherichia coli 0157, Staphylococcus-Micrococcus spp., Lactobacillus spp.,* laktik streptokoklar, küf, maya, *Salmonella spp.* ve *Listeria spp.* mikroorganizmaları yönünden analiz edilmiştir. Analiz edilen örgü peynirlerinde koliform bakteri, *Escherichia coli, Escherichia coli 0157, Staphylococcus-Micrococcus spp.,* küf, maya ve *Listeria spp.* kontaminasyonu oranı sırasıyla % 80.00, % 65.71, % 7.62, % 84.76, % 32.38, % 93.33 ve % 2.86 düzeyinde bulunmuştur. Örneklerin hiçbirinde *Salmonella spp.* tespit edilememiştir. *E. coli* ve *E. coli 0157* 'nin antibiyotiklere olan dirençleri ayrıca incelenmiştir. Örgü peynirlerinin üretim ve satış sırasında bazı mikroorganizmalar ile kontamine olabildiği sonucuna varılmıştır. Bu kontaminasyonlar da önemli halk sağlığı risklerine neden olabilir.

Anahtar sözcükler: *Örgü peyniri, Mikrobiyolojik özellikler, Kalite, Antibiyotik direnci*

INTRODUCTION

In Turkey, there are more than 130 types of cheese and its derivatives which differ in terms of regions and their production techniques. Örgü Cheese (Braided Cheese) is a special type of cheese that is specific to Southeastern Anatolia Region; it is produced using conventional methods primarily in Diyarbakir and in large parts of other cities such as Mardin, Siirt, Sanliurfa and Van provinces. Örgü Cheese constitutes the 60-65% of the cheese consumption in the region [1]. However, in the recent years, Örgü Cheese has been produced in many production facilities located in Thrace, Eastern Anatolia, and Central Anatolia regions and is offered in the market all around Turkey [1-3].

Especially, ewe's milk is preferred in the production of Örgü Cheese. Also goat's and cow's milk are used where ewe's milk is not available or available at scarce amounts. In the production of the Örgü Cheese with

İletişim (Correspondence)
☎ +90 412 2488020
✉ avural@dicle.edu.tr

conventional methods, the curd, which is formed as a result of fermentation of milks at milking temperature, is drained and the obtained curd is left for fermentation. After the pH has fallen to approximately 5, scalding at 70-80°C in 3% saline water for 5-6 minutes is performed. Scalded and melted cheese is kneaded to give it an örgü shape. Örgü Cheese can either be consumed freshly or be offered for consumption after it has been ripened in brine solutions containing salt by 10-12% [1-4]. Örgü Cheese, which is shaped as a braid, rich in terms of fat, homogenous and having elastic consistency, is accepted to be similar to the White Cheese (Feta Cheese) in terms of its composition, similar to the Circassian Cheese in terms of its taste, and similar to the Kashar, Abkhasian, and Maras Cheeses in terms of its production technology including the scalding operation in its manufacturing process [1].

Örgü Cheeses that are offered for consumption in Diyarbakir province are generally produced by conventional methods in enterprises without modern technology and under relatively low hygienic conditions; their compositions and quality vary depending on the experiences and working conditions of the masters performing the production. Because this non-standard production style is excessive, products with different compositions and quality are offered for sale. In the research studies conducted on the composition of the Örgü Cheese, it has been reported that total dry matter is 42.70-54.64%, fat is 14.72-17.84%, protein is 15.83-21.69%, ash is 6.43-8.01%, salt is 5.45-6.03%, and titrable acidity (% lactic acid) is 0.34-0.80 [2-5].

In this study it is aimed at determining the microbiological characteristics of brined Örgü Cheeses that are offered for consumption in Diyarbakir and evaluating the probable public health risks. Antibiotic resistances of *E. coli* and *E. coli O157* microorganisms that are detected in the Örgü Cheese samples are also tested.

MATERIAL and METHODS

Collection of the Sample

In Diyarbakir province, 105 Örgü Cheese samples that had been sold without packaging were collected from several markets and delicatessens into sterile sample collection bags at 250 g amounts. The analyses were started immediately after the samples were brought to the laboratory under cold conservation conditions (4°C).

Microbiological Analyses

In the process of microbiologic analyses of Örgü Cheese samples, 10 g sample taken under aseptic conditions was homogenized using 90 ml of 0.1% peptone water. Preparing decimal dilutions, the appropriate medium was inoculated by standard analysis methods. For the count of total mesophilic aerobic bacteria (TMAB), Plate Count Agar was inoculated using pour plate method and reproduced colonies were counted after 48 h of incubation at 37°C [6]. Violet Red Bile Agar (Oxoid CM107) was used for the purposes of isolating coliform bacteria. Red violet colored colonies of 2-3 mm diameter were counted after 24 hours of incubation at 37°C which was realized after inoculation with double layer pour plate inoculation method [7]. TBX agar (Oxoid CM945) was used for the purposes of *E. coli* isolation. After 4 h of incubation at 30°C and 18 h of incubation at 44°C green colored colonies were evaluated. Serologic tests were applied to all *E. coli* strains using Oxoid Dryspot *E. coli 0157* (DRO120M) test kit [8]. In order to count *Staphylococcus-Micrococcus spp.,* 48 h of incubation at 37°C was applied in the egg yolk telluride emulsion added Baird Parker Agar Base (Oxoid CM275 + SR54) medium [7]. Man-Rogosa Sharpe Agar (Oxoid CM361) was used in the counting process of lactic acid bacteria and after 48 hours of incubation at 30°C, gray-white colonies of 0.5-2.5 mm diameter were evaluated [7]. M17 Agar (Oxoid CM785) was used in the counting process of lactic streptococci and they were evaluated after 48 h of incubation at 37°C [8]. Potato Dextrose Agar (Oxoid CM139) was used in the counting process of mould and yeast and the counts were realized after 5 days of incubation at 25°C which started after inoculation by spread plate method [6].

For the purposes of *Salmonella spp.* pre-enrichment, 25 g Örgü Cheese sample was incubated for 24 h at 35°C by adding buffered peptone water (Merck 1.07228). Taking 1 ml of pre-enrichment liquid, this amount was kept in Tetrathionate Broth (Oxoid CM0029) and Selenite Cystine Broth (Oxoid CM0699) at 35°C for 24 h and as selective media, XLD agar (Oxoid CM0469) and Salmonella Shigella Agar (Oxoid CM0099) were inoculated. Biochemical and serologic tests were applied to the typical colonies that reproduced in the selective agar media after 24 h at 35°C. In the isolation process of *Listeria spp.,* by adding 225 ml of Buffered Listeria Selective Enrichment Broth (Oxoid CM897) into 25 g cheese sample, the test material was incubated for 48 h at 30°C. Oxford Listeria Selective Supplement (Oxoid SR0140) added Listeria Selective Agar Base (Oxford) (Oxoid CM0856) medium was inoculated as a selective medium and after 48 h of incubation at 35°C black-haloed, small, brown-black, and swollen colonies were assessed to be under suspicion of being *Listeria spp.* Gram staining, motility, catalase, oxidase, urease, esculin, voges-proskauer, methyl red, sugar, hemolysis, and

CAMP tests were also applied to these colonies [8,9,10].

Antibiotic Sensitivity Tests

Antibiotic sensitivity tests were applied to *E. coli* and *E. coli O157* bacteria that were isolated from the Örgü Cheese samples. Sensitivity tests of these bacteria to Cefazolin, Ciprofloxacin, Clindamycin, Erythromycin, Gentamicin, Ofloxacin, Oxacillin, Rifampin, Tetracycline, Trimethoprim + Sulfamethoxazole, and Vancomycin were conducted using "BD Phoenix™ 100 Automatic Microbiology Identification System" in accordance with the instructions of the manufacturer firm (BD Diagnostic Instrument Systems, Sparks, MD, USA).

Phoenix ID/AST Panel

"BD Phoenix™ 100 Automatic Microbiology Identification System" is a device which is designed for rapid identification (ID) of the bacteria and antimicrobial sensitivity tests (AST). In this system, 100 identification and antimicrobial sensitivity tests can be conducted. The ID part of the system contains a series of conventional, cromatogenic, and florogenic biochemical materials to determine the bacteria identification. Phoenix AST method is a bouillon based micro-dilution test which is used for antimicrobial sensitivity measurement. In the ID part of the device, there are 45 shafts and 2 fluorescent control shafts which contain dried chemical substrates. In the AST part, the device has 84 shafts and reproduction control shafts which contain dried antimicrobial agents. The device operates by locating the prepared pure cultures in the device according to the manufacturer firm's instructions and it performs the evaluations in full automatic system.

RESULTS

In this study, 105 Örgü Cheese samples were analyzed in terms of presence and contamination levels of TMAB, coliform bacteria, *E. coli, E. coli O157, Staphylococcus-Micrococcus spp., Lactobacillus spp.,* lactic streptococci, mould, yeast, *Salmonella spp.,* and *Listeria spp.* For the Örgü Cheese, the microbial contamination rates were given in *Table 1,* the presence of microorganisms was given in *Table 2,* and the resistance of *E. coli* and *E. coli O157* strains to various antibiotics were given in *Table 3.*

Table 1. *Microbial Contamination Rates in the Örgü Cheese Samples (percent, %)*
Tablo 1. *Örgü peyniri örneklerinde mikrobiyel kontaminasyon oranları (yüzde, %)*

Microorganism	Sample Number (n)	Contamination Rate	
		Positive (n)	%
Coliform bacteria	105	84	80.00
E. coli	105	69	65.71
E. coli O157	105	8	7.62
Staphylococcus-Micrococcus spp.	105	89	84.76
Mould	105	34	32.38
Yeast	105	98	93.33
Salmonella spp.	105	Non detectable	0.00
Listeria spp.	105	3	2.86

Table 2. *Presence of microorganisms in the Örgü Cheese samples (cfu/g)*
Tablo 2. *Örgü peyniri örneklerindeki mikroorganizma varlığı*

Microorganism	Sample Number (n)	Contamination Rate (cfu/g)		
		Minimum	Maximum	Average
TMAB	105	$1.0x10^{6}$	$2.4x10^{9}$	$1.6x10^{8}$
Coliform bacteria	105	$2.0x10^{1}$	$7.8x10^{5}$	$3.6x10^{4}$
E. coli	105	$1.0x10^{1}$	$4.9x10^{4}$	$5.0x10^{3}$
Staphylococcus-Micrococcus spp.	105	$2.0x10^{2}$	$1.0x10^{7}$	$1.4x10^{5}$
Lactobacillus spp.	105	$1.0x10^{4}$	$9.2x10^{7}$	$3.4x10^{6}$
Lactic streptococci	105	$1.0x10^{4}$	$1.4x10^{8}$	$3.7x10^{6}$
Mould	105	$1.0x10^{2}$	$2.6x10^{4}$	$2.2x10^{3}$
Yeast	105	$1.0x10^{2}$	$3.7x10^{5}$	$3.1x10^{4}$

Table 3. *Some antibiotics resistance of detected E. coli and E. coli 0157 bacteria in the orgu cheese*
Tablo 3. *Örgü peyniri örneklerinde tespit edilen E. coli and E. coli 0157 bakterilerinin bazı antibiyotiklere direnci*

Antibiotic	*E. coli* (n=69)						*E. coli* 0157 (n=8)					
	S		R		I		S		R		I	
	n	%	n	%	n	%	n	%	n	%	n	%
Amikacin	69	100.00	-	-	-	-	8	100.00	-	-	-	-
Amoxicillin-Clavulanate	69	100.00	-	-	-	-	8	100.00	-	-	-	-
Ampicillin	53	76.81	16	23.19	-	-	7	87.50	1	12.50	-	-
Cefazolin	69	100.00	-	-	-	-	8	100.00	-	-	-	-
Cefepime	59	85.51	-	-	10	14.49	6	75.00	-	-	2	25.00
Cefoperazone-Sulbactam	62	89.86	-	-	7	10.14	7	87.50	-	-	1	12.50
Cefotaxime	69	100.00	-	-	-	-	8	100.00	-	-	-	-
Cefoxitin	69	100.00	-	-	-	-	8	100.00	-	-	-	-
Ceftazidime	69	100.00	-	-	-	-	8	100.00	-	-	-	-
Ciprofloxacin	69	100.00	-	-	-	-	8	100.00	-	-	-	-
Gentamicin	69	100.00	-	-	-	-	8	100.00	-	-	-	-
İmipenem	69	100.00	-	-	-	-	8	100.00	-	-	-	-
Levofloxacin	69	100.00	-	-	-	-	8	100.00	-	-	-	-
Meropenem	69	100.00	-	-	-	-	8	100.00	-	-	-	-
Piperacillin	55	79.71	-	-	14	20.29	8	100.00	-	-	-	-
Piperacillin-Tazobactam	69	100.00	-	-	-	-	8	100.00	-	-	-	-
Trimethoprim-Sulfamethoxazole	49	71.01	20	28.99	-	-	6	75.00	2	25.00	-	-

S: Sensitive; R: Resistance; I: Intermediate

DISCUSSION

With this study, samples of Örgü Cheese, which were offered for consumption in Diyarbakir, were analyzed in terms of the presence of various microorganisms and their counts. Akyuz et al.[5], Ozdemir et al.[2], Aksu et al.[3], and Turkoglu et al.[4] have detected the count of TMAB in Örgü Cheeses as $1.6x10^6$ cfu/g, $1.0x10^7$ cfu/g, $3.9x10^5$ cfu/g, and 6.89 log cfu/g respectively. In our study the count of TMAB was detected to be $1.6x10^8$ cfu/g, which is higher than the results obtained by other researchers in their studies on the Örgü Cheese.

In this research, coliform bacteria contamination was detected to be 80% and the average count of coliform bacteria was $3.6x10^4$ cfu/g. In their study, Aksu et al.[3] have reported the count of coliform bacteria to be $3.2x10^2$ cfu/g and the contamination level to be 78%. In other research studies conducted on Örgü Cheeses, the count of coliform bacteria was reported to be $9.5x10^1$ cfu/g by Akyuz et al.[5], $3.7x10^2$ cfu/g by Ozdemir et al.[2], and 3.73 log cfu/g by Turkoglu et al.[4]. In this study of ours, *E. coli* and *E. coli O157* contamination were detected to be 65.71% and 7.62% respectively, where the count of *E. coli* was found to be $5.0x10^3$ cfu/g. Aksu et al.[3] reported *E. coli* contamination to be 34% and the count of *E. coli* to be 4.3x10 cfu/g. In several types of cheeses *E. coli O157:H7* level was detected to be 1% by Akkaya et al.[11] and Aslantas and Yildiz [12], 2% by Aksu et al.[13], 3.2% by D'Aubert et al.[14] and 3.33% by Gonul [15]. The results that we have found are also higher than these values.

E. coli and *E. coli O157* strains that were detected in this study were analyzed in terms of their resistance to various types of antibiotics. All *E. coli* strains isolated from the Örgü Cheese samples were determined to be sensitive to amikacin, amoxicillin-clavulanate, cefazolin, cefotaxime, cefoxitin, ceftazidime, ciprofloxacin, gentamicin, imipenem, levofloxacin, meropenem, and piperacillin-tazobactam. However, in addition to these antibiotics, *E. coli O157* strains were determined to be sensitive to piperacillin. In terms of resistance, 23.19% of *E. coli* and 12.50% of *E. coli O157* strains were detected to be resistant to ampicillin, and, their resistance to trimethoprim-sulfamethoxazole was detected to be 28.99% and 25.00% respectively. Strains of *E. coli* were detected to be semi-resistant to cefepime by 14.49%, to cefoperazone-sulbactam by 10.14%, and to piperacillin by 20.29%; however strains of *E. coli O157* were detected to be semi-resistant to cefepime by 25.00%, and to cefoperazone-sulbactam by 12.50%.

In this research, average counts of TMAB, coliform bacteria and *E. coli* in the analyzed Örgü Cheeses were found to be higher than the similar studies. It was considered to be originated from bad raw material, production conditions which were neither modern nor hygienic, unsuitable conditions storage, low salt concentration, non-hygienic equipment, and

contaminations induced by the environment and personnel.

Several researchers report *Staphylococcus aureus* borne intoxications arising from cheese consumption [16]. The Örgü Cheeses analyzed in our study were also analyzed in terms of *Staphylococcus-Micrococcus spp.* to give an idea about their hygienic quality. In this research, the contamination level of *Staphylococcus-Micrococcus spp.* in the Örgü Cheeses was found to be 84.76% and their count was found to be $1.4x10^5$ cfu/g. These results were higher than both the result of Ozdemir et al.[2] which was $2.2x10^1$ cfu/g and the result of Aksu et al.[3] which was $1.0x10^3$ cfu/g. The fact that the *Staphylococcus-Micrococcus spp.* count was high is assessed as the exposure of the Örgü Cheese to environmental and personnel related contaminations in the production and sale stages.

Lactic acid bacteria constitute the dominant flora of the cheese [17]. Average *Lactobacillus spp.* and lactic streptococci counts in the Örgü Cheeses offered in Diyarbakir for consumption were detected to be $3.4x10^6$ cfu/g and $3.7x10^6$ cfu/g, respectively. However Turkoglu et al.[4] and Ozdemir et al.[2] were detected the average count of lactic bacteria to be 6.78 log cfu/g and $1.0x10^7$ cfu/g, respectively. The results we have found are lower than those of these researchers.

Since moulds can develop in a very wide range of pH, water activity and temperatures, they develop in cheese easily and cause appearance, odor, and taste deformations. Hence, they are analyzed by many researchers in various cheeses [18,19]. On the other hand, yeasts lower the quality of the cheese by forming pores and they cause the deformation which is called early swelling. Therefore they are not desired to be present in the cheeses [20]. High mould and yeast counts are reported to be caused by not following the hygienic rules in the period from the production to the marketing of the cheese [21]. In this research, mould and yeast contamination in the Örgü Cheese samples were detected to be 32.38% and 93.33%, respectively. In their studies, Akyuz et al.[5], Ozdemir et al.[2], Aksu et al.[3], and Turkoglu et al.[4] reported the count of mould-yeast in the Örgü Cheeses as $1.7x10^5$ cfu/g, $1.0x10^5$ cfu/g, $4.9x10^4$ cfu/g, and 5.45 log cfu/g, respectively. In our study, the average mould count was detected to be $2.2x10^3$ cfu/g and the average yeast count was detected to be $3.1x10^4$ cfu/g. These results are lower than those results of other researchers. In the studies regarding mould and yeast counts, different results have been reported in terms of *Lactobacillus spp.* It is possible that this situation is originated from the production techniques, ripening period, and differences in conservation and sale conditions.

In this research, the counts of TMAB, coliform, *E. coli,* mould, yeast, and presence of *Listeria spp.* in the analyzed Örgü Cheeses are higher than the maximum allowable values provided in the Turkish Food Codex Microbiological Criteria Notification [22].

The number of research studies conducted on *Listeria spp.* and *Salmonella spp.* contaminations in Örgü Cheeses was limited. It has been reported that 29 of 60 mass intoxication events observed in the Europe after 1980 as a result of milk and dairy products consumption were originated from *Salmonella spp.* and 10 of these events were originated from *Listeria* [23]. *Listeria monocytogenes* was deemed responsible for 25 of 55 mass intoxication events in the United States of America and an intoxication case that caused the death of 48 people as a result of cheese consumption in Mexico [24,25]. In this research, although *Listeria spp.* contamination was detected to be 2.86%, *Salmonella spp.* were not detected in any of the samples.

Which is one of basic food items as cheese and raw milk is a good environment for the development of various microorganisms [26]. With this research determined that the Örgü Cheeses offered to the market for consumption in Diyarbakir were low quality and contaminated with pathogen. These contaminations also may cause important public health risks. We concluded that standardization of the Örgü Cheese production, the use of high quality raw materials, production in modern enterprises and hygienic conditions, conservation in cold, prevention of without package sales, good and hygienic sale applications, and vacuumed packaging or packaging under modified atmosphere will be effective in prevention of the probable dangers in terms of public health.

REFERENCES

1. Kamber U: Geleneksel Anadolu Peynirleri. Miki Matbaacılık, Ankara, 2005.

2. Özdemir S, Çelik Ş, Özdemir C, Sert S: Diyarbakırın Karacadağ yöresinde mahalli olarak yapılan örgü peynirinin mikrobiyolojik ve kimyasal özellikleri. *V. Süt ve Süt Ürünleri Sempozyumu*-Geleneksel Süt Ürünleri. Milli Prodüktivite Merkezi Yayınları, s. 154-166, Ankara, 1998.

3. Aksu H, Çolak H, Vural A, Erkan ME: Diyarbakır Bölgesinde üretilen örgü peynirlerde mikrobiyolojik ve kimyasal özellikler üzerine bir araştırma. *YYÜ Vet Fak Derg,* 10 (1-2): 8-11, 1999.

4. Türkoğlu H, Ceylan ZG, Dayısoylu KS: The microbiological and chemical quality of orgu cheese produced in Turkey. *Pakistan J Nutr,* 2 (2): 92-94, 2003.

5. Akyüz N, Tutşi MF, Mengel Z, Ocak E, Altun İ: Örgü peynirinin üretim tekniği, bazı mikrobiyolojik ve kimyasal özellikleri. *V. Süt ve Süt Ürünleri Sempozyumu,* Geleneksel

Süt Ürünleri. Milli Prodüktivite Merkezi Yayınları, 328-337, Ankara, 1998.

6. Anonymous: Bacteriological Analytical Manual. AOAC Int, Gaithersburg, 2001.

7. Harrigan WF: Laboratory Methods in Food Microbiology. Academic Press, New York, 1998.

8. Anonymous: The Oxoid Manual. 9th ed. Published by Oxoid Limited, Hampshire, 2006.

9. Mc Lauchlin J: Listeria monocytogenes, recent advances in the taxonomy and epidemiology of Listeriosis in humans. *J App Bacteriol,* 63, 1-11, 1987.

10. Schmidt-Wolf G, Seeliger HPR, Schretten-Brunner A: Menschliche Listeirose. Erkrankuugen in der Bundesrepublik Deutschland, 1969-1985. *Zentralbl Bakteriol Mikrobiol Hyg,* 472-486, 1987.

11. Akkaya L, Alişarlı M, Kara R, Telli R: Afyonkarahisar'da tüketime sunulan çiğ süt ve peynirlerde *E. coli O157:H7* varlığının belirlenmesi. *YYÜ Vet Fak Derg,* 18 (1): 1-5, 2007.

12. Aslantaş Ö, Yıldız P: Kars yöresinde hayvansal kaynaklı gıdalarda *Escherichia coli O157:H7* izolasyonu. *Vet Bil Derg,* 18 (1-2): 107-111, 2002.

13. Aksu H, Arun ÖÖ, Aydın A, Ugur M: *Escherichia coli O157:H7* 'nin hayvansal kökenli çeşitli gıda maddelerinde varlığı. *Pendik Vet Mikrobiyol Derg,* 30 (2): 77-81, 1999.

14. D'Aubert S, Cantoni C, Sarti A: Detection of *Escherichia coli O157:H7* in foods of animal origin. *Arch Vet Ital,* 46 (3): 67-72, 1995.

15. Gönül ŞA: Çiğ süt ve peynir örneklerinde Enterohemorajik *E. coli*'ye (O157:H7) rastlanma sıklığı. *Kükem Derg,* 20 (2): 69-73, 1997.

16. Şimşek O, Arıcı M: Peynirlerde bulunabilen patojen mikroorganizmalar ve bunlardan kayanaklanan hastalıklar. *II. Milli Süt ve Ürünleri Sempozyumu-Geleneksel Süt Ürünleri.* Milli Prodüktivite Merkezi Yayınları, s.154-166, Ankara, 1991.

17. Kurt A: Süt Teknolojisi. Atatürk Ünv Ziraat Fak Yay, No: 573, Erzurum, 1996.

18. Kılıç S, Gönç S: İzmir tulum peynirinin mikrobiyolojik özellikleri üzerine araştırmalar. *Ege Üniv Ziraat Fak Derg,* 27 (3): 169-185, 1990.

19. Kurt A, Çağlar A, Çakmakçı S: Erzincan tulum peynirinin mikrobiyolojik özellikleri. *Turk J Vet Anim Sci,* 16, 41-50, 1991.

20. Dığrak, M, Yılmaz Ö, Çelik S, Özçelik S: Elazığ'da satışa sunulan taze beyaz peynirlerin mikrobiyolojik kalitesi ve yağ asitleri analizi. *Turk J Biol,* 20, 221-230, 1996.

21. Uğur A: Muğla halk pazarında satışa sunulan ev yapımı peynirlerin mikrobiyolojik özellikleri. *Ekoloji,* 10 (40): 3-8, 2001.

22. Anonymous: Türk Gıda Kodeksi (TGK) Mikrobiyolojik Kriterler Tebliği. 06.02.2009 tarih ve 27133 sayılı Resmi Gazete. Tebliğ No: 2009/6, Ankara, 2001.

23.De Buyser ML, Dufour B, Maire M, Lafarge V: Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. *Int J Food Microbiol,* 67 (1-2): 1-17, 2001.

24. Linnan MJ, Mascola L, Lou XD, Goulet V, May S, Salminen C, Hird DW, Yonekura ML, Hayes P, Weaver R, Audurier A, Plikaytis BD, Fanin SL, Kleks A, Broome CV: Epidemic listeriosis associated with Mexican-style cheese. *N Engl J Med,* 319, 823-828, 1988.

25. Anonymous: Preliminary FoodNet data on the incidence of foodborne illness selected sites. United States-1999, *MMWR,* 49 (10): 201-205, 2000.

26. Baz E, Gülmez M, Güven A, Sezer Ç, Duman B: Kars ilinde satışa sunulan çiğ süt ve taze beyaz peynirlerin Koliform grubu bakteri, *E. coli ve E. coli O157:H7* yönünden incelenmesi. *Kafkas Univ Vet Fak Derg,* 9 (2): 165-167, 2003.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S59-S63, 2010
DOI:10.9775/kvfd.2009.1376

RESEARCH ARTICLE

Kefal Balığı Sucuklarında Duyusal ve Besin Kompozisyonun Belirlenmesi

Nermin BERİK * Dilek KAHRAMAN *

* Çanakkale Onsekiz Mart Üniversitesi, Su Ürünleri Fakültesi, Avlama ve İşleme Teknolojisi Anabilim Dalı, TR-17100 Çanakkale - TÜRKİYE

Makale Kodu (Article Code): KVFD-2009-1376

Özet

Bu çalışma kefal balığı *(Mugil cephalus)*'ndan sucuk üretmek amacıyla yapılmıştır. Kefal balıkları taze olarak balık halinden alınmıştır. Farklı iki grup sucuk formülasyonu kullanılmıştır. Birinci sucuk grubu; %100 balık etinden, ikinci sucuk grubu %75 balık eti ile %25 dana etinden oluşturulmuştur. Her iki grupta da olgunlaştırılan sucuklar, derin yağda kızartma tekniği uygulanarak pişirilmiştir. Çiğ ve pişmiş sucukların duyusal ve besin kompozisyonundaki değişimler incelenmiştir. Duyusal analiz, pH değerleri ve olgun sucukların besin değerlerinde, iki sucuk grubu arasında farklılık tespit edilmemiştir (P>0.05). Kızartılmış sucukların protein ve yağ miktarı; %100 balık eti içeren sucuklarda, dana eti karıştırılanlara oranla daha yüksek bulunmuştur (P<0.05). Sonuç olarak kefal balığından tüketicinin alışık olduğu lezzette sucuk üretilebilmektedir. Kızartma uygulamasının besin kompozisyonundaki etkisi, nitelik bakımından kabul edilebilir bulunmuştur. Böylece bilinen sucuk uygulamalarına balık etinin de karıştırılmasının uygun olduğu saptanmıştır. Balık sucuğu üretimi için çalışılması önerilmektedir.

Anahtar sözcükler: *Besin kompozisyonu, Balık sucuğu, Duyusal analiz, Protein, Yağ*

Determination of Sensory and Nutrient Composition at Mullet Fish Sausage

Summary

The aim of this study was produced sausage from mullet fish *(Mugil cephalus).* Mullet fish were bought freshly from fish market. Two different groups' sausages were formulated. Primary sausage group, 100% fish meat and the second group, 75% fish meat with 25% calf meat were made by mixing. Each group maturations sausages were cooked with deep fat-frying techniques. Freshly and cooked sausages was investigated of sensory changes and nutrient composition. Sensory analyses, pH values and mature sausages' nutrient composition of between two sausages groups were not differents P>0.05). The cook sausages were including of 100% fish meat of protein and fat values higher than calf meat sausages (P<0.05). As a result, will be produced sausage our traditional taste in accordance with mullet fish; often the preferred frying application was established that acceptable in the view of nutrient composition creates changes. In addition to, we are fair enough foresight that fish meat can to be in with calf meat sausage such as poultry applications of consumer habits.

Keywords: *Nutrient composition, Fish sausage, Sensory analysis, Protein, Lipid*

GİRİŞ

Günümüzde, hazır gıdaların tüketicinin damak tadına uygun ve ekonomik olmasının yanı sıra sağlıklı bir şekilde üretilmesi sağlanmaktadır. Sağlıklı beslenmek için protein gereksiniminin üçte birinin hayvansal kaynaklı olması gerekmektedir. Zengin protein, mineral madde, vitamin ve doymamış yağ içeriği su ürünlerinin, özelikle de balıkların insan beslenmesindeki önemini artırmaktadır [1]. Gıdaların hayvansal kaynaklı olması sağlıklı beslenme gerekliliğini daha önemli hale getirmiş ve hem halkın damak tadına uygun hem de ekonomik ürünler oluştu-

İletişim (Correspondence)
☎ +90 286 2180018/2057
✉ nberik@yahoo.com

rulma çabalarını arttırmıştır.

Su ürünleri özellikle denize kıyısı olan ülkelerde farklı şekillerde tüketilirken, Türkiye'de taze tüketim alışkanlığı, işlenmiş ürünlerden fazladır. İşlenmiş ürünlerin de çoğu yurt içinde tüketilmeyip, yurt dışına gönderilmektedir. Bunun nedeni tüketicinin taze ve işlenmemiş ürünün sağlıklı olduğuna inanması ve damak alışkanlığıdır. Buna bağlı olarak av sezonu dışında halk balık etinden yoksun kalmakta veya av yasakları delinerek türlerin korunması güçleşmektedir. İşlenmiş su ürünlerinin ülkemizdeki tüketimini arttırmak; hem her mevsim ve her bölgede su ürünü tüketilmesini, hem de balıkların üremesini ve gelecek nesillerin de su ürünlerinden yararlanmasını sağlayacaktır.

İşlenmiş su ürünlerinde iyi bir planlama ile güvenli, ekonomik ve tüketicinin beklentilerine uygun lezzette ürünler geliştirilmektedir. Özelikle tüketimi daha az olan balıklar farklı işleme teknikleriyle değerlendirilip, severek tüketilen ürünler oluşturulmaktadır. Bu ürünler içerisinde balık sucuğu [2,3] ve balık sosisi de [4-6] yer almaktadır.

Bu çalışmada, denizlerimizde yaygın dağılım göstermesine karşın tüketimi az olan kefal balıklarından, sucuk yapılabilirliği araştırılmıştır.

MATERYAL ve METOT

Çalışmada Balık Hali'nden taze olarak temin edilen toplam 5 kg ağırlığındaki kefal balıkları *(Mugil cephalus)* kullanılmıştır. Balıkların iç organları çıkartılıp temizlendikten sonra fileto haline getirilmişlerdir. Fileto halindeki etler kıyma makinesinden geçirilmişlerdir. Elde edilen kıyma teknolojiye uygun olarak çeşitli baharatlar ile karıştırılmış ve iki grup sucuk hamuru yapılmıştır. I. Grup sucuk hamuru; %100 balık etinden, II. Grup sucuk hamuru ise; %75 balık eti ile %25 dana etinden karışımından yapılmıştır *(Tablo 1)*. Sucuk hamurları mayalanmaları için 24 saat süreyle +4°C'de muhafaza edilmiştir.

Mayalanan sucuk hamurları, 36 kalibre yapay (kollajen) sucuk kılıflarına doldurulmuşlardır. Dolumu sonrası sucuklar; nem, sıcaklık ve hava akımı kontrollü şartlar altında altı gün boyunca olgunlaştırılmıştır *(Tablo 2)*. Olgulaşmış ürünler bir gün buzdolabında +4°C sıcaklıkta muhafaza edildikten sonra duyusal ve kimyasal analizleri yapılmıştır.

Duyusal Analizler

Duyusal analizler on panelist tarafından gerçekleştirilmiştir. Panelistler çiğ sucuklarda, kesit yüzey rengi ve görünüş, her iki grupta da pişmiş sucuklarda tat ve aroma, tekstür, genel beğeni açısından değerlendirme yapmışlardır. Her iki sucuk grubunun örnekleri, iki dakika süreyle kızartılarak panelistlere sunulmuştur. Panelistlerin değerlendirmelerinde ise; 1-3 (çok kötü - kabul edilemez), 4-5 (orta), 6-7 (iyi), 8-9 (çok iyi) puan aralığındaki hedonik skala kullanılmıştır [7].

Kimyasal Analizler

Kimyasal analizler üç paralelli olarak gerçekleştirilmiştir. Çiğ balık etinde, sucuk hamurunda, olgunlaşmış balık sucuğunda ve pişirilmiş (yağda kızartma) sucuk örneklerinde; pH [8], su [9], protein [10], yağ [11], ve kül [9] analizleri yapılmıştır. Karbohidrat ve enerji değerleri ise matematiksel yöntemlerle hesaplanmıştır [12].

İstatistik Analizler

Çalışma sonucunda elde edilen bulguların Microsoft Excel ve SPSS 16.0 paket programlarında istatistik hesapları yapılmıştır.

Tablo 1. *Sucuk formülasyonu*
Table 1. *Sausage formulation*

Sucuğun Bileşimi	Miktarı (%)
Et (Balık, Balık + Dana)	85.75
Yağ*	5.00
Tuz	1.75
Kırmızı biber	1.70
Zencefil	0.55
Yenibahar	0.25
Sarımsak	2.00
Toz şeker	0.52
Nişasta	0.90
Karabiber	0.50
Tarçın	0.55
Kimyon	0.50
Sodyum nitrit	0.01
Potasyum sorbat	0.02
Toplam	100.00

** Yağ bileşimi = ayçiçek yağı ½ + kuyruk yağı ½*

Tablo 2. *Kefal balığı sucuklarının olgunlaştırma ortam koşulları*
Table 2. *Mullet fish sausage of ripening environment conditions*

Zaman (Gün)	Rutubet (%)	Sıcaklık (°C)	Hava Akımı (m/sn)
1	90	22	1-2
2	87	20	1-2
3	84	19	1-2
4	81	18	1-2
5	78	17	1-2
6	75	15	1-2

BULGULAR

Çalışmada taze kefal balıklarından elde edilen I. ve II.

grup sucukların organoleptik analizleri sonucunda elde edilen puanların ortalama sonuçları *(Tablo 3)* incelendiğinde, iki ürün grubu arasında istatistiksel açıdan bir fark bulunmamıştır (P>0.05).

Çalışmada pH değeri balık etinde 5.75 ve kırmızı ette 5.74 bulunmuştur. Birinci ve ikinci grubun pH değerleri sırasıyla hamurlarında 5.70 ve 5.71, olgun sucuklarında 5.34 ve 5.36, pişmiş sucuklarında ise 5.30 ve 5.33 olarak tespit edilmiştir. Olgunlaşmış sucuk ve pişmiş sucuk örneklerindeki pH değerlerinin sucuk hamurlarına göre daha düşük olduğu tespit edilmiştir (P<0.05).

Besin değeri analizleri bulgularımıza göre; hammadde (balık ve kırmızı et) ve ürün (olgunlaşmış) ile tüketim (pişirme) aşamalarında sırasıyla su oranlarında azalma; protein, yağ, kül ve karbohidrat oranlarında ise artış saptanmıştır *(Tablo 4). B*alık ve kırmızı etin besin değerleri incelendiğinde istatistiksel açıdan tümü farklı bulunmuştur (P<0.05). Her iki sucuk grubunda örneklerin su değerleri arasındaki farklılıklar (sucuk hamuru, olgunlaşmış sucuk ve pişirilmiş sucuk ürünleri arasında) önemli bulunmuştur (P<0.05). Su içeriği bakımından I. sucuk grubu ile II. sucuk grubunda, hamur ve olgunlaşmış çiğ ürünler farklılık göstermemiştir (P>0.05). Protein ve yağ değerlerinin, her iki sucuk grubunda da (sucuk hamuru, olgun sucuk ve pişirilmiş sucuk ürünleri arasında) anlamlı olduğu görülmektedir (P<0.05). Karbohidrat ve kül bulgularının, iki sucuk grubu arasında istatistiksel açıdan önemli (P<0.05); iki gruptaki sucuk hamuru, olgunlaşmış çiğ sucuk ve kızartılmış ürünler arasında ise önemsiz olduğu tespit edilmiştir (P>0.05).

Tablo 3. *Sucuk gruplarının duyusal analiz sonuçları*
Table 3. *The sensory result of groups of sausages*

Duyusal Özellikler (Çiğ Sucuk)	**I-B Grubu**	**II-B Grubu**
Kesit Yüzey Rengi	8.5±0.3 ^{a}	8.3±0.3 ^{a}
Kesit Yüzey Görünüşü	8.8±0.2 ^{a}	8.1±0.2 ^{a}
Genel Beğeni	8.7±0.2 ^{a}	8.2±0.3 ^{a}
Duyusal Özellikler (Pişmiş Sucuk)	**I-C Grubu**	**II-C Grubu**
Tat ve Aroma	8.4±0.2 ^{a}	7.8±0.2 ^{a}
Tekstür	8.2±0.2 ^{a}	7.8±0.2 ^{a}
Genel Beğeni	8.3±0.1 ^{a}	7.8±0.1 ^{a}

** Ortalama ±standart hata n = 6, **B:** Olgunlaşmış Çiğ Sucuk, **C:** Pişmiş (yağda kızartılmış) Sucuk, 1-3 (çok kötü - kabul edilemez), 4-5 (orta), 6-7 (iyi), 8-9 (çok iyi) (7)*
Aynı satırda aynı yüzde ürün değerleri arasında farklı üstel harflerle ifade edilen değerler arasındaki farklar istatistiksel açıdan önemlidir (P<0.05)

TARTIŞMA ve SONUÇ

Duyusal analiz bulgularına *(Tablo 3)* göre; I. grubun kesit yüzey rengi ve yüzey görünüşü II. gruptan yüksek değerde bulunmasına karşın istatistiksel açıdan önemli bulunmamıştır (P>0.05). Tüketici tarafından çok bilinmemekle beraber, balık etinin sucuk olarak değerlendirildiği çalışmalar bulunmaktadır. Sazan [2] ve gümüş [3] balıklarından sucuk üretilerek duyusal ve besin kompozisyonu araştırılmıştır. Çalışmamızda kefal etinden elde edilen sucuğun genel beğeni puanları çok iyi olarak değerlen-

Tablo 4. *Sucuk gruplarının besin değeri analiz sonuçları*
Table 4. *Nutrient value result of groups of sausages*

Besin Değeri	**Balık Eti**	**Kırmızı Et**	**I. Grup (%100 Balık)**			**II. Grup (%75 Balık + %25 Dana)**		
			I-A Grubu	**I-B Grubu**	**I-C Grubu**	**II-A Grubu**	**II-B Grubu**	**II-C Grubu**
Su	77.45±0.01 $^{+}$	68.97±0.02 *	71.93±0.21 ^{aA}	50.35±0.15 ^{bA}	28.76±0.02 ^{cB}	70.30±0.01 ^{aA}	51.68±0.20 ^{bA}	33.91±0.08 ^{cA}
Protein	15.97±0.00 $^{+}$	14.10±0.01 *	15.18±0.01 ^{cA}	24.83±0.00 ^{bA}	33.70±0.04 ^{aA}	14.22±0.01 ^{cA}	22.39±0.14 ^{bA}	31.94±0.00 ^{aB}
Yağ	2.43±0.21 $^{+}$	10.82±0.10 *	6.72±0.11 ^{cB}	15.66±0.14 ^{bA}	24.34±0.08 ^{aA}	8.44±0.01 ^{cA}	16.79±0.19 ^{bA}	20.06±0.01 ^{aB}
Kül	1.11±0.08 $^{+}$	0.60±0.05 *	2.87±0.05 ^{cA}	3.64±0.02 ^{bA}	4.12±0.07 ^{aA}	2.36±0.02 ^{cA}	3.46±0.01 ^{bA}	5.03±0.10 ^{aA}
Karbohidrat	2.76±0.00 $^{+}$	4.51±0.02 *	3.30±0.27 ^{bA}	5.52±0.32 ^{aA}	9.08±0.09 ^{aA}	3.68±0.03 ^{cA}	5.68±0.27 ^{bA}	9.17±0.19 ^{aA}

** Ortalama±standart hata n=6, **A:** Sucuk hamuru, **B:** Olgunlaşmış sucuk, **C:** Kızartılmış sucuk*
Aynı satırda balık ve kırmızı et grupları arasında farklı küçük üstel işaretlerle ifade edilen değerler arasındaki farklar istatistiksel açıdan önemlidir (P<0.05)
Aynı satırda aynı ürün grup değerleri arasında farklı küçük üstel harflerle ifade edilen değerler arasındaki farklar istatistiksel açıdan önemlidir (P<0.05)
Aynı satırda farklı ürün grupları arasındaki farklı büyük üstel harflerle ifade edilen değerler arasındaki farklar istatistiksel açıdan önemlidir (P<0.05)

dirilmiştir. I.C sucuk grubunun tat, aroma ve tekstür değeri II.C sucuk grubundan yüksek bulunmasına karşın istatistiksel olarak önemli farklılık tespit edilememiştir (P>0.05). Literatürlere ve bulgularımıza göre sucuk grupları arasındaki duyusal farklılıklar; balık türlerine, kullanılan katkı maddelerine ve bunların etkinliğine, etteki doğal floraya ve olgunlaştırma koşullarındaki farklılıklara bağlı olarak değişmektedir.

Çalışmamızda pH miktarı I. ve II. sucuk grupları arasında istatiksel açıdan önemli farklılıklar saptanmamıştır (P>0.05). I.A grubunda ortalama 5.71 ve II.A grubunda ortalama 5.70 pH miktarı elde edilmiştir. Sazan [2] ve gümüş [3] balığı sucuk çalışmalarında ise pH değeri sazan sucuğunda 5.77, gümüş sucuğunda 6.12 olarak bildirilmiştir. I.B grubunda ortalama 5.36 ve II.B grubunda ortalama 5.34 pH değeri elde edilmiştir. Literatür bulgularına göre çalışmamızdaki pH sonuçları daha düşük olarak bulunmuştur. Laktik asit bakterileri pH'ı düşürerek bakteri ve patojenlerin gelişimini engelleyerek bozulmayı önlemektedir [13]. pH değerinin olgunlaşma süresince düşmesi ortamdaki laktobasil miktarının artışından kaynaklandığı düşünülmektedir.

Kefal balığının besin kompozisyonu ile ilgili çalışmalarda %74.53 su, %16.23 protein ve %1.16 kül [14]; %76 su, %19.5 protein ve %1.40 kül olarak bildirilmiştir [15]. Pasifik kefalinde cinsiyetlere göre filetolarında besin değerleri incelenmiş ve ortalama yağ değerleri dişi kefallerde %3.39, erkeklerde ise %3.78 olarak bildirilmiştir [16]. Çalışmamızda ise benzer olarak kefal filetolarında %77.45 su, %15.97 protein, %2.43 yağ, %1.11 kül ve %2.76 karbohidrat saptanmıştır. Kırmızı et karışımı ile yapılan sucuk çalışmasında [17] kırmızı ette ortalama %18.5 protein, %11.85 yağ ve 5.84 pH ölçülmüştür. Çalışmamızda ise II. grup sucukların yapımı sırasında kullanılan kırmızı ette bulunan %68.97 su, %14.10 protein, %10.82 yağ ve 5.74 pH ölçülmüştür. Bilinçli tüketici, sağlıklı yaşam için uygunluğu ve ekonomik olması gibi nedenlerle beyaz eti daha fazla tercih etmektedir. Balık eti ise dengeli olarak içerdiği esansiyel amino asitler ve yağ asitleri nedeniyle diğer seçeneklerin önüne geçmektedir [18,19].

Çalışmamızdaki I. ve II. sucuk gruplarının su miktarı incelendiğinde *(Tablo 4)* Farklı iki sucuk grubu arasında anlamlı bir farklılık tespit edilememiştir (P>0.05). Bununla birlikte su miktarının I.A grubuna göre II.A grubunda düşük bulunmasının nedeni kırmızı ete göre balık etinin daha fazla su içeriğine sahip olmasıyla açıklanabilir. Sazan balığında yapılan çalışmada su miktarı birinci gün ortalama %50.20 [2], gümüş balığında yapılan sucuk çalışmasında ise ortalama %53.02 olarak bildirilmiştir [3]. Çalışmamızda da benzer şekilde su miktarları I.B grubunda ortalama %50.35, II.B grubunda ise ortalama %51.68 olarak saptanmıştır. I.B ve I.C sucuk gruplarının su miktarı II.B ve II.C gruplarından daha düşük bulunmuştur (P>0.05). Bu durum II. sucuk gruplarında balık eti oranının azaltılmasıyla ilişkilendirilebilir. Saptanan su miktarlarının sucuk kalitesine olumsuz etkisi bulunmamaktadır.

İnsan ve hayvan beslenmesinde proteinin vazgeçilmez önemi vardır. Hayvansal proteinler yapıtaşı olarak da görev yaptıkları için düzenli olarak tüketilmeleri gerekmektedir. Balık eti protein miktarı *(Tablo 4)* I. ve II. sucuk grupları arasında istatistiksel açıdan farklık bulunmamıştır (P>0.05). I.A grubunda bulunan protein miktarı II.A grubundan yüksek çıkmış olup, bu durum kırmızı ete oranla balık etinin daha fazla protein içermesiyle açıklanabilir. Sazan balığından sucuk yapımı çalışmasında [2] olgunlaşmış sucukların protein miktarı ortalama %21.02; gümüş balığı sucuklarında [3] ise, ortalama %20.05 olarak bildirilmiştir. Çalışmamızda protein miktarı I.B sucuk grubunda ortalama %22.39 ve II.B grubunda ise ortalama %24.83 olarak tespit edilmiştir Protein miktarı literatür bulgularına göre daha yüksek bulunmuştur. Elde edilen yüksek değer çalışmamızda kullanılan balığın türü ve sucuk hamurunun formülasyonunda düşük miktarda yağ kullanılarak eksikliğin balık eti ile tamamlanmasıyla açıklanabilir.

Yağ miktarı bulgularımıza göre *(Tablo 4)* I. ve II. sucuk grupları arasında bir farklılık görülmemektedir (P>0.05). I.A sucuk grubunun yağ miktarı II.A sucuk grubundan düşük bulunmuştur. Bunun nedeni balığın kırmızı ete oranla daha düşük yağ içeriğine sahip olmasından kaynaklanmaktadır. Sazan balığı [2] sucuklarında yağ miktarı ortalama %27.82, gümüş balığı sucuklarında [3] ortalama %26.86 olarak bildirilmiştir. Çalışmamızda ise yağ miktarı I.B grubunda ortalama %16.79 ve II.B grubunda %15.66 olarak elde edilmiştir. Yağ miktarının diğer çalışmalara oranla düşük olmasının nedeni formülasyon oluşturulurken yağ miktarının daha düşük tutulmasından kaynaklanmaktadır. Kefal balığından sucuk üretirken damak tadına uygun olması kadar, doymuş yağ oranının düşük olmasına özen gösterilmiştir. Böylece sağlıklı beslenmeye uygun olan sucuk üretimi amaçlanmıştır. Bu nedenle çalışmamızda kullanılan yağın yarısı katı, sıvı olan diğer yarısı ise ayçiçek yağıdır. Bir başka çalışmada da ayçiçek yağı kullanılarak üretilen frankfurter tipi sosislerin kolesterol miktarı daha düşük bulunduğu bildirilmiştir [20].

Kül miktarı bulgularına göre *(Tablo 4)* I. ve II. sucuk grupları arasında istatistiksel açıdan farklılıklar bulunmamıştır (P>0.05). Bununla birlikte kül miktarının I.A ve I.B (%100 balık eti) gruplarında, II.A ve II.B (%75 balık eti) gruplarına göre yüksek bulunması balık etindeki mineral madde miktarının fazla olmasıyla ilişkilendirilebilir. Çalışmada I.B sucuk grubunda ortalama %3.46 ve II.B sucuk grubunda ise ortalama %3.64 kül tespit edilmiştir. Kızar-

tılan ürünlerde ise II.C (%75 balık eti) grubunun daha yüksek kül içermesi balık et dokusunun boşluklu et yapısı nedeniyle pişirme işlemi sırasında daha fazla su kaybetmesinden kaynaklanmaktadır. Balık sucukları için pişirme süresi daha kısa uygulanmalıdır.

Karbohidrat miktarı bulguları *(Tablo 4)* I. ve II. sucuk grupları arasında istatistiksel açıdan farklılıklar bulunmuştur (P<0.05). Çalışmamızda I. sucuk grubunun karbohidrat miktarı II. sucuk grubundan düşük olup, bunun nedeni balık etinin kırmızı ete kıyasla az da olsa düşük karbohidrat içermesidir.

Sonuç olarak, analiz bulguları incelendiğinde uygun teknolojiler ve katkı maddeleri ile kefal balığı etinden sucuk üretilebileceği saptanmıştır. Çalışmaların değerlendirilmeyen farklı balıklarla sürdürülmesi; hem ekonomik açıdan katkı sağlayacak hem de halkın besin gereksinimi önemli ölçüde giderebilecektir. Bölgelere göre ucuz ve nitelikli hammaddeler seçilerek bu çalışmaların çeşitlendirilmesi ve sürdürülmesi önerilmektedir.

KAYNAKLAR

1. Samsun N, Samsun O, Kalaycı F: Sinop bölgesinde (Karadeniz) avlanan Kalkan (*Scophthalmus maeoticus* Pallas, 1811) balığının et verimi ile protein ve yağ oranlarının mevsimsel değişimi. *Fırat Üniv Fen Müh Bil Der,* 17 (4): 629-635, 2005.

2. Arslan A, Dinçoğlu HA, Gönülalan Z: Fermented *Cyprinus carpio* L. sausage. *Turk J Vet Anim Sci,* 25, 667-673, 2001.

3. Arslan A, Dinçoğlu HA, Gönülalan Z: Gümüş balığından fermente sucuk üretimi üzerine deneysel çalışmalar. *Kafkas Univ Vet Fak Derg,* 7 (1): 47-54, 2001.

4. Raksakulthai N, Chantikul S, Chaiyawat M: Production and storage of chinese style fish sausage from hybrid clarias catfish. *Kasetsart J Nature Sci,* 38, 102-110, 2004.

5. Gülyavuz HG: Balık etlerinde sosis yapımı üzerinde bir araştırma. *Yüksek Lisans Tezi.* Akdeniz Üniv Fen Bil Enst, Isparta, 1991.

6. Dinçer MT: Alabalık filetosu kullanarak balık sosisi üretimi ve soğuk muhafaza (4±2°C) şartlarında kalite özelliklerinde meydana gelen değişimlerin incelenmesi. *Doktora Tezi.* Ege Üniv Fen Bil Enst, İzmir, 2008.

7. Gök V: Antioksidan kullanımının fermente sucukların bazı kalite özellikleri üzerine etkileri. *Doktora Tezi.* Ankara Üniv Fen Bil Enst, Ankara, 2006.

8. Landvogt A: Errors in pH measurement of meat and meat products by dilution effects. *Proceedings of the 37th International Congress of Meat Sci. and Techn. Kulmbach, Germany*, 1991.

9. Hortwitz W: Official methods of analysis of aoac international (Oma). Washington: Association of Official Analytical Chemists. 2000.

10. Nielsen SS: Food Analysis. New York: Kluwer Academic/ Plenum Publishers. pp. 134-135, 2003.

11. Folch J, Lees M, Sladane-Stanley GHA: Simple method for the isolation and purification of total lipids from animal tissue. *J Biol Chem,* 226 (1): 497-509, 1957.

12. Keskin H: Gıda Kimyası. İstanbul Üniv Yay, Sayı: 1980, Kimya Fak. No: 21, s. 1046, 1975.

13. Hu Y, Xia W, Ge C: Effect of mixed starter cultures fermentation on the characteristics of silver carp sausages. *World J Microbiol Biotechnol,* 23, 1021-1031, 2007.

14. Şengör GF, Akkuş S, Maliki RH: A research on chemical composition and cholesterol contents of various seafoods (in Turkish). *X. Ulusal Su Ürünleri Sempozyumu,* Adana, 137-150, 1999.

15. Gülyavuz H, Ünlüsayın M: Seafoods Processing Tecnology (in Turkish). Süleyman Demirel Üniv Eğirdir Su Ürünleri Fak, Isparta, s. 366, 1999.

16. Erdem ME, Kalaycı F, Samsun N: Sinop kıyılarında avlanan pasifik kefali (Mugil so-iuy, basilevsky, 1855) filetolarında besin içeriklerinin dağılımı. *Ege Univ J Fish Aquatic Sci,* 23 (1/3): 421-424, 2006.

17. Sarıçoban C: Sığır etine farklı oranlarda karıştırılan yumurta tavuğu etinin Türk tipi sucuk üretiminde kullanılabilme imkanları üzerine bir araştırma. *Yüksek Lisans Tezi.* Selçuk Üniv Fen Bil Enst, Konya, 2000.

18. Brown A: Understanding Food. Fish and Shellfish. Wadsworth/Thomson Learning, USA, pp.165-186, 2007.

19. Baysal A: Beslenme. Hatipoğlu Yayınevi, Ankara, 2002.

20. Ertaş AH, Karabaş G: Ayçiçek yağı ile frankfurter tipi sosis üretimi üzerinde arastırma. *Tr J Agricult Forest,* 22, 235-240, 1998.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S65-S72, 2010
DOI:10.9775/kvfd.2009.1470

RESEARCH ARTICLE

Sodyum Laktat İlavesinin Taze Gökkuşağı Alabalığından (*Oncorhynchus mykiss* W.) Yapılan Köftelere Etkisi

Gülsüm ÖKSÜZTEPE * Özlem EMİR ÇOBAN ** Hüsnü Şahan GÜRAN ***

* Fırat Üniversitesi Veteriner Fakültesi, Gıda Hijyeni ve Teknolojisi Bölümü, TR-23119 Elazığ - TÜRKİYE
** Fırat Üniversitesi Su Ürünleri Fakültesi, Avlama ve İşleme Teknolojisi, TR-23119 Elazığ - TÜRKİYE
*** Dicle Üniversitesi Veteriner Fakültesi, Gıda Hijyeni ve Teknolojisi Bölümü, TR-21100 Diyarbakır - TÜRKİYE

Makale Kodu (Article Code): KVFD-2009-1470

Özet

Bu araştırmada, %0.5 (A), %1 (B) ve %2 (C) oranında sodyum laktat ilave edilerek hazırlanmış taze gökkuşağı alabalığı (*Oncorhynchus mykiss* W.) köftelerinde hazırlama ve muhafaza sırasında meydana gelen mikrobiyolojik, kimyasal ve duyusal değişimler incelenmiştir. Toplam mezofilik aerob bakteri sayısı muhafaza süresine bağlı olarak sodyum laktat ilaveli gruplarda kontrol grubuna göre nispeten daha az artış göstermiştir (P<0.05). Koliform grubu bakteriler, *Staphylococcus - Micrococcus* ve maya ve küf sayısı tüm gruplarda muhafazanın ilk gününden itibaren artmaya başlamıştır. İstatistiksel olarak, C grubundaki *Staphylococcus - Micrococcus* ve maya ve küf sayılarının diğer gruplardan daha az olduğu görülmüştür (P<0.05). pH, kuru madde, kül ve tuz değerleri bakımından muhafaza sıcaklıklarına göre örnekler arasında fark tespit edilememiştir (P>0.05). TVB-N ve TBA değerleri tüm örneklerde muhafaza süresince giderek artmıştır. TVB-N ve TBA miktarları bakımından gruplar arasında önemli farklılıkların olduğu (P<0.05) belirlenmiştir. Balık köftelerinin muhafazanın başlangıcında duyusal açıdan daha fazla beğenildiği, ileri muhafaza günlerinde ise beğeni düzeyinin giderek azaldığı saptanmıştır (P<0.05). Sonuç olarak, ilave edilen sodyum laktat miktarı arttıkça ürünün dayanma süresinin arttığı ve köfte hamuruna %2 oranında sodyum laktat ilavesinin ürünün duyusal özelliklerinde istenmeyen bir değişime neden olmadığı aksine duyusal olarak belirlenen raf ömründe belirgin bir iyileşmeye neden olduğu görülmüştür. Yine, %2 oranında sodyum laktat ilave edilen örneklerin +4±1°C'de 16 gün boyunca yenilebilir niteliğini koruduğu gözlemlenmiştir.

Anahtar sözcükler: *Sodyum laktat, Alabalık, Köfte, Mikrobiyolojik kalite, Kimyasal kalite*

The Effect of Addition of Sodium Lactate in Fish Balls made from Fresh Rainbow Trout (*Oncorhynchus mykiss* W.)

Summary

In this study, microbiological, chemical and sensory changes of fish balls made from rainbow trout (*Oncorhynchus mykiss* W.) treated with addition of sodium lactate at different percentages [0.5% (group A), 1% (group B) and 2% (group C)] were analyzed during their storage. Total mesophilic aerobic bacteria count of fish balls was determined 4.11 $\log_{10}$ cfu/g on day 0. The count relatively decreased until day 2 and then constantly increased in all groups during the remaining storage. Statistical analyses showed that treatment with sodium lactate was effective on total mesophilic aerobic bacteria count of fish balls (P<0.05). The numbers of coliform, *Staphylococcus - Micrococcus* and yeast and mould increased in all groups as from beginning of the storage. Numbers of *Staphylococcus - Micrococcus* and yeast and mould in group C was found to be lower from other groups (P<0.05). It was not seen significant difference between groups in terms of pH, dry matter, ash and salt levels (P>0.05). TVB-N and TBA values gradually increased in all groups during the storage period. Significant differences was found between groups in point of TVB-N and TBA values (P<0.05). Although fish balls were found to be desirable from sensorial point of view at the beginning of the storage, their sensory attributes gradually decreased during the storage period (P<0.05). It was seen that addition of sodium lactate at 2% to fish balls increased the shelf life of the product while it did not have negative effect on sensory attributes of the product. In conclusion, it was observed that fish balls with addition of 2% sodium lactate were found to be acceptable up to 16 days at +4±1°C.

Keywords: *Sodıum lactate, Rainbow trout, Meat balls, Microbiology quality, Chemical quality*

İletişim (Correspondence)
☎ +90 424 2370000/3965
✉ gulsumoksuztepe@hotmail.com

GİRİŞ

Yaşamın devamı ve sağlıklı beslenmemiz için gerekli olan gıdaların hijyenik ve ekonomik olmasının yanı sıra, protein, yağ, karbonhidrat, vitaminler ve mineral maddeleri dengeli ve yeterli bir şekilde içermesi de arzu edilmektedir. Bu isteğe cevap veren en önemli gıda gruplarından biri de su ürünleri olup, bu gıda grubu içinde ön sırayı balık almaktadır [1]. Su ürünleri, özellikle balık ve balık etinden elde edilen ürünler günümüz şartlarında gerek sağlık yönünden gerekse çabuk bozulabilir özelliği nedeniyle diğer hayvansal kaynaklı gıdalardan daha önemli bir yere sahiptir. Balıklardan daha fazla yararlanabilmek için avlanmayı takiben kısa süre içinde tüketilmeli veya uygun koşullarda muhafaza edilerek tüketiciye en iyi kalitede ulaştırılması sağlanmalıdır [2,3]. Balık eti değişik şekillerde işlenerek soframıza girmektedir. Özellikle oteller ve lokantalar için işlenmiş balık ürünleri değişik aroma ve alternatifler sunduğu için bu gibi yerlerde aranan ürünler haline gelmiştir. Bu ürünlerden biri de balık köftesidir.

Balık köftesi, balık etinin temizlenip, haşlanıp kıyma haline getirildikten sonra baharat ilavesiyle elde edilen bir balık ürünüdür. Balık köftesinin soğukta (4±1°C) muhafazasıyla ilgili yapılan bir çalışmada [4], örneklerin 8. güne kadar kalite özelliğini koruduğu ancak 10 günlük muhafazadan sonra bozulduğu saptanmıştır. Konu ile ilgili yapılan diğer çalışmada ise [5] çiğ ve haşlanmış hamsi balığından yapılmış balık köftelerinin 4±1°C'de muhafazası sırasında 9 günlük raf ömrüne sahip olduğu belirlenmiştir.

Gıda maddelerinin mikrobiyolojik güvenliğini sağlamak ve raf ömrünü artırmak için organik asitler ve türevleri doğal alternatifler olarak gösterilmektedir [6,7]. Laktik asit ve tuzu olan laktatlar, ette ve bir çok fermente gıda da doğal olarak bulunmaları, yüksek etkime gücüne sahip olmaları, tüketiciler için sağlık riski oluşturmamaları, ürünün duyusal niteliklerini değiştirmemeleri nedeniyle ürünlerin mikrobiyolojik güvenirliliğini artırmak amacı ile katkı maddesi olarak önerilmektedir [6,8]. Organik asitler hücre içerisine alındıklarında dissosiye olurlar ve inhibisyona yol açacak biçimde hücre içi pH'sını düşürerek metabolizmayı olumsuz yönde etkilerler [9].

L (+) laktik asidin tuzu olan laktatlar ilave edildiği gıda maddesinin su aktivitesini (a_w) düşürerek ve spesifik etki göstererek prezervatif olarak etkili olmaktadırlar [7,8,10]. Laktatların antimikrobiyel ajan olarak et ve et ürünlerinde bir çok patojen ve bozulma yapıcı mikroorganizmaya karşı etkili olduğu bildirilmiştir [11-13]. Laktatların antimikrobiyel aktivitelerinin yanı sıra ürünlerin renk, lezzet ve tekstür gibi duyusal özelliklerini iyileştirdiği ve antioksidant olarak etkili olduğu da ileri sürülmüştür [13-15].

Bu araştırma, sodyum laktatın taze gökkuşağı alabalığı *(Oncorhynchus mykiss)* etinden yapılan balık köftelerinin mikrobiyolojik, kimyasal ve duyusal özellikleri üzerine etkisini belirlemek için yapılmıştır.

MATERYAL ve METOT

Materyal

Bu çalışmanın materyalini oluşturan taze gökkuşağı alabalıkları *(Oncorhynchus mykiss)* Devlet Su İşleri Dokuzuncu Bölge Müdürlüğünün Keban Tesislerinden temin edilmiştir. Yaklaşık olarak 3-4 kg ağırlığında olan balıkların önce derileri soyulmuş, daha sonra başları kesilerek iç organları temizlenmiş ve filetoları çıkarılarak kaynar suda bir-iki dakika haşlanmıştır. Et kılçıklardan temizlendikten sonra, ayna delik çapı 3 mm olan kıyma makinesinden geçirilerek kıyma haline getirilmiştir.

Köftelerin Formülasyonu ve Hazırlanması

1 kg balık eti (suda haşlanmış), 20 g tuz, 10 g zeytinyağı, 3 adet patates (haşlanmış - 54 g), 5 g kimyon, 5 g yedibahar, 5 g karabiber, 2 dilim ekmek (somun ekmeğinin içi - 18 g), 2 adet kuru soğan (rendelenmiş - 20 g), 1 demet maydanoz (43 g). Elde edilen kıyma baharat karışımına (karabiber, yenibahar, kimyon), tuz, zeytinyağı ve katkı maddeleri (haşlanmış patates, somun ekmeğinin içi, soğan, maydanoz, yumurta) ilave edilerek karıştırılmıştır. Karışımdan 20-25 g ağırlığında koparılıp elle şekil verilerek önce çırpılmış yumurtaya sonra galeta ununa bulandıktan sonra 5 cm eninde ve 10 cm boyunda olan strafor tabaklara yerleştirilerek streç film ile kaplanmıştır. Örnekler 4 gruba ayrılmıştır. Birinci grup kontrol grubu (Grup K) olarak belirlenmiştir. Aynı karışımdan hazırlanan köftelere %0.5 (Grup-A), %1 (Grup-B) ve %2 (Grup-C) oranında sodyum laktat ilave edilerek 3 köfte grubu daha hazırlanmıştır. Hazırlanan köfte örnekleri 4±1°C'de muhafazaya alınmıştır. Çalışma 3 tekerrürlü olarak yapılmıştır. Örnekler hazırlama aşamasında (fileto) ve muhafazanın belirli günlerinde (0, 2, 4, 6, 8, 10, 12, 14 ve 16. günler) mikrobiyolojik ve kimyasal yönden incelenmiştir. Ayrıca, belirtilen muhafaza günlerinde her grup örnekten birer paket alınarak aseptik şartlar altında açılan köfte örnekleri yağda kızartıldıktan sonra 5 kişilik panel grubu tarafından duyusal olarak analiz edilmiştir.

Metot

Mikrobiyolojik analizler için, köfte örnekleri bir parçalayıcının (Stomacher 400) özel torbasında 5 g tartılmış ve üzerine steril %0.1'lik peptonlu sudan 45 ml ilave edilerek parçalayıcıda homojen hale getirilmiştir. Böylece örneğin 10^{-1} (1/10)'lik dilüsyonu hazırlanmıştır. Bu dilüsyondan aynı seyrelticiyi kullanmak suretiyle örneğin 10^{-6}'ya

kadar diğer seyreltileri hazırlanmıştır. Örneklerin her seyreltisinden 1'er ml kullanılarak iki seri halinde plak dökme metoduyla ekimleri yapılarak inkübasyon süresi sonunda 30-300 koloni içeren plaklar değerlendirilmiştir [1,16].

Örneklerdeki toplam mezofilik aerob mikroorganizmaların sayımı için Plate Count Agar (PCA) (30±1°C'de 72 saat) [16], koliform grubu bakterilerin sayımı için Violet Red Bile Agar (VRB) (30±1°C'de 24 saat) [15], *Staphylococcus - Micrococcus* mikroorganizmaların sayımları için Mannitol Salt Agar (MSA) (37±1°C'de 36-48 saat) [17], besi yeri kullanılmıştır. Maya ve küf sayımı % 10'luk tartarik asit ilave edilerek pH'sı 3,5'e düşürülmüş Potato Dextrose Agar (PDA) (21±1°C'de 5 gün) besi yerinde yapılmıştır [18].

Örneklerin pH değerleri, pH metre (EDT, GP 353) ile saptanmıştır [19]. Kuru madde miktarları Türk Standardları Enstitüsü'nün [20] önerdiği metoda göre yapılmıştır. Tuz miktarları Mohr metoduna göre tespit edilmiştir [21]. TVB-N miktarının belirlenmesinde, Varlık ve ark.'nın [1] bildirdiği spektrofotometrik yöntem uygulanmıştır. TBA sayısı ise, 1000 g örnekteki malonaldehit miktarı üzerinden hesaplanmıştır [22].

Duyusal analizler için kızartılmış olan köfte örnekleri renk, koku, gevreklik, lezzet, tuzluluk ve görünüş yönünden incelenmiştir. Değerlendirme toplam 30 puan üzerinden yapılmıştır [23].

İstatistiksel Analiz

Çalışma üç bağımsız tekrardan oluşturuldu. Mikrobiyolojik veriler $\log_{10}$ kob/g çevrildi ve 3 x 4 x 4 x 10 (tekrar sayısı x uygulama x muhafaza sıcaklığı x örnekleme günleri) faktöriyel dizaynı kullanılarak değerlendirildi. Veriler, SAS programı kullanılarak ana etkiler (uygulama, muhafaza sıcaklığı ve örnekleme günleri) ile uygulama, muhafaza sıcaklığı ve örnekleme günleri arasında üçlü değişkenler arası interaksiyonlar yönünden varians analizine tabi tutuldu. General Linear Models (GLM) prosedürlerine göre, en düşük kareler ortalamaları Fisher's Least significant difference (LSD) testi kullanılarak ayrıştırıldı ve bunda istatistiksel önem seviyesi 0.05 olarak kabul edildi [24].

BULGULAR

Bu çalışmada, farklı oranlarda sodyum laktat ilaveli taze alabalık etinden yapılan köfte örneklerinin +4±1°C'de muhafazası sırasında mikrobiyolojik, kimyasal ve duyusal özelliklerinde meydana gelen değişimler incelenmiştir. Deneysel köfte örneklerinin mikrobiyolojik analiz bulguları *Tablo 1*'de, kimyasal analiz bulguları *Tablo 2*'de ve duyusal analiz sonucunda belirlenen organoleptik özellikleri ise *Tablo 3*'te gösterilmiştir.

TARTIŞMA ve SONUÇ

Örneklerin yapımında kullanılan filetoda 5.28 $\log_{10}$ kob/g seviyesinde tespit edilen toplam mezofilik aerob mikroorganizma sayısı muhafazanın 2. gününden itibaren artmaya başlamıştır. Kontrol grubunda muhafazanın 8. gününde 8.83 $\log_{10}$ kob/g seviyesine, A grubunda 10.

Tablo 1. *4±1°C'de muhafaza edilen köfte örneklerinin mikrobiyolojik analiz bulguları ($\log_{10}$kob/g)*
Table 1. *Microbiological analyses results of fish balls during storage at 4±1°C ($\log_{10}$ cfu/g)*

Mikroorganizma	Fileto	Örnek Tipi	Muhafaza Süresi (Gün)									
			0	2	4	6	8	10	12	14	16	18
Total Mezofilik Aerob	5.28	K	4.11 ^{a}	4.09 ^{a}	4.98 ^{a}	7.95 ^{a}	8.83 ^{a}	AY				
		A	5.26 ^{b}	4.78 ^{b}	4.91 ^{b}	6.89 ^{b}	7.93 ^{b}	8.79 ^{a}	AY			
		B	5.13 ^{b}	4.54 ^{b}	4.77 ^{b}	6.72 ^{c}	7.51 ^{c}	7.89 ^{b}	8.90 ^{a}	AY		
		C	4.85 ^{c}	4.20 ^{c}	4.56 ^{c}	5.65 ^{d}	6.18 ^{d}	6.49 ^{c}	6.92 ^{b}	7.06 ^{a}	8.80 ^{a}	AY
Koliform	2.40	K	3.80 ^{a}	4.75 ^{a}	5.16 ^{a}	5.84 ^{a}	6.92 ^{a}	AY				
		A	3.42 ^{a}	4.48 ^{a}	4.88 ^{b}	4.95 ^{b}	5.78 ^{b}	6.37 ^{a}	AY			
		B	3.29 ^{a}	4.14 ^{a}	4.49 ^{b}	4.61 ^{b}	5.08 ^{b}	5.23 ^{a}	6.44 ^{a}	AY		
		C	3.14 ^{a}	3.29 ^{b}	3.52 ^{c}	3.82 ^{c}	4.30 ^{c}	4.61 ^{a}	5.11 ^{b}	6.83 ^{a}	7.90 ^{a}	AY
Staphylococcus - Micrococcus	2.06	K	3.94 ^{a}	4.01 ^{a}	4.84 ^{a}	5.82 ^{a}	6.85 ^{a}	AY				
		A	3.37 ^{a}	3.57 ^{b}	3.76 ^{b}	4.87 ^{b}	5.59 ^{b}	6.19 ^{a}	AY			
		B	3.18 ^{a}	3.39 ^{b}	3.58 ^{b}	4.85 ^{bc}	5.22 ^{bc}	5.76 ^{bc}	6.45 ^{a}	AY		
		C	3.11 ^{a}	3.24 ^{b}	3.35 ^{b}	3.91 ^{c}	4.21 ^{c}	4.54 ^{c}	5.32 ^{b}	6.82 ^{a}	7.25 ^{a}	AY
Maya ve Küf	1.56	K	2.22 ^{a}	2.35 ^{a}	2.70 ^{a}	2.84 ^{a}	3.90 ^{a}	AY				
		A	2.11 ^{a}	2.24 ^{a}	2.55 ^{a}	2.73 ^{a}	2.96 ^{b}	3.43 ^{a}	AY			
		B	2.08 ^{ab}	2.15 ^{ab}	2.35 ^{b}	2.46 ^{ab}	2.60 ^{bc}	3.20 ^{ab}	4.03 ^{a}	AY		
		C	1.63 ^{b}	1.74 ^{b}	1.80 ^{b}	1.88 ^{b}	1.96 ^{c}	2.68 ^{b}	3.57 ^{b}	4.23 ^{a}	4.90 ^{a}	AY

a,b,c: *Aynı sütunda farklı harflerle gösterilen ortalamalar diğerinden farklıdır (P<0.05)*
K: *Kontrol,* ***A:*** *%0.5 Sodyum laktat ilaveli,* ***B:*** *%1 Sodyum laktat ilaveli,* ***C:*** *% 2 Sodyum laktat ilaveli,* ***AY:*** *Analiz yapılmadı*

Tablo 2. *4±1°C'de muhafaza edilen köfte örneklerinin kimyasal analiz bulguları*
Table 2. *Chemical analyses results of fish balls during storage at 4±1°C*

Değer	Fileto	Örnek Tipi	Muhafaza Süresi (Gün)									
			0	2	4	6	8	10	12	14	16	18
pH	6.79	K	6.12 ^{a}	6.40 ^{a}	6.46 ^{a}	6.38 ^{a}	6.28 ^{a}	AY				
		A	6.17 ^{a}	6.39 ^{a}	6.44 ^{a}	6.41 ^{a}	6.32 ^{a}	6.30 ^{a}	AY			
		B	6.26 ^{a}	6.42 ^{a}	6.43 ^{a}	6.34 ^{a}	6.31 ^{a}	6.28 ^{a}	6.25 ^{a}	AY		
		C	6.30 ^{a}	6.46 ^{a}	6.49 ^{a}	6.40 ^{a}	6.37 ^{a}	6.35 ^{a}	6.29 ^{a}	6.22 ^{a}	6.19 ^{a}	AY
Kuru Madde (%)	24.94	K	36.96 ^{a}	35.43 ^{a}	32.96 ^{a}	33.65 ^{a}	32.89 ^{a}	AY				
		A	35.41 ^{a}	34.34 ^{a}	34.53 ^{a}	32.00 ^{a}	32.23 ^{a}	34.94 ^{a}	AY			
		B	36.19 ^{a}	36.18 ^{a}	33.82 ^{a}	32.25 ^{a}	32.22 ^{a}	36.02 ^{a}	34.73 ^{a}	AY		
		C	32.45 ^{a}	35.44 ^{a}	32.92 ^{a}	30.90 ^{a}	30.75 ^{a}	36.85 ^{a}	36.17 ^{a}	34.46 ^{a}	35.50 ^{a}	AY
Kül (%)	1.86	K	2.61 ^{a}	2.65 ^{a}	2.24 ^{a}	2.45 ^{a}	2.78 ^{a}	AY				
		A	2.53 ^{a}	2.70 ^{a}	2.73 ^{a}	2.56 ^{a}	2.63 ^{a}	2.59 ^{a}	AY			
		B	2.52 ^{a}	2.50 ^{a}	2.67 ^{a}	2.45 ^{a}	2.59 ^{a}	2.36 ^{a}	2.77 ^{a}	AY		
		C	2.59 ^{a}	2.54 ^{a}	2.43 ^{a}	2.68 ^{a}	2.71 ^{a}	2.77 ^{a}	2.35 ^{a}	2.56 ^{a}	2.90 ^{a}	AY
TVB-N (mg/100 g)	8.20	K	8.16 ^{a}	11.11 ^{a}	14.23 ^{a}	18.81 ^{a}	22.56 ^{a}	AY				
		A	7.59 ^{a}	10.39 ^{a}	13.55 ^{a}	16.16 ^{b}	20.76 ^{a}	23.65 ^{a}	AY			
		B	7.25 ^{a}	9.98 ^{b}	11.24 ^{b}	15.21 ^{b}	18.43 ^{b}	21.52 ^{b}	23.11 ^{a}	AY		
		C	7.13 ^{a}	9.63 ^{b}	10.72 ^{c}	12.86 ^{c}	15.08 ^{c}	17.34 ^{c}	19.67 ^{b}	23.57 ^{a}	26.75 ^{a}	AY
TBA (mg/1.000 g)	0.62	K	0.76 ^{a}	1.28 ^{a}	2.75 ^{a}	3.59 ^{a}	5.54 ^{a}	AY				
		A	0.79 ^{a}	1.17 ^{a}	2.48 ^{a}	3.35 ^{a}	4.04 ^{b}	5.27 ^{a}	AY			
		B	0.74 ^{a}	1.17 ^{a}	1.95 ^{b}	2.93 ^{b}	3.88 ^{c}	4.13 ^{b}	5.88 ^{a}	AY		
		C	0.68 ^{a}	0.87 ^{a}	1.41 ^{b}	2.06 ^{b}	2.59 ^{d}	3.42 ^{c}	4.84 ^{b}	5.57 ^{a}	5.77 ^{a}	AY
Tuz (%)	0.89	K	3.58 ^{a}	3.21 ^{a}	3.31 ^{a}	3.65 ^{a}	3.33 ^{a}	AY				
		A	3.52 ^{a}	3.35 ^{a}	3.37 ^{a}	3.29 ^{a}	3.44 ^{a}	3.29 ^{a}	AY			
		B	3,70 ^{a}	3.40 ^{a}	3.33 ^{a}	3.38 ^{a}	3.58 ^{a}	3.22 ^{a}	3.41 ^{a}	AY		
		C	3.45 ^{a}	3.48 ^{a}	3.27 ^{a}	3.16 ^{a}	3.29 ^{a}	3.45 ^{a}	3.42 ^{a}	3.37 ^{a}	3.25 ^{a}	AY

a,b,c: *Aynı sütunda farklı harflerle gösterilen ortalamalar diğerinden farklıdır (P<0.05)*
K: *Kontrol,* ***A:*** *%0.5 Sodyum laktat ilaveli,* ***B:*** *%1 Sodyum laktat ilaveli,* ***C:*** *% 2 Sodyum laktat ilaveli,* ***AY:*** *Analiz yapılmadı*

günde 8.79 $\log_{10}$ kob/g seviyesine, B grubunda 12. günde 8.90 $\log_{10}$ kob/g seviyesine ve C grubunda ise 16. günde 8.80 $\log_{10}$ kob/g seviyesine çıkmıştır *(Tablo 1)*. Türk Gıda Kodeksi Mikrobiyolojik Kriterler Tebliği'ne [25] göre tüm gıda maddelerindeki toplam mezofilik aerob bakteri sayısı kabul edilebilir sınır değeri 10^6 kob/g'dır. Buna göre sonuçlar değerlendirildiği zaman kontrol grubunun muhafazanın 8. gününden, A grubunun 10. günden, B grubunun 12. günden ve C grubunun ise 16. günden itibaren bozulduğu görülmüştür. Elde edilen bulgular, balık köftesinin 4±1°C'de muhafazasıyla raf ömrünün 8. güne kadar devam ettiğini muhafazanın 10. gününden itibaren ise bozulduğunu tespit eden Gökoğlu'nun [4] bulgularıyla benzerlik arz etmektedir. İstatistiksel olarak veriler değerlendirildiğinde, kullanılan laktat oranı artıkça toplam mezofilik aerob bakteri sayısında kontrol grubuna göre daha yavaş bir artış olduğu gözlemlenmiştir (P<0.05). Yine tablo incelendiğinde %2'lik laktat oranının bu grup bakterilerin sayısında yaklaşık 1 $\log_{10}$ kob/g değerinde azalmaya neden olduğu görülmektedir *(Tablo 1)*. Elde edilen bu bulgu Çetin ve Bostan'ın [26] bulgularıyla benzerlik arz etmektedir.

Koliform bakteriler temiz sularda avlanan balıkların deri ve kaslarında bulunmazlar. Çünkü işlem görmemiş balık eti steril kabul edilmektedir. Bu grup bakterilerin varlığı, balığın ya fekal kontaminasyonlu sulardan avlandığını, ya da avlandıktan sonra uygulanan işlemlere bağlı olarak bulaştığını gösterir. Jay, Saunders ve Shewan'a göre; fekal kontaminasyonun belirticisi olarak kabul edilen koliformların balıklardaki sayılarının en fazla $2.0x10^2$ kob/g; $2.5x10^2$ kob/g; ya da $1.6x10^3$ kob/g olabileceği önerilmektedir [27-29]. Bu çalışmada, filetoda 2.40 $\log_{10}$ kob/g bulunan koliform bakteriler muhafazanın 0. gününden itibaren artış göstererek kontrol grubunda 8. günde 6.92 $\log_{10}$kob/g, A grubunda 10.günde 6.37 $\log_{10}$kob/g, B grubunda 12. günde 6.44 $\log_{10}$kob/g ve C grubunda ise 16. günde 7.90 $\log_{10}$kob/g olarak en yüksek seviyeye ulaştı *(Tablo 1)*. İstatistiksel olarak değerlendirildiğinde, laktat oranı arttıkça koliform grubu bakteri sayısı bakımından B (%0.05 oranında sodyum laktat) ile C (%1 oranında sodyum laktat) grubu arasında pek bir farklılık olmadığı ancak kontrol dahil diğer gruplar arasında önemli farklılıklar olduğu görülmüştür *(Tablo 1)* (P<0.05).

Tablo 3. *4±1°C muhafaza edilen köfte örneklerinin duyusal analiz bulguları*
Table 3. *Sensory analyses results of fish balls during storage at 4±1°C*

Özellikler	Örnek Tipi	Muhafaza Süresi (Gün)								
		2	4	6	8	10	12	14	16	18
Renk	K	4.88 ^{a}	4.84 ^{a}	4.75 ^{a}	4.00 ^{a}	AY				
	A	4.88 ^{a}	4.79 ^{a}	4.38 ^{a}	4.11 ^{a}	4.00 a	AY			
	B	4.88 ^{a}	4.84 ^{a}	4.68 ^{a}	4.60 ^{a}	4.40 a	4.17 ^{a}	AY		
	C	5.00 ^{a}	4.92 ^{a}	4.88 ^{a}	4.70 ^{a}	4.52 a	4.44 ^{a}	4.34 ^{a}	4.28 ^{a}	AY
Görünüş	K	4.88 ^{a}	4.84 ^{a}	4.75 ^{a}	4.60 ^{a}	AY				
	A	4.92 ^{a}	4.90 ^{a}	4.88 ^{a}	4.68 ^{a}	4.40 ^{a}	AY			
	B	4.98 ^{a}	4.92 ^{a}	4.88 ^{a}	4.70 ^{a}	4.59 ^{a}	4.27 ^{a}	AY		
	C	5.00 ^{a}	4.92 ^{a}	4.88 ^{a}	4.80 ^{a}	4.70 ^{a}	4.64 ^{a}	4.34 ^{a}	4.04 ^{a}	AY
Koku	K	5.00 ^{a}	4.75 ^{a}	4.75 ^{a}	4.70 ^{a}	AY				
	A	4.92 ^{a}	4.84 ^{a}	4.70 ^{a}	4.63 ^{a}	4.48 ^{a}	AY			
	B	5.00 ^{a}	4.86 ^{a}	4.80 ^{a}	4.75 ^{a}	4.68 ^{a}	4.00 ^{a}	AY		
	C	5.00 ^{a}	4.92 ^{a}	4.90 ^{a}	4.85 ^{a}	4.70 ^{a}	4.50 ^{a}	3.84 ^{a}	2.50 ^{a}	AY
Gevreklik	K	4.70 ^{a}	4.67 ^{a}	4.43 ^{a}	4.20 ^{a}	AY				
	A	4.79 ^{a}	4.70 ^{a}	4.62 ^{a}	4.25 ^{a}	3.34 ^{a}	AY			
	B	4.87 ^{a}	4.84 ^{a}	4.70 ^{a}	4.63 ^{a}	4.17 ^{a}	3.14 ^{a}	AY		
	C	5.00 ^{a}	5.00 ^{a}	4.88 ^{a}	4.63 ^{a}	4.43 ^{a}	3.69 ^{a}	3.24 ^{a}	2.50 ^{a}	AY
Lezzet	K	4.41 ^{a}	4.38 ^{a}	4.28 ^{a}	4.25 ^{a}	AY				
	A	4.59 ^{a}	4.42 ^{a}	4.35 ^{a}	4.25 ^{a}	3.17 ^{a}	AY			
	B	4.60 ^{a}	4.51 ^{a}	4.45 ^{a}	4.63 ^{a}	3.74 ^{a}	3.14 ^{a}	AY		
	C	4.71 ^{a}	4.59 ^{a}	4.75 ^{a}	4.70 ^{a}	3.99 ^{a}	3.77 ^{a}	3.34 ^{a}	2.34 ^{a}	AY
Genel Beğeni Düzeyi	K	4.63 ^{a}	4.54 ^{a}	4.38 ^{a}	4.15 ^{a}	AY				
	A	4.71 ^{a}	4.66 ^{a}	4.63 ^{a}	4.54 ^{a}	3.88 ^{a}	AY			
	B	4.83 ^{a}	4.70 ^{a}	4.67 ^{a}	4.63 ^{a}	4.13 ^{a}	3.84 ^{a}	AY		
	C	4.94 ^{a}	4.83 ^{a}	4.88 ^{a}	4.70 ^{a}	4.49 ^{a}	3.97 ^{a}	3.17 ^{a}	2.50 ^{a}	AY

a,b,c: *Aynı sütunda farklı harflerle gösterilen ortalamalar diğerinden farklıdır (P<0.05)*
K: *Kontrol,* ***A:*** *%0.5 Sodyum laktat ilaveli,* ***B:*** *%1 Sodyum laktat ilaveli,* ***C:*** *% 2 Sodyum laktat ilaveli,* ***AY:*** *Analiz yapılmadı*

Staphylococcus'lar doğada yaygın olarak bulunurlar. Ancak, deniz ürünleri doğal olarak *Staphylococcus* mikroorganizmalarını içermezler. Bu mikroorganizmaların 100 kob/g'dan fazla olması insanlardan kaynaklanan bulaşmayı gösterir [30]. Filetoda 2.06 $\log_{10}$kob/g olarak saptanan *Staphylococcus - Micrococcus* sayıları tüm gruplarda muhafazanın ilk gününden itibaren artmaya başlamıştır. Kontrol grubunda 8. günde 6.85 $\log_{10}$kob/g, A grubunda 10. günde 6.19 $\log_{10}$kob/g, B grubunda 12. günde 6.45 $\log_{10}$kob/g ve C grubunda ise 16. günde 7.25 $\log_{10}$kob/g olarak en yüksek seviyeye çıkmıştır *(Tablo 1)*. İstatistiksel olarak kontrol grubunda elde edilen bulguların diğer gruplardan farklı olduğu görülmüştür. Sodyum laktatın %2'lik oranında kullanıldığı C grubundaki *Staphylococcus - Micrococcus*'ların sayılarının ise diğer gruplardan 1'er $\log_{10}$kob/g değer olarak daha az bulunduğu tespit edilmiştir *(Tablo 1)* (P<0.05).

Maya ve küfler, balıklarda normal flora içerisinde bulunmazlar. Bunlar genellikle toprak orijinli olup, balıklar avlandıkları anda, sudan veya avlanma sonrası kullanılan alet ve malzemelerden bulaşmaktadırlar [27,30]. Maya ve küf sayısı filetoda 1.56 $\log_{10}$kob/g olarak belirlenmiştir. Bu sayı kontrol dahil tüm gruplarda muhafazanın ilerlemesine bağlı olarak artış göstermiştir ve %2 oranında sodyum laktat ilaveli olan C grubu hariç diğer gruplarda aynı seviyelerde seyrettiği görülmüştür *(Tablo 1)*. İstatistiksel olarak C grubunun diğerlerinden daha az sayıda maya ve küf içerdiği ve bu değerlerin önemli olduğu bulunmuştur (P<0.05).

Örneklerin yapımında kullanılan filetoda pH değeri 6.79 olarak belirlenmiştir. Tespit edilen bu değer tüm köfte örneklerinde 0. günde ve muhafazanın 2. gününde nispeten azalmıştır. Muhafazanın 4. gününde tüm gruplarda yükselme gösterdikten sonra muhafazanın sonuna kadar tekrar azalmıştır *(Tablo 2)*. Farklı sodyum laktat oranları göz önüne alındığında, örnekler arasında istatistiksel olarak fark bulunamamıştır (P>0.05). Muhafazanın sonunda bütün köfte gruplarındaki pH değerlerinin 6.19-6.30 arasında seyrettiği görülmüştür. pH ile ilgili bulgularımız Yanar ve Fenercioğlu'nun [31] sazan etinden yaptıkları köftelerde tespit ettikleri 6.1 ile 6.3 değeri ile Akkuş ve ark.'nın [5] hamsi etinden yaptıkları köftelerde tespit ettikleri 6.3 ile 7.7 değerlerinden farklılık arz etmektedir. Bu durum, kullanılan farklı balık türlerinden,

katılan katkı maddelerinden ve uygulanan farklı ısıl işlemlerinden kaynaklanmış olabilir.

Bu çalışmada filetoda %24.94 olarak tespit edilen kuru madde miktarı tüm köfte örneklerinde 0. gün ile muhafazanın başlangıç gününde artış göstermiştir. Muhafazanın 4. gününden itibaren muhafazanın sonuna kadar tüm gruplarda dalgalanmalar şeklinde seyrederek ortalama olarak %32.00 ile %36.85 arasında farklı değişimler göstermiştir *(Tablo 2)*. Kuru madde miktarı bakımından gruplar arasında istatistiksel olarak fark görülmemiştir (P>0.05).

Kül miktarı filetoda %1.86 olarak belirlenmiştir. Bu değer muhafazanın 2. gününe kadar artış gösterdikten sonra tüm gruplarda muhafaza süresi boyunca dalgalanmalar göstermiştir *(Tablo 2)*. Kül miktarı bakımından gruplar arasındaki farkın önemsiz olduğu bulunmuştur (P>0.05).

TVB-N başlıca balıkta bulunan bakterilerin ve endojen enzimlerin etkisi ile TMA ve amonyaktan ibarettir [32]. Balık ve diğer su ürünlerinin muhafazasında süreye bağlı olarak TVB-N değerinin yükseldiği bildirilmektedir. Huss [33], taze balığın içerdiği TVB-N miktarını 5-20 mg/100 g, taze kabul edilebilir sınır değerini 30-40 mg/100 g olarak bildirmektedir. Varlık ve ark.[1] ise, TVB-N değerlerinde göre kalite sınıflandırmasını, 25 mg/100 g'a kadar "çok iyi", 30 mg/100 g'a kadar "iyi", 35 mg/100 g'a kadar "pazarlanabilir", 35 mg/100 g'dan fazlasını "bozulmuş" olarak değerlendirmektedir. Yine aynı araştırmacılar, tatlı su balıklarında TVB-N ile ilgili tüketilebilirlik sınır değerini 32-36 mg/100 g olarak belirtmektedirler. Pastoriza ve ark.[34], TVB-N'nin kabul edilebilir sınır değerini 35 mg/100 g, Ariyani [35] 30 mg/100 g olarak bildirmektedirler. Bu çalışmada, örneklerin yapımında kullanılan filetoda ortalama olarak 8.20 mg/100 g miktarında tespit edilen TVB-N değerleri, tüm köfte örneklerinde 0. günde kısmen azalmıştır. Daha sonraki günlerinde ise tüm örneklerde muhafazanın sonuna kadar sürekli artış göstermiştir. TVB-N miktarları kontrol grubunda muhafazanın 8. gününde 22.56 mg/100 g, A grubunda 10. günde 23.65 mg/100 g, B grubunda 12. günde 23.11 mg/100 g ve C grubunda ise 16. günde 26.75 mg/100 g olarak saptanmıştır *(Tablo 2)*. Saptanan bu değerler bazı araştırmacıların [1,33,34,35] önerdiği değerlerin oldukça altındadır. Bu çalışmada, kontrol grubu 10. günde, A grubu 12. günde, B grubu 14. günde ve C grubu ise 18. günde duyusal olarak tamamen bozulduğu belirlenmiştir. Buradan da anlaşılacağı üzere, TVB-N miktarlarına göre balık köftelerinin değerlendirilmesi uygun düşmemektedir. Çünkü önerilen değerler işlem görmemiş balıklar için verilmiştir. Ayrıca, balık köftesi farklı katkı maddelerinin ilavesiyle hazırlanan bir üründür. TVB-N miktarları sazan etinden hazırlanmış köftelerde muhafaza süresince 10.52-13.78 mg/100 g [31]; hamsi kıymasından hazırlanmış köftelerde ise 16.68-42.93 mg/100 g [5] tespit edilmiştir. Bu bulgular bizim bulgularımızla (9.63-26.75 mg/100 g) uyuşmamaktadır. Bulguların uyumsuzluğu, farklı balık türlerinden ve farklı muhafaza koşullarından kaynaklanabilir. Yapılan istatistiksel analizde, gruplar arasındaki farkın önemli olduğu bulunmuştur (P<0.05).

Ürünün bozulmasına neden olan etkenlerden biri de yağ oksidasyonudur. Okside olmuş ürünlerde acımsı bir tat ve sarı bir renk oluşmaktadır. Yağ oksidasyonunu ifade eden kriterlerden biri de tiyobarbiturik asit (TBA) sayısıdır. TBA miktarlarının değişiminde balığın türü, yağ miktarı, mevsim vs. gibi faktörlerin etkili olduğu bildirilmektedir [36]. TBA sayısı çok iyi bir materyalde 3'ten az olmalı, iyi materyalde ise 5'ten fazla olmamalı, tüketilebilirlilik sınır değeri ise 7-8 arasında bulunmalıdır [1,37]. Bu çalışmada filetoda 0.62 mg malonaldehit/1000 g değerinde bulunan TBA sayısı tüm köfte örneklerinde muhafazanın başından sonuna kadar sürekli artış göstermiştir. Tüm gruplarda muhafaza süresince TBA değerleri 0.87-5.88 mg malonaldehit/1000 g değerleri arasında seyretmiştir. Bu değerlerin önerilen tüketilebilirlik sınır değerlerinden (7-8 mg malonaldehit/1000 g) oldukça düşük olduğu görülmüştür. Konu ile ilgili yapılan bazı araştırmalarda [31,38] TBA sayısının, sazan köftelerinin 6 ay süreyle -20°C'de muhafazası sırasında 0.6 ile 2.2 mg malonaldehit/kg ve gökkuşağı alabalığından hazırlanan ve 21 gün süreyle +4±1°C'de muhafazaya alınan balık burgerlerde ise 0.11-1.45 mg malonaldehit/kg olduğu bildirilmektedir. Her iki çalışmadaki bulgular, muhafaza sıcaklığı ve süresi göz önüne alındığında, bu çalışmada elde edilen değerlerden nispeten farklıdır. Bu durumun, farklı balık türlerinden ve farklı işleme şekillerinden kaynaklandığı söylenebilir. Elde edilen veriler istatistiksel olarak değerlendirildiğinde, TBA miktarı bakımından gruplar arasında önemli farklılıkların olduğu (P<0.05) belirlenmiştir.

Tuz miktarı filetoda %0.89 olarak saptanmıştır. Bu değer muhafazanın 2. gününe kadar tüm örneklerde artış gösterdikten sonra muhafaza süresi boyunca dalgalanmalar şeklinde seyretmiştir *(Tablo 2)*. Tuz miktarı bakımından gruplar arasındaki farkın önemsiz olduğu görülmüştür (P>0.05). Yapılan bir çalışmada [34], gökkuşağı alabalığı filetosunda tuz miktarı %0.45 ve bu filetodan hazırlanan farklı balık burgerlerde ise %0.78 - %0.88 oranında tuz saptandığı bildirilmektedir. Aynı çalışmada tuz miktarı bakımından gruplar arasında fark tespit edilemediği de belirtilmektedir. Ancak, adı geçen araştırmada elde edilen değerler bizim bulgularımızdan (%3.16 - %3.65) oldukça düşüktür. Bu durum adı geçen ürünün farklı şekilde hazırlanmasından kaynaklanmış olabilir.

Laktatların et ürünlerinde beklenen en önemli etkisi

raf ömründe artış sağlamasıdır. Çalışmamızda da köfte hamuruna laktat ilavesi duyusal olarak belirlenen raf ömründe belirgin bir iyileşme sağlamıştır. Köfte hamuruna katılan %2 laktat ilavesi ürünün renk, koku, görünüş ve tadında istenmeyen bir değişime neden olmamıştır. Bulgularımız sodyum laktat üzerinde yapılan bazı araştırmacıların [13,15,26] bulgularıyla uyum içerisindedir. Kontrol grubunun 10. günden, A grubunun 12. günden, B grubunun 14. günden ve C grubunun ise 18. günden itibaren duyusal olarak tüketilebilirlik özelliklerini kaybettikleri görülmüştür. Dolayısıyla sodyum laktat oranı arttıkça ürünün raf ömrünün uzadığı gözlemlenmiştir. Ancak ilave edilen laktat oranı bakımından ise gruplar arasındaki farkın istatistiksel olarak önemsiz olduğu tespit edilmiştir (P>0.05).

Taze gökkuşağı alabalığından (*Oncorhynchus mykiss* W.) hazırlanan köftelerin üretiminde sodyum laktatın kullanılmasıyla ürünün duyusal özelliklerinin (renk, koku ve lezzet) etkilenmediği ancak mikrobiyel gelişmeyi yavaşlatarak raf ömrünü arttırdığı ve köfte hamuruna %2 oranında sodyum laktat ilavesinin ürünün duyusal özelliklerinde istenmeyen bir değişime neden olmadığı görülmüştür. Yine, %2 oranında sodyum laktat ilave edilen örneklerin 4±1°C'de 16 gün boyunca yenilebilir niteliğini koruduğu gözlemlenmiştir. Böylece gıda maddelerinin mikrobiyolojik güvenliğini sağlamak ve raf ömrünü artırmak için organik asitler ve türevlerinden doğal alternatifler olarak ne derece faydalanabileceğimiz kısmen de olsa belirlenmiştir.

KAYNAKLAR

1. Varlık C, Erkan N, Özden Ö, Mol S, Baygar T: Su Ürünleri İşleme Teknolojisi. 7. Baskı, İstanbul Üniversitesi Basımevi, İstanbul, 2004.

2. Anonim: Su Ürünleri Kalite Kontrol El Kitabı. Türkiye Cumhuriyeti Tarım ve Köyişleri Bakanlığı Koruma ve Kontrol Genel Müdürlüğü, Ankara, 2000.

3. Yıldırım Ö, Ergün S, Yaman S, Türker A: Effects of two seaweeds (*Ulva lactuca* and *Enteromorpha linza*) as a feed additive in diets on growth performance, feed utilization, and body composition of rainbow trout *(Oncorhynchus mykiss). Kafkas Univ Vet Fak Derg,* 15 (3): 455-460, 2009.

4. Gökoğlu N: Balık köftesinin soğukta depolanması. *Gıda Derg,* 19 (3): 217-222, 1994.

5. Akkuş Ö, Varlık C, Erkan N, Mol S: Çiğ ve haşlanmış balık etinden yapılmış köftelerin bazı kalite parametrelerinin incelenmesi. *Turk J Vet Anim Sci,* 28, 79-85, 2004.

6. Koos JT: Preservation of food products with natural ingredients. *Food Marc Technol,* 3, 5-11, 1992.

7. Cubina I: Natural lactic acid L(+) and lactates in the food industry. *5th International Congress on Food Industry* "New Aspects on Food Processing". Kuşadası, Turkey. 23-28 April, Proceedings, pp.106-108, 1995. 1995.

8. Wit De JC, Rombouts FM: Antimicrobial activity of sodium lactate. *Food Microbiol,* 7, 113-120, 1990.

9. Baird-Parker AC: Organic acids. **In,** Elliot RP, Baird-Parker AC, Bryan FL, Christian JHB, Clark DS, Olson JCB, Roberts TA (Eds): Microbiological Ecology of Foods. pp. 126-135,T.A. Academic Press Inc., , New York, 1980.

10. Buric MC, Koos JT: Natrium lactate in fleischprodukten. *Fleischwirtschaft,* 70 (11): 1266-1268, 1990.

11. Houtsma PC, De Wit JC, Rombouts FM: Minimum inhibitory concentration (MIC) of sodium lactate for pathogens and spoilage organisms occurring in meat product. *Int J Food Microbiol,* 20, 247-257, 1993.

12. Miller RK, Acuff G: Sodium lactate affects pathogens in cooked beef. *J Food Sci,* 59 (1): 15-19, 1994.

13. Brewer MS, Rostogi BK, Argoudelis L, Sprouls GK: Sodium lactate / Sodium chloride effects on aerobic plate counts and color of aerobically packaged ground pork. *J Food Sci,* 60 (1): 58-62, 1995.

14. Lamkey JW, Leak FW, Tuley WB, Johnson DD, West RL: Assessment of sodium lactate addition to fresh pork sausage. *J Food Sci,* 56 (1): 220-223, 1991.

15. Papadopaulos LS, Miller RK, Ringer LJ, Cross HR: Sodium lactate effect on sensory characteristics, cooked meat color and chemical composition. *J Food Sci,* 56 (3): 621-635, 1991.

16. Harrigan WF: Laboratory Methods in Food Microbiology, 3rd ed., Academic Press, London, 1998.

17. British Standards Institution: Methods of Microbiological Examination of for Dairy Purposes. B.S.4285, British Standards Institution, London, 1968.

18. Oxoid: The Oxoid Manual. 50th ed., Oxoid Ltd. Hampshire, 1982.

19. Association Official Analytical Chemists: Official Methods of Analysis of the Association of Official Analytical Chemists. 15th ed., Association Official Analytical Chemists (AOAC), Washington, DC, 1990.

20. Türk Standardları Enstitüsü: Et ve Et Mamülleri Rutubet Miktarı Tayini. TS 1743, Türk Standardları Enstitüsü, Ankara, 1974.

21. Tolgay Z, Tetik İ: Muhtasar Gıda Kontrolu ve Analizleri Klavuzu. Ege Matbaası, Ankara, 1964.

22. Tarladgis BG, Watts BM, Younnathan MT, Dugan LR: A distillation method for the quantative determination of malonaldehyde in rancid foods. *J Am Oil Chem Soc,* 37, 44-48, 1960.

23. Kurtcan Ü, Gönül M: Gıdaların duyusal değerlendirilmesinde puanlama metodu. *Ege Univ Müh Fak Derg,* 5, 137-146, 1987.

24. Statistical Analyses System: SAS, Inst. Inc. Cary. 8. Version, North Caroline, US, 1999.

25. Türk Gıda Kodeksi: Gıdalarda Bakılması Gereken Mikroorganizmalar. Ankara, 2004.

26. Çetin B, Bostan K: Hazır köftelerin mikrobiyolojik kalitesi ve raf ömrü üzerine sodyum laktatın etkisi. *Turk J Vet Anim Sci,* 26, 843-848, 2002.

27. Jay JM: Modern Food Microbiology. 5th ed., Chapman & Hall, New York, 1996.

28. Saunders GC: Microbiological Standards for Foodstuffs,

Food Legislation Surveys. British Food Manufacturing Industries Research Association, Leatherhead, 1983.

29. Shewan JM: The microbiology of fish and fishery products. A progress report. *J Appl Bacteriol,* 34, 299-315, 1971.

30. Göktan D: Gıdaların Mikrobiyal Ekolojisi, Cilt 1: Et Mikrobiyolojisi. Ege Üniversitesi Basımevi, İzmir, 1990.

31. Yanar Y, Fenercioğlu H: Sazan *(Cyprinus carpio)* etinin balık köftesi olarak değerlendirilmesi. *Turk J Vet Anim Sci,* 23, 361-365, 1999.

32. Lannelongue M: Storage characteristics of fresh packed in modified atmosphere containing CO_2. Master Thesis, T and M University Collage Station, TX, USA, 1980.

33. Huss HH: Quality and Quality Changes in Fresh Fish. Food and Agriculture Organization Fisheries Technical Paper-348, Food and Agriculture Organization of United Nations, Rome, 1995.

34. Pastoriza L, Sampedro G, Herrera JJ, Cabo ML: Effect of carbon dioxide on microbiol growth and quality of salmon slices. *J Sci Food Agric,* 72 (3): 348-352, 1996.

35. Ariyani F: Quality changes of sardines *(Sardinella neopilchardus)* at Indonesian ambient temperature. *Int J Agri Sci (IJAS),* 1 (1): 21-28, 2000.

36. Ruiz-Capillas C, Moral A: Correlation between biochemical and sensory quality indices in hake stored in ice. *Food Res Int,* 34 (5): 441-447, 2001.

37. Sinnhuber RO, Yu TC: 2-Thiobarbituric acid method for the measurement of rancidity in fishery products. II. The quantitative determination of malonaldehyde. *Food Technol,* 1, 9-12, 1958.

38. Taşkaya L, Çaklı Ş, Kışla D, Kılınç B: Quality changes of fish burger from rainbow trout during refrigerated storage. *J Fish and Aqu Sci,* 20 (1-2): 147-154, 2003.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S73-S80, 2010
DOI:10.9775/kvfd.2009.1475

RESEARCH ARTICLE

Microbiological and Chemical Properties of Bonito Fish *(Sarda sarda)* Fillets Packaged with Chitosan Film, Modified Atmosphere and Vacuum [1]

Gonca ALAK * Sükriye ARAS HISAR * Olcay HISAR *
Güzin KABAN ** Mükerrem KAYA **

[1] *This work has been supported by the Scientific and Technological Research Council of Turkey (TUBITAK), Project no: 107 O 606*
* Fisheries Department, Agricultural Faculty, Atatürk University, TR-25240 Erzurum - TURKEY
** Food Engineering Department, Agricultural Faculty, Atatürk University, TR-25240 Erzurum - TURKEY

Makale Kodu (Article Code): KVFD-2009-1475

Summary

A study was undertaken to determine the effect of chitosan film or vacuum and modified atmosphere (MA) (100% CO_2) packing on microbial (psychrotorophic, mesophilic aerobic, lactic acid bacteria, Enterobacteriaceae and Pseudomonas counts) and chemical [(pH, total volatile bases nitrogen (TVB-N) and lipid oxidation (TBARS)] properties of Atlantic bonito (Sarda sarda) fillets stored at 4±1°C for 15 days. Growth of aerobic bacteria in fillet packaged with chitosan film was slower than in the fillets of control and vacuum groups during storage. Chitosan group had the lowest average pH value among the treatments. However, over-wrap of the fish fillets with chitosan film did not significantly retard the increase in the TVB-N content and TBARS values. In conclusion, it can be advised that chitosan film is suitable for extending the self life with strong antimicrobial effect.

Keywords: *Chitosan film, Fatty fish, Modified atmosphere packaging, Vacuum packaging*

Palamut *(Sarda sarda)* Filetolarının Kimyasal ve Mikrobiyolojik Özellikleri Üzerine Kitosan Film ile Kaplama, Modifiye Atmosfer ve Vakum Ambalajlamanın Etkisi

Özet

Bu çalışma kitosan kaplamanın veya modifiye atmosfer ve vakum ambalajlamanın 4±1°C'de 15 gün boyunca depolanan palamut balığı (Sarda sarda) filetolarının mikrobiyolojik (psikrotrofik, mezofilik aerobik, laktik asit, Enterobacteriaceae ve Pseudomonas bakterileri sayıları) ve kimyasal (pH, TVB-N ve TBARS) parametreleri üzerine etkilerini belirlemek amacıyla yapılmıştır. Kitosan film ile paketlenmiş filetolarda aerobik bakterilerin gelişimi, kontrol ve vakum gruplarına göre daha yavaş olduğu bulunmuştur. Tüm muamele grupları içerisinde en düşük pH değeri kitosan ile kaplı numunelerde belirlenmiştir. Buna rağmen kitosan ile kaplı filetolarda; TVB-N ve TBARS değerlerindeki değişimin önemli oranda yavaşlamadığı bulunmuştur. Sonuç olarak, kitosan film güçlü antimikrobiyal etkisinden dolayı raf ömrünün uzatılmasında kullanılabilir.

Anahtar sözcükler: *Kitosan kaplama, Yağlı balık, Modifiye atmosfer paketleme, Vakum paketleme*

INTRODUCTION

Chemical deterioration and microbial spoilage may cause losses up to 25% of gross primary agricultural and fishery products in every year [1]. MAP offers multiple advantages to the fish industry and the consumer. Various atmospheres have been examined in fish packaging [2-6]. Oxygen, nitrogen and carbon dioxide are the most usual gases used in MAP [7,8]. Since, different concentration of CO_2 has antimicrobial effects on bacteria; it inhibits the

İletişim (Correspondence)
☎ +90 442 2311426
✉ galak@atauni.edu.tr

growth of microorganisms during the logarithmic phase and extends the lag phase [9]. Statham [10], explains that "weak acids are known to have antimicrobial activity in their undissociated form; therefore carbonic acid is unique as a microbial inhibitor since at pH values near neutrality at least one half of the acid is in the undissociated form. The pH value for the first dissociation is 6.37 yielding hydrogen and bicarbonate ions".

Natural and synthetic agents can also control the deterioration of fish [11,12], however the usage of artificial preservatives in food disturbs consumers [13]. Therefore, there is scope for new methods for food safety which have a natural or 'green' image. Use of edible coatings and films, especially on highly perishable unmodified and/or fresh foods is one possibility for preventing or delaying spoilage.

There is an increasing interest in edible films due to concern over the disposal of conventional synthetic plastic material. While degradation of plastics requires a long time, this process in edible films from renewable agriculture products occurs readily after their disposal. Moreover, they can be an effective tool in extending shelf-life of the food.

Many different substances, used as film, act as barriers to oxygen but not to water, this being one of the factors limiting the compounds suitable for such use. Other important characteristics include antioxidant, binding, and texturizing properties. Antimicrobial activity by certain substances is another extremely important factor [14].

Chitosan is a cationic polysaccharide obtained by deacetylation of citin, which is chiefly in the exo-skeletons of crustaceans [15]. Chitosan is of interest as potential edible film component because of its numerous technological and physiological properties [16,12]. First of all, abundant commercial supplies are available. Some studies have been conducted on the use of chitosan in foods related to its antimicrobial activity and its ability to form protective films [17,18], texturizing [19], binding action [20] and antioxidant activity [21]. Therefore, the present study was undertaken to determine the microbiological and chemical properties of bonito fish *(Sarda sarda)* fillets packaged with chitosan film, vacuumed and modified atmosphere (MA) (100% CO_2) stored at 4°C.

MATERIAL and METHODS

Film Preparation

Low viscous chitosan was purchased from Sigma-Aldrich (Israel). Chitosan solution (1.5%, w/v) was prepared by dissolving 7.5 g of chitosan in 500 ml of acetic acid solution (1.5%, v/v). To achieve complete dispersion of chitosan, the solution was stirred overnight at room temperature. After the chitosan was dissolved completely, the solution was filtered with cheese cloth (mesh size of the cheese cloth was around 1 mm square) by vacuum aspiration to remove impurities. The final film forming solution was cast onto flat, level Teflon-coated glass plates. After drying the films at room temperature for at least 72 h, they were the peeled from plates. Dried films were conditioned at 50% RH and 25°C for 48 h prior to testing.

Preparation, Packaging and Storage of Samples

A total of 50 Atlantic bonito caught from the Black Sea and transferred to the market on ice in 24 h were purchased at market with an average weight of 300 g and then transferred to the Meat Processing Laboratory at Atatürk University in Erzurum. They were decapitated and filleted by hand. Two fillets were obtained from each fish by removing the head and bone, 100 fillets in total. The fillets were divided into 4 groups. The first batch was wrapped with stretch film (Sigma) and used as control group. The 2nd group was vacuum packaged in film bags with 15x25 cm OPA/EVOH/PE (Oriented Polyamid-EVOH-Polyetilen, UPM-Kymmene Walki Films, Finland) and with low gas permeability (oxygen transmission rate of 5 cm^3/m^2/days atm at 23°C, nitrogen transmission rate of 1cm^3/m^2/days atm at 23°C, carbon dioxide transmission rate of 23 cm^3/m^2/days atm at 23°C and water vapour transmission rate of 15 g/m^2/days atm at 38°C). The 3rd group was also placed the film bags and MA packaged by using a packaging machine (Multivac A 300/16, Sepp Haggenmuller, D 87787 Wolfertschwenden, Germany). The final gas/product ratio was about 2:1 (v/w) for MAP condition. The composition of gas mixture was adjusted as 100% CO_2 by a commercial company (Karbogaz, Istanbul, a partner of PRAXAIR, Danbury). The 4th group was wrapped with the chitosan film. Fillets in all 4 groups were stored at 4±1°C for 15 days and subjected to microbial and chemical analyses on the 0, 3, 6, 9, 12 and 15 days of storage period. All experiments and measurements were carried out in triplicate.

pH Value

The pH values were recorded by using a Schott model pH meter (Schott, Lab Star pH) after homogenization of each 10 g fish muscle sample in 100 ml distilled water.

Total Volatile Base Nitrogen (TVB-N)

A vapor distillation method was used for total volatile bases nitrogen (TVB-N) estimation [22]. The results were expressed as mg TVBN/100 g.

Lipid Oxidation

Lipid oxidation, measured as Thiobarbituric acid reactive substances (TBARS) values, was determined according to Lemon [23].

Microbial Analysis

25 g fish muscle was removed aseptically and homogenized for 1 min in a Stomacher 400 (Lab Stomacher Blander 400-BA7021, Sewardmedical) bag containing 0.85% NaCl solution. Further decimal dilutions were made and then 0.1 ml of each dilution was pipetted onto the surface of plate count agar (PCA, Merck) [24]. PCA plates were then incubated for 7 days at 10°C for psychrotorophic bacteria count and for 2 days at 37°C for mesophilic bacteria count. Enterobacteriaceae was determined in Violet-Red-Bile-Glucose agar (VRBG-agar, Merck) plates incubated anaerobically at 30°C for 2 days. Lactic acid bacteria were determined in MRS Agar (Oxoid) plates incubated anaerobically at 30°C for 72 h. Finally, Pseudomonas counts were determined using Pseudomonas agar base (Oxoid) supplemented with C-F-C (Cetrimide-Fucidin-Cephloridine) selective supplement (Oxoid) incubated at 25°C for 48 h. All counts were expressed as $\log_{10}$ CFU/g. In addition, McMeekin [25], reported that usually a "specified reactive level" should be used to show unacceptable levels in food products, therefore, a horizontal line was used in each figure to show these spoilage levels in the present study.

Statistical Analysis

Data were checked for normal distributions with normality plots prior to one-way analysis of variance (ANOVA), and followed by Duncan's multiple range test to determine significant differences among means at P=0.05 level [26,27].

RESULTS

The counts of all determined microbiological indicators except lactic acid were significantly (P<0.05) affected by application of the three packing and especially chitosan film. Duncan comparison test of the average of significant differences in the variance analysis of the values determined in the bonito fillets at various storage times are presented in *Table 1* and *2*.

The results of Duncan test showed that the number of total aerobic mesophilic bacteria count was higher in control group than that of chitosan group *(Table 1)*. The highest total aerobic mesophilic bacteria count was determined on day 15 during the storage time *(Table 2)*. The interaction of storage time x treatment resulted in a significant effect on total aerobic mesophilic bacteria count (P<0.05). After the 6th days, total aerobic mesophilic bacteria counts in the fillets of control group reached above than 10^6 CFU/g. However, growth of aerobic bacteria in fillet packaged with chitosan film was slower than in the fillets of control and vacuum groups during storage and mesophilic bacteria count did not reach 10^6 CFU/g in chitosan group during the experimental period *(Fig. 1a)*.

Psychrotrophic, *Pseudomonas* and *Enterobacteriaceae* bacteria counts were higher in the control group

Table 1. *Microorganisms in bonito fillets packaged with air, vacuum, chitosan film and MA (log cfu/g)*
Tablo 1. *Kontrol, vakum, kitosan ve MA ambalajlanan Palamut balığı filetolarındaki mikroorganizmalar (log cfu/g)*

Treatments	Total Aerobic Mesophilic Bacteria	Psychrotrophic	*Pseudomonas*	*Enterobacteriaceae*	Lactic Acid Bacteria
Control	5.65±1.70 ^{a}	6.90±1.60 ^{a}	7.10±2.08 ^{a}	4.24±1.30 ^{c}	3.61±0.72 ^{a}
VP	5.13±1.42 ^{ab}	6.04±1.15 ^{ab}	5.82±1.45 ^{ab}	3.75±0.83 ^{bc}	4.11±1.10 ^{a}
Chitosan	4.01±0.85 ^{b}	5.64±1.09 ^{b}	5.57±1.48 ^{b}	2.56±0.65 ^{a}	3.52±0.59 ^{a}
MAP	4.74±1.30 ^{ab}	5.06±0.88 ^{b}	5.16±1.20 ^{b}	3.13±0.70 ^{ab}	3.70±0.75 ^{a}

(a-c) Any two means in the same column having the same letters are not significantly different at P<0.05

Table 2. *The influence of storage time on microbiological status of bonito fillets (log cfu/g)*
Tablo 2. *Depolamanın Palamut balığı filetolarında mikrobiyal duruma etkisi*

Storage Time (day)	Total Aerobic Mesophilic Bacteria	Psychrotrophic	*Pseudomonas*	*Enterobacteriaceae*	Lactic Acid Bacteria
0	2.75±0.16 ^{a}	4.18±0.03 ^{a}	3.47±0.26 ^{a}	2.05±0.05 ^{a}	2.84±0.07 ^{a}
3	4.28±0.37 ^{b}	5.20±0.77 ^{b}	4.63±0.79 ^{b}	3.17±0.68 ^{b}	2.96±0.39 ^{a}
6	4.84±0.58 ^{bc}	5.61±0.88 ^{b}	6.10±1.04 ^{c}	3.36±1.01 ^{b}	3.62±0.67 ^{b}
9	5.44±1.36 ^{cd}	6.62±1.27 ^{c}	6.98±1.31 ^{cd}	3.55±1.06 ^{bc}	3.94±0.43 ^{bc}
12	5.94±1.29 ^{d}	6.68±1.06 ^{c}	7.07±1.20 ^{cd}	4.00±1.03 ^{bc}	4.22±0.52 ^{c}
15	6.04±1.08 ^{d}	7.19±0.97 ^{c}	7.23±0.92 ^{d}	4.38±0.72 ^{c}	4.83±0.66 ^{d}

(a-d) Any two means in the same column having the same letters are not significantly different at P<0.05

than those of the chitosan and MAP groups *(Table 1)*. The highest Psychrotrophic, *Pseudomonas* and *Enterobacteriaceae* bacteria counts were determined at the last day of experiment *(Table 2)*. Those bacteria counts in fillets treated with different applications increased with length of storage at 4°C.

MAP statistically demonstrated lower counts of *Enterobacteriaceae* as compared to the control group (P<0.05) and chitosan film had the best inhibition in all groups *(Fig. 1d)*. pH values (a), TVB-N (b) and TBARS level (c) of bonito fillets with chitosan film or VP, MAP at 4°C in *Fig. 2*.

Table 3. *Some chemical properties of bonito fillets packaged with air, vacuum, chitosan film and MA*
Tablo 3. *Kontrol, vakum, kitosan ve MA ambalajlanan Palamut balığı filetolarındaki bazı kimyasal parametreler*

Treatments	pH	TVB-N (mg/100 g)	TMA (mg/100 g)	TBARS (µmol/kg)
Control	6.33±0.30 ^{a}	36.64±25.56 ^{a}	8.60±7.35 ^{a}	43.12±20.70 ^{ab}
VP	6.13±0.22 ^{b}	28.17±17.17 ^{a}	6.77±5.58 ^{a}	26.89±8.87 ^{a}
Chitosan	6.02±0.14 ^{b}	24.66±14.36 ^{a}	5.80±4.32 ^{a}	58.04±36.15 ^{b}
MAP	6.08±0.14 ^{b}	27.41±15.88 ^{a}	6.31±4.87 ^{a}	28.03±8.82 ^{a}

(a-b) Any two means in the same column having the same letters are not significantly different at P<0.05

Table 4. *The influence of storage time on some chemical status of bonito fillets*
Tablo 4. *Depolamanın Palamut balığı filetolarında bazı kimyasal parametreler üzerine etkisi*

Storage Time (day)	pH	TVB-N (mg/100 g)	TMA (mg/100 g)	TBARS (µmol/kg)
0	5.99±0.04 ^{a}	9.69±1.34 ^{a}	0.85±0.03 ^{a}	12.75±0.27 ^{a}
3	5.98±0.09 ^{a}	10.93±1.63 ^{a}	1.10±0.14 ^{a}	26.72±3.11 ^{ab}
6	6.02±0.11 ^{ab}	23.82±1.15 ^{b}	5.99±0.28 ^{b}	35.71±12.38 ^{b}
9	6.18±0.15 ^{bc}	31.30±7.66 ^{b}	7.28±1.37 ^{b}	40.10±12.13 ^{bc}
12	6.23±0.32 ^{c}	44.65±12.26 ^{c}	11.19±3.11 ^{c}	57.92±27.52 ^{cd}
15	6.44±0.20 ^{d}	54.92±12.76 ^{d}	14.79±3.30 ^{d}	60.93±32.89 ^{d}

(a-b) Any two means in the same column having the same letters are not significantly different at P<0.05

Psychrotrophic bacteria counts in the fillets of control group reached above than 10^7 CFU/g after the 6th day of storage time. However, growth of psychrotrophic bacteria in fillet packaged with chitosan film and MAP was slower than in the fillets of control group during storage and psychrotropic bacteria in the chitosan group count reached 10^7 CFU/g at the last day of experiment (15th day) *(Fig. 1b)*.

The effect of storage time x treatment on *Pseudomonas* count was important (P<0.05). *Pseudomonas* count was below 10^7 CFU/g in fillets packaged under 100% CO_2 and with chitosan film on day [15]. It reached to 10^7 CFU/g in control and vacuum packaged samples in day 6 and 9, respectively. *Pseudomonas* counts in fillets sealed with chitosan film were approximately 1 and 2 log units lower in control fillets *(Fig. 1c)*.

The use of chitosan film, vacuum and MAP during the storage time inhibited Enterobacteriaceae bacteria growth, however the differences were not significant between vacuum and control group (P>0.05). The groups packaged with chitosan film and

Treatment had no significant effect on lactic acid bacteria count *(Table 1)*. In contrast, lactic acid bacteria count was affected by storage *(Table 2)*. Lactic acid bacteria growth was slower in chitosan than the other treatments *(Fig. 1d)*. Some chemical properties of bonito fillets packaged with air, vacuum, chitosan film and MA *(Table 3)* and the influence of storage time on some chemical status of bonito fillets *(Table 4)*.

DISCUSSION

The antimicrobial properties of chitosan have been documented both *in vitro* and in situ against a number of food spoilage and pathogenic microorganisms such as *Staphylococcus aureus, Pseudomonas aeruginosa, Shigella dysenteriae, Bacillus cereus, Proteus vulgaris, Escherichia coli, Vibrio spp.* and *Salmonella typhimurium* [12,28-31]. López-Caballero [28], reported that the coating, a blend of chitosan dissolved in acid acetic and gelatin, was observed to exert an inhibitory effect on the gram-negative flora. Ouattara [31], also determined that the antimicrobial films with a chitosan matrix were able to

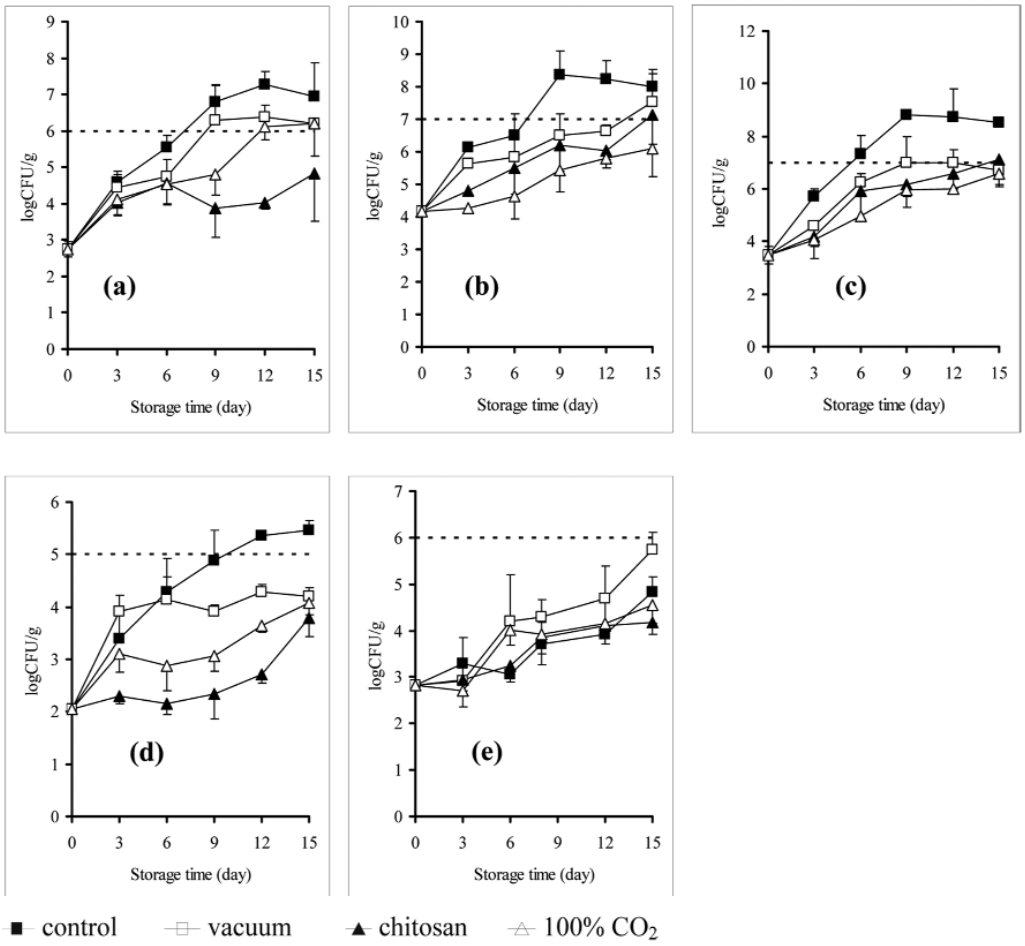

Fig 1. *Mesophilic* ***(a),*** *psychrotrophic* ***(b),*** *Pseudomonas* ***(c),*** *Enterobacteriaceae* ***(d)*** *and lactic acid bacteria* ***(e)*** *counts on bonito fillets with chitosan film or VP, MAP at 4°C. Upper areas of horizontal lines are unacceptable in each figure. Each value represents mean ±SD (n=3)*

Şekil. 1. *Palamut balığında Mezofilik* ***(a),*** *psikrotrofik* ***(b),*** *Pseudomonas* ***(c),*** *Enterobacteriaceae* ***(d)*** *and laktik asit bakteri* ***(e)*** *değerleri*

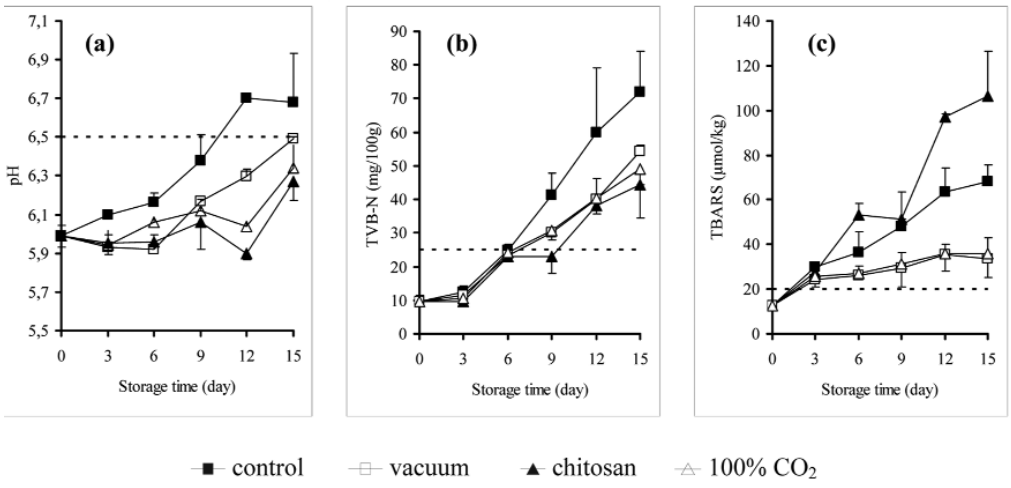

Fig 2. *pH values* ***(a),*** *TVB-N* ***(b)*** *and TBARS level* ***(c)*** *of bonito fillets with chitosan film or VP, MAP at 4°C. Upper areas of horizontal lines are unacceptable in each figure. Each value represents mean ±SD (n=3)*

Şekil. 2. *Palamut balığında kitosan, vakum, MA ve kontrol pH* ***(a),*** *TVB-N* ***(b)*** *TBARSl* ***(c)*** *değerleri*

inhibit the growth of Enterobacteriaceae. The reason for antimicrobial action of chitosan may be due to its ability to absorb nutrients of bacteria and thus inhibition of their growth [32] or its interaction with negatively charged microbial cell membranes, resulting in increased permeability of the membranes [33]. The antimicrobial effect of chitosan depends on several factors including in pH value of product and storage temperature. This effect is more pronounced in substrate with pH values lower than 6.3 which is pK_a value of chitosan [33]. Taking into account that pH values of the fillets wrapped with chitosan film ranged between 5.94 and 6.34 during the storage period *(Fig. 2a),* it shows that growth inhibition of microorganism would be stronger if some acidifying substance (acetic acid) was added. With regards to influence of storage temperature, it was reported that low temperatures such as the 4°C used in this study were optimum for enhancing antimicrobial activity of the chitosan [34].

Our results do not support the conclusions of Jeon [35] and Roller [34] that chitosan shows generally a stronger bactericidal effect on Gram-positive such as LAB than Gram-negative bacteria. In contrast, we observed a higher resistance of Gram positive bacteria. On the other hand, Lee [36] reported that chitosan showed a bifidogenic effect at concentration between 0.1 and 0.5% and it had growth stimulation effect on *Lactobacillus casei* and *Lactobacillus brevis* at 0.1%. However, contrary, it was determined that chitosan caused a decrease in bacteroides, bifidobacteria and clostridia [37]. Differences in inhibitory or stimulation effects obtained by different authors are probably due to variations in experimental materials and conditions, such as the methods used, chitosan applied, or the medium pH.

Storage time x treatment interaction had significant effects ($P<0.05$) on pH values. It slightly decreased until day 3 for all samples except control group, whereas after 3 days there was a gradual increase *(Fig. 2a).* This is associated mostly with increase of Gram-negative bacteria populations [38] such as Pseudomonas and *Enterobacteriaceae* cause protein and amino acid degradation resulting in formation of ammonia and consequent pH increase [39]. The lowest average pH value was obtained from fillets wrapped with chitosan in the present study. This can be attributed that release of acetic acid is limited from chitosan matrix. Since Ouattara [31], reported that 2-22% of acetic acid remained in chitosan after 168 h of storage.

While treatment had no significant effect on total volatile bases nitrogen (TVB-N), an index of spoilage ($P>0.05$), storage time x treatment interaction had significant effects on TVB-N value ($P<0.05$) *(Fig. 2b).* On the 9th day of storage, TVB-N values of the control, vacuum and MAP groups were above 25 mg/100 g. In contrast, TVB-N values decreased distinctly by wrapping chitosan film. Similarly, Jeon [18], reported reduction of 35-50% in the formation TVB-N at the end of a 12 days storage period using whole cod fillets and different types of soluble chitosan coatings.

Atlantic bonito is a fatty fish (10% fat content) species; therefore oxidative changes are very important in the lipids, which are highly vulnerable to oxidation. Storage time x treatment interaction had significant effects ($P<0.05$) on TBARS values. While the TBARS values of vacuum and modified atmosphere groups were close during the storage, they increased rapidly after day 3 in control and chitosan groups *(Fig. 2c).* The anti-oxidative effect of the chitosan film was not observable in fish, possibly because the acid used for solubilization affected the antioxidative feature of chitosan film in the present study. Since, it was reported that the acidic condition contribute to lipid peroxidation by maintaining iron as ferrous ion [35] and iron catalysed oxidation has been reported to be pH-sensitive and to be most active under acidic conditions [40]. In our previous study, it was reported that TBARS values appeared to be negatively affected by the application of the different concentration of lactic acid [41].

Similarly to our results, it was reported that minimum TBARS values were recorded in trout fillet packaged with MA (100% CO_2) and vacuum [42]. However, Jeon [18], found lower content of TBARS in chitosan-coated herring and cod samples than those of the uncoated samples throughout the storage time [28]. In addition effectiveness of chitosan of different viscosity (14 cP, 57 cP and 360 cP) on oxidative stability of cooked, comminuted flesh of herring (Clupea harengus), was investigated by Kamil [21], who observed that among the different viscosity chitosans, 14 cP chitosan was more effective than the higher viscosity chitosans in preventing lipid oxidation in the herring flesh model system.

In conclusion, the chitosan film has the potential to be used as active biodegradable film with strong anti-microbial effects. However, further works are needed to investigate the effect of the chitosan film on rancidity in lean fish and the combined application of acidic natural antioxidants and chitosan during the preparing chitosan film due to the high TBARS values recorded in Atlantic bonito fillets.

REFERENCES

1. Baird-Parker TC: The Microbiological Safety and Quality of Food, Aspen Publishers Inc, Gaithersburg, 2000.

2. Barnett HJ, Conrad JW, Nelson RW: Use of laminated high and low density polyethylene flexible packaging to store trout (Salmo gairdneri) in a modified atmosphere. *J Food Protect,* 50, 645- 651, 1987.

3. Farber JM: Microbial aspects of modified-atmosphere packaging technology-a review. *J Food Protect,* 54, 58-70, 1991.

4. Parkin KL, Wells MJ, Brown WD: Modified atmosphere storage of rockfish fillets. *J Food Sci,* 47, 181- 184, 1981.

5. Reddy NR, Shreiber CL, Buzard KS, Skinner GE, Armstong DJ: Shelf life of fresh tilapia fillets packaged in high barrier film with modified atmospheres. *J Food Sci,* 59, 260-264, 1994.

6. Stammen K., Gerdes D, Caporaso F: Modified atmosphere packaging of seafood. *Int J Food Sci Nutr,* 29, 301-331, 1990.

7. Gimenez B, Roncales P, Beltran JA: Modified atmosphere packaging of filleted rainbow trout. *J Agric Food Chem,* 82, 1154-1159, 2002.

8. Randell K, Ahvenainen R, Hattula T: Effect of the gas/product ratio and CO_2 concentration on the shelf-life of MA packed fish. *Packaging Technol Sci,* 8, 205- 218, 1995.

9. Church N: MAP fish and crustaceans-sensory enhancement. *Food Sci Technol Today,* 12, 73-83, 1998.

10. Statham JA: Modified atmosphere storage of fisheries products: The state of the art. *Food Technol Aust,* 36, 233-239, 1984.

11. Lindley MG: The impact of food processing on anti-oxidants in vegetable oils, fruits and vegetables. *Trends Food Sci Technol,* 9, 336-340, 1998.

12. Shahidi F, Arachchi JKV, Jeon YJ: Food applications of chitin and chitosans. *Trends Food Sci Technol,* 10, 37-51, 1999.

13. Tassou CC, Drosino EH, Nychas GJE: Inhibition of resident microbial flora and pathogen inocula on cold fresh fish fillets in olive oil, oregano, and lemon juice under modified atmosphere or air. *J Food Protect,* 59, 31-34, 1995.

14. Borderías AJ, Sánchez-Alonso I, Pérez-Mateos M: New applications of fibres in foods: Addition to fishery products. *Trends Food Sci Technol,* 16, 458-465, 2005.

15. No HK, Park NY, Lee SH, Meyers SP: Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *Int J Food Microbiol,* 74, 65-72, 2002.

16. Koide SS: Chitin-chitosan: Properties, benefits and risks. *Nutr Res,* 18, 1091-1101, 1998.

17. Cuero RG: Antimicrobial action of exogenous chitosan, Birkhauser, Basel, 1999.

18. Jeon YJ, Kamil JYVA, Shahidi F: Chitosan as an edible invisible film for quality preservation of herring and Atlantic cod. *J Agri Food Chem,* 20, 5167-5178, 2002.

19. Benjakul S, Visessanguan W, Phatchrat S, Tanaka M: Chitosan affects transglutaminase-induced surimi gelation. *J Food Biochem,* 27, 53-66, 2003.

20. No HK, Lee KS, Meyers SP: Correlation between physico-chemical characteristics and binding capacities of chitosan products. *J Food Sci,* 65, 1134-1137, 2000.

21. Kamil JYVA, Jeon YJ, Shahidi F: Antioxidative activity of chitosans of different viscosity in cooked comminuted flesh of herring *(Clupea harengus). Food Chem,* 79, 69-77, 2002.

22. Anonymous: Unterschung von Lebensmitteln Bestimmung des Gehaltes von flqchtigen stickstoffhaltigen Basen (TVB-N) in Fischen und Fischerzeugnissen Referenzerfahren. Amtliche Sammlung von Untersuchunsverfahren nach 35 LMBG, 80, 1988.

23. Lemon DW: An improved TBA test for rancidity. New Series Circular, Halifax, Nova Scotia. 1975.

24. González-Fandos E, Villarino-Rodríguez A, García-Linares MC, García-Arias MT, García-Fernández MC: Microbiological safety and sensory characteristics of salmon slices processed by the sous vide method. *Food Cont,* 16, 77-85, 2005.

25. McMeekin TA, Olley JN, Ross T, Ratkowsky DA: Predictive Microbiology. Theory and Application. Research Studies Press, 1993.

26. Duncan DB: Multiple range and multiple F-tests. *Biometrics,* 11, 313-323, 1971.

27. Ergün G, Aktaş S: ANOVA modellerinde kareler toplamı yöntemlerinin karşılaştırılması. *Kafkas Univ Vet Fak Derg,* 15 (3): 481-484, 2009.

28. López-Caballero ME, Gómez-Guillén MC, Pérez-Mateos M, Montero P: A chitosan-gelatin blend as a coating for fish patties. *Food Hydrocolloid,* 19, 303-311, 2005.

29. Arashisar S, Hisar O, Kaban G, Kaya M, Alak G: Effect of chitosan coating on chemical and microbiological properties of Atlantic bonito *(Sarda sarda)* fillets. *J Food Sci Technol, 46,* 380-383, 2009.

30. Sudarshan NR, Hoover DG, Knorr D: Antibacterial action of chitosan. *Food Biotechol,* 6, 257-272, 1992.

31. Ouattara B, Simard RE, Piette G, Begin A, Holley RA: Inhibition of surface spoilage bacteria in processed meats by application of antimicrobial films prepared with chitosan. *Int J Food Microbiol,* 62, 139-142, 2000.

32. Knorr D: Recovery and utilization of chitin andchitosan in food processing waste management, *Food Technol,* 45, 114-122, 1991.

33. Helander IM, Nurmiaho-Lassila EL, Ahvenainen R, Rhoades J, Roller S: Chitosan disrupts the barrier properties of the outer membrane of gram-negative bacteria. *Internat J Food Microbiol,* 71, 235-244, 2001.

34. Roller S, Covill N: The antimicrobial properties of chitosan in mayonnaise and mayonnaise-based shrimp salads. *J Food Protect,* 63, 202-209, 2000.

35. Jeon YJ, Park PJ, Kim SK: Antimicrobial effect of chitooligosaccharides produced by bioreactor. *Carbohydr Polym,* 44, 71-76, 2001.

36. Lee BJ, Hendricks DG: Antioxidant effect of L-carnosine on liposomes and beef homogenates. *J Food Sci,* 62, 241-244, 1997.

37. Vernazza CL, Gibson GR, Rastall RA: *In vitro* fermentation of chitosan derivatives by mixed cultures of human fecal bacteria. *Carbohydr Polym,* 60, 539-545, 2005.

38. Verma SP, Sahoo J: Improvement in the quality of ground chevon during refrigerated storage by tocopherol acetate preblending. *Meat Sci,* 56, 403-413, 2000.

39. Nychas GJE, Drosinos E, Board RG: Chemical changes in

stored meat, Blackie Academic and Professional, London. 1998.

40. Liu HP: Catalysts of lipid peroxidation in meats. 1: Linoleate peroxidation catalyzed by MetMb or Fe(ii)-EDTA. *J Food Sci,* 35, 590-592, 1970.

41. Arashisar Ş, Hisar O, Yanık T: The effects of lactic acid on chemical properties of rainbow trout *(Oncorynchus mykiss)* fillets. *J Food Sci Technol,* 3, 495-497, 2005.

42. Arashisar Ş, Hisar O, Kaya M, Yanik T: Effects of modified atmosphere and vacuum packaging on microbiological and chemical properties of rainbow trout *(Oncorynchus mykiss)* fillets. *Int J Food Microbiol,* 97, 209-124, 2004.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S81-S86, 2010
DOI:10.9775/kvfd.2009.1520

RESEARCH ARTICLE

Set Type Yoghurt Production by Exopolysaccharide Producing Turkish Origin Domestic Strains of *Streptococcus thermophilus* (W22) and *Lactobacillus delbrueckii ssp. bulgaricus* (B3)

Ayşe GÜRSOY * Fügen DURLU-ÖZKAYA ** Filiz YILDIZ * Belma ASLIM ***

* Ankara University, Faculty of Agriculture, Department of Dairy Technology, TR-06110 Dışkapı, Ankara - TURKEY
** Gazi University, Tourism Faculty, TR-06830 Gölbaşı, Ankara - TURKEY
*** Gazi University, Faculty of Science and Art, Department of Biology, TR-06500 Ankara - TURKEY

Makale Kodu (Article Code): KVFD-2009-1520

Summary

Lactobacillus delbrueckii spp. bulgaricus (B3) and *Streptococcus thermophilus* (W22) with high exopolysaccharide (EPS) production were combined with commercial culture in various percentages and used in yoghurt production. Lactic acid and acetaldehyde contents, titratable acidity, tyrosine, viscosity and consistency values, and EPS level were measured throughout storage for twenty one day with 10-day intervals. The yoghurt sample produced with commercial culture only (control) had the highest level of acidity. Tyrosine content of the yoghurt produced by wild strains (sample D) was higher than that of others, whereas acetaldehyde and EPS levels were lower. The domestic strains did not influence the viscosity of yoghurt significantly (P>0.01). On contrary, the gel firmness of yoghurts produced with *Lactobacillus delbrueckii spp. bulgaricus* (B3) was lower than the other samples. The yoghurt sample made by isolated strains only had the highest viscosity values. Yoghurt produced by using isolated strains was found not to be acceptable by the panellists.

Keywords: *Exopolysaccharides, Yoghurt, Streptococcus thermophilus, Lactobacillus delbrueckii ssp. bulgaricus*

Ekzopolisakkarit Üretimi Yüksek Yerli *Streptococcus thermophilus* (W22) ve *Lactobacillus delbrueckii ssp. bulgaricus* (B3) Suşları ile Set Tip Yoğurt Üretimi

Özet

Bu çalışmada, yoğurt üretiminde ekzopolisakkarit üretimi yüksek olan yerli *Lactobacillus delbrueckii ssp. bulgaricus* (B3) ve *Streptococcus thermophilus* (W22) bakterileri ticari kültürle farklı oranlarda karıştırılarak kullanılmıştır. Üretilen yoğurtlar 21 gün süreyle buzdolabı koşullarında depolanmış ve 10 gün arayla analize tabi tutulmuştur. Deneme örneklerinde laktik asit, asetaldehit, titrasyon asitliği, tirozin değeri ve ekzopolisakkarit miktarı belirlenmiş; ayrıca konsistens ve viskozite ölçümleri de alınmıştır. Sadece ticari kültürün kullanıldığı kontrol yoğurdunun asitliği diğer örneklerden daha yüksek düzeyde bulunmuştur. Sadece izole suşların kullanıldığı örnek ise tirozin içeriği açısından diğerlerinden daha yüksek değer vermiştir. Buna karşın bu örneğin asetaldehit ve ekzopolisakkarit içeriği daha düşük çıkmıştır. Yerli suşlar yoğurdun viskozitesini etkilememiş, *Lactobacillus delbrueckii ssp. bulgaricus* (B3)'ün kullanıldığı yoğurtta pıhtı sıkılığı daha düşük bulunmuştur. Sadece izole suşlar kullanılarak üretilen yoğurdun (D örneği) viskozitesi diğerlerinden bir miktar yüksek bulunmuş ve söz konusu örnek yapılan duyusal değerlendirme sonucunda panelistlerce beğenilmemiştir.

Anahtar sözcükler: *Ekzopolisakkarit, Yoğurt, Streptococcus thermophilus, Lactobacillus delbrueckii ssp. bulgaricus*

İletişim (Correspondence)
☎ +90 312 5961353
✉ gursoy@agri.ankara.edu.tr

INTRODUCTION

Lactic acid bacteria (LAB) have been extensively studied in the food industry, not only in view of their ability to acidify and hence preserve food products from spoilage, but as well for their contribution to the organoleptic properties of the final fermented food [1,2]. Among them a combination of *Streptococcus thermophilus* and *Lb. delbrueckii ssp. bulgaricus* which grow synergistically are widely used as starter cultures for the production of yoghurt [3-5]. It is widely known that some LAB like *Streptococcus thermophilus* and *Lb. delbrueckii ssp. bulgaricus* secrete EPS [2,3,6,7]. The use of cultures producing EPS play an important industrial role in the texture development of yoghurts and other fermented milks, low fat cheeses and dairy desserts [3,6,8].

In general, the food industry is particularly interested in natural viscosifiers and texture enhancers, the so-called biothickeners. These are mostly plant carbohydrates (e.g., locust bean gum, starch, alginate, pectin and guar gum), animal hydrocolloids (e.g., gelatin and casein), or bacterial biopolymers (e.g. xanthan and gellan) [1]. However most of the plant carbohydrates used are chemically modified to improve their rheological properties. Their use is hence strongly restricted, especially in the European Union (E numbers) [6]. The use of cultures producing EPS increases resistance of yoghurt coagulum to thermal and physical shocks, and play an important role in achieving satisfactory firmness and apparent viscosity of yoghurt [1,8]. EPS also effective in protecting microbial cells against phagocytosis, phage attacks, antibiotics, toxic compounds, osmotic stress and bacteriocins [2,9-11]. With these considerations in mind, it was aimed to produce set-type yoghurt by using EPS producing Turkish origin domestic strains of *Streptococcus thermophilus* and *Lb. delbrueckii ssp. bulgaricus,* and to evaluate the quality of the resulting products.

MATERIAL and METHODS

Culture and Growth Conditions

The strains of *Lb. delbrueckii ssp. bulgaricus* B3 and *Streptococcus thermophilus* W22 used in this study were isolated from village type yoghurt by Aslim et al.[12]. The strains isolated were identified as *Lb. delbrueckii ssp. bulgaricus* B3 and *Streptococcus thermophilus* W22 on the basis of morphological and biochemical characteristics, and 16S rDNA sequence analysis were done [2]. The criteria for selection of the strains were based on their quantity of EPS production. Each strain was grown in skim milk (10 g/100 ml) and incubated at 40°C for 18 h. In addition, the control yoghurt was produced incorporating a commercial blend of starter culture coded TM-081 (Rhodia-EZAL group).

Production of Yoghurt

Fresh cow milk was obtained from the Ankara University, Faculty of Agriculture, Department of Dairy Technology. The gross composition of raw milk was: 7.38±0.53 titratable acidity (°SH), 6.55±0.09 pH, 3.08±0.06 total fat and 12.66±0.22 total solids. The raw milk was heated up to 40°C and skimmilk powder was added so that total solids content was increased to 15%. After homogenization at 60°C at 200 kg/cm^2, milk was heat treated at 85°C for 20 min and cooled to 42°C, and then divided in four equal portions. Each portion was inoculated with 3% (v/v) commercial starter (A); 1.5% (v/v) commercial starter plus 1.5% (v/v) *Lb. delbrueckii ssp. bulgaricus* B3 (B); 1.5% (v/v) commercial starter plus 1.5% (v/v) *Streptococcus thermophilus* W22 (C); 1.5% (v/v) *Lb. delbrueckii ssp. bulgaricus* B3 and 1.5% (v/v) *Streptococcus thermophilus* W22 (D). Inoculated milk was, then, incubated at 42°C for 4 to 5 h until pH 4.6 was attained. Following incubation, the samples were stored at 4°C for 21 days. Analyses were performed in the experimental yoghurts after 1, 11 and 21 days of storage. The experiment was repeated three times in duplicate.

Chemical Analysis

The dry matter, titratable acidity and total fat contents were determined by the methods given in Turkish national standard for yoghurt [13]. The spectrophotometric methods were followed for the analysis of lactic acid [14] and tyrosine contents [15]. Acetaldehyde content was determined according to Lees and Jago [16]. The firmness of yoghurt curd was determined by a penetrometer (Stanhope-Seta Surrey, England) using the cone-form penetration body with an apical angle of 45°C, and a weight of 72.5 g. The depth of penetration was measured at 5 s at a product temperature of 7±1°C. HAAKE-Viscometer VT 181/VT 24 was employed for determination of viscosity. Sensory properties were evaluated by 10 experienced panellists according to the scoring sheet given in Turkish national standard [13]. Analysis of variance was performed using MINITAB [17], and the results were analyzed as a randomized plot design. Means were compared by the least significant differences method [18,19].

Isolation and Quantification of EPS

The yoghurt samples were boiled at 100°C for 10 min. After cooling, they were treated with 17 ml/100 ml of 85% trichloracetic acid solution and centrifuged [20].

After removal of the cells and protein by centrifugation, the EPS was precipitated with ethanol. The EPS was recovered by centrifugation at 4°C at 14000 rpm for 20 min. Total EPS (expressed as mg l^{-1}) was estimated in each sample by phenol-sulphuric method [21] using glucose as a standard [22].

RESULTS

Total solids of the samples A, B, C and D were as follows respectively (g 100 g^{-1}); 14.60±0.103, 14.51±0.108, 14.51±0.106, 14.48±0.092 at the beginning of storage period. The fat contents of the samples (g 100 g^{-1}) were found to be 3.03±0.033, 2.96±0.088, 3.00±0.000, 3.00±0.000 respectively.

Table 1 describes some chemical properties of yoghurt during the storage. Lactic acid value and the titratable acidity of yoghurt samples increased throughout the storage period. There was no significant difference between the samples with regard to titratable acidity and lactic acid (P>0.01).

There was a significant difference (P<0.01) between yoghurt samples in terms of tyrosine contents of the samples. The highest tyrosine value was obtained from the sample D, where local strains of yoghurt bacteria with high EPS production were used. This sample was followed by sample B (including *Lb. delbrueckii ssp. bulgaricus* B3).

No significant difference was noted between the samples with regard to acetaldehyde levels (P>0.01). In all samples, the highest acetaldehyde content was recorded on the 11th day of storage, and then decreased.

EPS producing starters are used to improve physical properties of yoghurt. In this study, it was observed that there was no statistically significant difference between the samples with regard to EPS concentrations (P>0.01). However, the highest EPS concentrations was recorded on the 11th day of storage from the sample B (256 mg/L). The sample D had the lowest EPS concentrations (175 mg/L). Given the average of EPS values, it was shown that sample B (219 mg/L) and C (220 mg/L) were relatively higher than the sample A (196 mg/L).

Table 1. *Some chemical and physical properties of yoghurt samples*
Tablo 1. *Yoğurt örneklerinin fiziksel ve kimyasal özellikleri*

Properties	Storage (Day	Treatments			
		A	B	C	D
Titratable Acidity (ºSH)	1 11 21	49.25±2.88 55.40±2.85 56.39±3.07	47.47±1.71 52.74±2.52 54.26±2.75	47.02±3.58 50.67±4.87 52.36±5.45	45.87±3.69 48.00±3.73 50.05±4.80
Lactic Acid (g/100g)	1 11 21	0.83±0.34 0.89±0.41 0.98±0.84	0.82±0.39 0.87±0.41 0.90±0.27	0.79±0.61 0.84±0.53 0.86±0.77	0.76±0.49 0.75±0.62 0.79±0.83
Tyrosine Value (mg/g)	1 11 21	0.278±0.017 ^{ab} 0.312±0.027 ^{ab} 0.400±0.011 ^{ab}	0.291±0.017 ^{b} 0.356±0.022 ^{b} 0.379±0.028 ^{b}	0.272±0.013 ^{ab} 0.300±0.030 ^{ab} 0.346±0.015 ^{ab}	0.344±0.024 ^{a} 0.410±0.064 ^{a} 0.471±0.026 ^{a}
Acetaldehyde (ppm)	1 11 21	17.05±4.70 22.68±2.17 18.87±0.72	18.41±4.32 23.55±1.61 16.57±1.47	14.92±4.92 21.60±2.68 14.96±0.17	12.21±1.65 16.78±0.95 15.25±1.41
EPS (mg/l)	1 11 21	206±13.5 200±13.7 181±42.7	215.67±7.67 265.30±17.6 176.30±68.2	243.33±6.17 251.00±31.9 166.70±37.4	188.0±22.01 174.7±30.80 169.7±73.60
Penetrometer Value (x1/10 mm)	1 11 21	306.33± 6.33 280.00±10.40 286.00± 5.69	309.67±7.84 276.00±12.30 279.30±10.10	305.33±07.80 286.70±14.70 285.00±13.70	311.33±9.53 282.70±13.50 286.30±14.30
Viscosity (cP)	1 11 21	1441.7±98.2 1683.0±10.1 1758.0±13.1	1275±94.6 1475±13.9 1575±13.9	1308.3±79.5 1508.0±21.4 1558.0±20.4	1275.0±14.40 1536.7±7.26 1512.7±7.22

A: Commercial culture (3%)
B: Commercial culture (1.5%) plus isolated Lb. delbrueckii ssp. bulgaricus B3 (1.5%)
C: Commercial culture (1.5%) plus isolated Streptococcus thermophilus W22 (1.5%)
D: Isolated Lb. delbrueckii ssp. bulgaricus B3 (1.5%) plus isolated Streptococcus thermophilus W22 (1.5%)
a, b, ab, Means within the same row with different superscripts differ significantly (P<0.01)

The good quality of yoghurt is characterized with high firmness and low whey separation. In the present study, there was no significant difference between the yoghurt samples in terms of gel firmness (P>0.01). The EPS(+) strains seemed to be ineffective on gel firmness.

In the yoghurt samples, the viscosity measurements were in good correlation with the gel firmness readings. It is thought that the slight increase in viscosity was caused by the EPS(+) strains.

Given the results of sensory evaluation *(Table 2),* we observed that there was no difference between the samples in terms of apperance and odour. On examination of structure and texture, the samples B and C, in which strains of *Lb. delbrueckii ssp. bulgaricus* B3 along with commercial culture and *Streptococcus thermophilus* W22 were used had higher scores than the others. The samples B and C most were given higher scores by the panel group, whereas sample D, was found to be too sweet.

Table 2. *Organoleptic properties of yoghurt samples (Mean ± SE)*
Tablo 2. *Yoğurt örneklerinin duyusal nitelikleri (ortalama ± standart hata)*

Treatments	Storage (Day)	Appearance (Max 5 Points)	Body and Texture (Max 5 Points)	Odour (Max 5 Points)	Taste (Max 5 Points)
A	1	5.000±0.000	4.233+0.122	4.933±0.067	4.000±0.115
	11	4.933±0.067	3.933±0.067	5.000±0.000	3.667±0.167
	21	4.933±0.067	3.767±0.145	4.600±0.231	3.500±0.252
B	1	4.867±0.133	4.300±0.252	5.000±0.000	4.200±0.306
	11	5.000±0.000	4.233±0.033	5.000±0.000	4.167±0.186
	21	4.633±0.186	3.933±0.067	4.700±0.153	3.933±0.296
C	1	4.933±0.067	4.467±0.033	5.000±0.000	4.467±0.333
	11	5.000±0.000	4.300±0.153	5.000±0.000	4.133±0.133
	21	4.400±0.100	3.967±0.203	4.700±0.153	3.933±0.067
D	1	5.000±0.000	4.133±0.133	5.000±0.000	3.833±0.203
	11	4.933±0.067	4.167±1.012	5.000±0.000	3.833±0.441
	21	4.833±0.167	3.900±0.265	4.833±0.167	3.467±0.808

A: Commercial culture (3%)
B: Commercial culture (1.5%) plus isolated Lb. delbrueckii ssp. bulgaricus B3 (1.5%)
C: Commercial culture (1.5%) plus isolated Streptococcus thermophilus W22 (1.5%)
D: Isolated Lb. delbrueckii ssp. bulgaricus B3 (1.5%) plus isolated Streptococcus thermophilus W22 (1.5%)

DISCUSSION

The development of acidity is important not only form milk fermentation, but also necessary for a well-balanced aroma, texture and flavor of yoghurt. Acid development by domestic strains was poor (especially sample D) in comparison with the commercial starter. Özer and Atasoy [23] used viscous and non-viscous commercial strains of *Lb. delbrueckii ssp. bulgaricus* (B3) and *Stretococcus thermophilus* (CH-1) in yoghurt production. The authors determined the levels of titratable acidity and lactic acid in yoghurt produced by viscous culture as 44°SH and 0.62 g 100 g^{-1} respectively. These figures were reported to be 53.4°SH and 0.77 g 100 g^{-1} in yoghurt produced by non-viscous culture.

Although lactic acid bactreria are generaly weakly proteolytic compared with other groups of bacteria such as Bacillus, Proteus, Pseudomonas, and coliforms, some strains of LAB show high proteolytic activity in many fermented dairy products [24]. Proteolytic activity of the starter used contributes to texture development of the final product. Proteolytic ability of lactic acid bacteria is dependent on species and strains. It is known that the proteolytic activity of *Lb. delbrueckii ssp. bulgaricus* is higher than that of *Streptococcus thermophilus.* In our research, the highest tyrosine value was obtained from the sample D. Özer and Atasoy [23], found that yoghurt produced by viscous cultures provided a higher level of tyrosine than that of yoghurt produced by non-viscous cultures. Proteolytic activities of starter bacteria may have some adverse effects on fermented milk. In yoghurt, production of bitter peptides is largely attributed to proteolysis by *Lb .delbrueckii ssp. bulgaricus* during storage.

Yoghurt sample produced with viscous and/or domestic culture had less acetaldehyde level than those manufactured with mixed or only commercial yoghurt bacteria. The acetaldehyde production ability of EPS(-) strains of yoghurt bacteria is high,

whereas acetaldehyde production capacity in ropy or viscous strains of the yoghurt bacteria decreases considerably [23,25,26].

It was reported that the amount of EPS produced by the LAB ranged from 150 to 600 mg/L, depending on strains under optimal culture conditions [6,9,27]. In sample D, where only domestic strains were, incorporated it was seen that the amount of EPS was lower than those of the other samples. Similar results were obtained by Marshall and Rawson [28], who produced yoghurt, using strains of *Streptococcus thermophilus* and *Lb. delbrueckii ssp. bulgaricus* with and without exopolysaccharide production ability and showed that the lowest level of EPS was obtained from the sample in which EPS(+) strains were used together. On the other hand, Bouzar et al.[29] showed that mixed cultures of a non-ropy strains of *Streptococcus thermophilus* and different ropy strains of *Lb. delbrueckii ssp. bulgaricus* produced EPS at a faster rate.

The firmness of the gel and the ability to retain the water are influenced by the structure of the gel formed or the type of the culture used [11]. In the present study, the EPS(+) strains seemed to be ineffective on gel firmness. In many studies, it was stated that the higher the EPS level in yoghurt produced by ropy strains, the lower the curd firmness. The EPS(+) could interfere with the association between casein micelles resulting in a less firm coagulum [28,30,31]. The gel firmness of the samples increased during the cold storage. In contras, Güzel-Seydim et al.[32] stated that penetrometer values of all samples decreased significantly during storage.

Several strains which are capable of forming EPS, were isolated and analyzed [33]. In present study, viscosity of samples produced by the domestic strains was found to be higher than that of control sample. Some researcher explained that, there was no clear correlation between the EPS concentration and viscosity, and polysaccharide type was far more important than EPS quantity in terms of viscosity. It was reported that the formation of protein strand and protein-protein bond development is partly prevented by excessive formation of polysaccharide flaments attached to the protein matrix and thus reducing rigidity of the yoghurt gel [9,28,30,34].

The primary function of lactic cultures used to manufacture of yoghurt is to produce lactic acid required for the formation of the coagulum. Additionally they produce volatile compounds reponsible for the typical flavour and texture of yoghurt. Careful selection of the strains employed and good monitoring throughout the manufacturing process of yoghurt are, therefore, essential to control the metabolic end-products, final pH, flavour and aroma and texture efficiently. According the results obtained, the yoghurt sample made by domestic starter strains only had a negative result, especially in terms of sensory qualities. The use of combined cultures of commercial starter and *Lb. delbrueckii ssp. bulgaricus* B3 and/or *Streptococcus thermophilus* W22 may offer an alternative to yoghurt processors. Our findings are crucial for the dairy industry to be used domestic strains as starter culture and to be taken place in the culture collections.

REFERENCES

1. Vaningelgem F, Zamfir M, Mozzi F, Adriany T, Vancanneyt M, Swings J, De Vuyst L: Biodiversity of Exopolysaccharides produced by *Streptococcus thermophilus* strains is reflected in their production and their molecular and functional characteristics. *Appl Environ Microb,* 70, 900-912, 2004.

2. Durlu-Özkaya F, Aslim B, Ozkaya MT: Effect of exo-polysaccharides (EPSs) produced by *Lactobacillus delbrueckii ssp. bulgaricus* strains to bacteriophage and nisin sensitivity of the bacteria. *LWT-Food Sci Technol,* 40, 564-568, 2007.

3. De Vuyst L: Technology aspects related to the application of functional starter cultures. *Food Technol Biotech,* 38, 105-112, 2000.

4. Hassan AN, Corredig M, Frank JF: Capsule formation by starter cultures affects the viscoelastic properties of yoghurt during structure formation. *J Dairy Sci,* 85, 716-720, 2002.

5. Tarakçı Z: Influence of kiwi marmalade on the rheology characteristics, color values and sensorial acceptability of fruit yogurt. *Kafkas Univ Vet Fak Derg,* 16, 173-178, 2010.

6. De Vuyst L, Vanderveken F, Van de Ven S, Degeest B: Production by and isolation of exopolysaccharides from *Streptococcus thermophilus* grown in a milk medium and evidence for their growth-associated biosynthesis. *J Appl Microbiol,* 84, 1059-1068, 1998.

7. Welman AD, Maddox IS: Exopolysaccharides from lactic acid bacteria: Perspectives and challenges. *Trends Biotechnol,* 21, 269-274, 2003.

8. Ngoufack ZF, El-Noda AN, Tchouanguep FM, El-Soda M: Effect of ropy and capsular exopolysaccharides producing strain of *Lactobacillus plantarum* 162RM on characteristics and functionally of fermented milk and soft Kareish type cheese. *Afr J Biotechnol,* 3, 512-518, 2004.

9. De Vuyst L, Degeest B: Heteropolysaccharides from lactic acid bacteria. *FEMS Microbiol Rev,* 23, 153-177, 1999.

10. Sutherland IW: Polysaccharides for microbial exopolysaccharides. *Carbohyde Polym,* 38, 310-328, 1999.

11. Ruas-Madiedo P, Hugenholtz J, Zoon P: An overview of the functionality of exopolysaccharides produced by lactic acid bacteria. *Int Dairy J,* 12, 163-171, 2002.

12. Aslim B: Bazı laktik asit bakterilerinin EPS üretimlerinin belirlenmesi. TUBITAK Proje No: TBAG-2090 (IDIT 129), Ankara, 2005.

13. TSE (Türk Standartları Enstitüsü): Yoğurt, (TS 1331), Ankara, Turkey, 1989.

14. Steinsholt K, Calbert HE: A rapid colorimetric method for determination of lactic acid in milk and milk products. *Milchwissenschaft,* 31, 402-408, 1960.

15. Tunail N: A study on some important physiological properties of lactic acid bacteria isolated from White brined cheese used as starter. *Associate Proffesor Ship Thesis,* Ankara, 1978.

16. Lees GL, Jago GR: Methods for the estimation of acetaldehyde in cultured dairy products. *Aust J Dairy Technol,* 24, 181-185, 1969.

17. Ryan BF, Joiner BL, Ryan TA Jr: Minitab Handbook. PWS-KENT Publishing Company, Boston, USA, 1985.

18. Düzgüneş O, Kesici T, Kavuncu O, Gürbüz F: Araştırma ve Deneme Metotları (İstatistik Metotları II). Ankara Üniv Ziraat Fak Yayınları, 1021, Ankara Üniv Basımevi, Ankara, 1987.

19. Ergün G, Aktaş S: ANOVA modellerinde kareler toplamı yönteminin karşılaştırılması. *Kafkas Univ Vet Fak Derg,* 15, 481-484, 2009.

20. Frengova GI, Simova ED, Beshkova DM, Simov ZI: Production and monomer composition of exopolysaccharides by yoghurt starter cultures. *Can J Microbiol,* 46, 1123-1127, 2000.

21. Dubois M, Gilles KA, Hamilton JK, Pebers PA, Smith F: Colorimetric Method for determination of sugars and related substances. *Anal Chem,* 28, 350-356, 1956.

22. Torino MI, Taranto MP, Sesma F, De Valdez GF: Heterofermentative pattern and exopolysaccharide production by *Lactobacillus helveticus* ATCC 15807 in response to environmental pH. *J Appl Microbiol,* 91, 846-852, 2001.

23. Özer B, Atasoy F: Effects of addition of amino acids, treatment with ß-galactosidase and use of of heat-shocked cultures on the acetaldehyde level in yoghurt. *Int J Dairy Technol,* 55, 166-170, 2002.

24. Rajagopal SN, Sandine WE: Associative growth and proteolysis of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in skim milk. *J Dairy Sci,* 73, 894-899, 1990.

25. Robinson RK, Tamime AY, Chubb LW: Acetaldehyde as an indicator of flavour intensity in yoghurt. *The Milk Industry,* 79, 4-6, 1977.

26. Tamime AY, Robinson RK: Yoghurt Science and Technology. 3rd ed., Woodhead Published Ltd., Cambridge, England, 1999.

27. Duboc P, Mollet B: Applications of exopolysacharides in dairy industry. *Int Dairy J,* 11, 759-768,2001.

28. Marshall VM, Rawson HL: Effect of exopolysaccharide-producing strains of thermophilic lactic acid bacteria on the texture of the stirred yoghurt. *Int J Food Sci Tech,* 34, 137-143, 1999.

29. Bouzar F, Cerning J, Desmazeaud M: Exopolysaccharide production and texture-promoting properties of mixed-strain starter cultures in yoghurt production. *J Dairy Sci,* 80, 2310-2317, 1997.

30. Hassan AN, Frank JF, Schmidth KA, Shalabi SI: Textural properties of yoghurt made with encapsulated nonropy lactic cultures. *J Dairy Sci,* 79, 2098-2103, 1996.

31. Hess SJ, Roberts RF, Ziegler GR: Rheological properties of nonfat yoghurt stabilized using *Lactobacillus delbrueckii ssp. bulgaricus* producing exopolysaccharide or using commercial stabilizer system. *J Dairy Sci,* 80, 252-263, 1997.

32. Güzel-Seydim ZB, Sezgin E, Seydim AC: Influence of exopolysaccharide producing cultures on the quality of plain set type yoghurt. *Food Control,* 16, 205-209, 2005.

33. Grobben GJ, Chin-Joe I, Kitzen V, Boels IC, Boer F, Sikkema J, Smith MR, De Bont JAM: Enhancement of exopolysaccharide production by *Lactobacillus delbrueckii subsp. bulgaricus* NCFB 2772 with a simplified defined medium. *Appl Environ Microb,* 64, 1333-1337, 1998.

34. Sebastiani H, Zelger G: Texture formation by thermophilic lactic acid bacteria. *Milchwissenschaft,* 53, 15-20, 1998.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S87-S91, 2010
DOI:10.9775/kvfd.2009.1639

RESEARCH ARTICLE

Determination of Aflatoxin M1 Levels in Some Cheese Types Consumed in Erzurum - Turkey

Meryem AYDEMİR ATASEVER * Gülşah ADIGÜZEL *
Mustafa ATASEVER * Korhan ÖZTURAN **

* Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Ataturk University, TR-25240 Erzurum - TURKEY
** Nineth Army Cops "A" Type Food Control Detachment Commandery, TR-25200 Erzurum - TURKEY

Makale Kodu (Article Code): KVFD-2009-1639

Summary

In this study the total 304 cheese samples (85 white cheese, 75 kashar cheese, 62 civil cheese, 82 cream cheese) were examined in terms of AFM1. The AFM1 content and concentrations of the samples were researched by competitive ELISA method. Determinable limit was 50 ng/kg and it was determined that white cheese samples included 82.4% AFM1, kashar cheese samples 80%, civil cheese samples 19.4% and cream cheese samples 84.2%. According to European Commission limit (250 ng/kg), the sample incidence exceeding the acceptable limits were 27.1%, 34.7%, 17.1% in white cheese, kashar cheese and cream cheese samples, respectively. The sample ratio exceeding the limits regulated by Turkish Food Codex (500 ng/kg) was determined in white cheese, kashar cheese and cream cheese samples as 16.5% (14/85), 14.7% (11/75) and 6.1% (5/82) respectively, any sample exceeding these limits was not met in civil cheese samples. As understood from these results, high AFM1 level determined in some cheese types is an important problem threatening the public health in Turkey.

Keywords: *AFM1, Cheese, ELISA*

Türkiye (Erzurum)'de Tüketilen Bazı Peynir Çeşitlerinde Aflatoxin M1 Seviyesinin Belirlenmesi

Özet

Bu çalışmada toplam 304 peynir örneği (85 beyaz peynir, 75 kaşar peynir, 62 civil peynir, 82 krem peynir) AFM_1 yönünden incelendi. Örneklerin AFM_1 içeriği ve konsantrasyonu kompetitiv ELISA metoduyla araştırıldı. AFM_1'in belirlenebilir limiti 50 ng/kg olup, beyaz peynir örneklerinin %82.4, kaşar peynir örneklerinin %80, civil peynir örneklerinin %19.4 ve krem peynir örneklerinin ise %84.2'ünde bu maddeye rastlandı. Avrupa Komisyonu'na göre AFM_1 yönünden yasal limitleri (250 ng/kg) aşan numune oranı beyaz peynir, kaşar peynir ve krem peynir örneklerinde sırasıyla %27.1, %34.7, %17.1 olarak belirlendi. Türk Gıda Kodeksi'ne göre yasal limitleri (500 ng/kg) aşan numune oranı beyaz peynir, kaşar peynir ve krem peynir örneklerinde sırasıyla %16.5 (14/85), %14.7 (11/75) ve %6.1 (5/82) olarak belirlenirken civil peynir numunelerinde sözkonusu limitleri aşan numuneye rastlanmadı. Bu sonuçlardan anlaşılacağı gibi yüksek AFM_1 düzeyi Türkiye'de halk sağlığını tehdit eden önemli bir problemdir.

Anahtar sözcükler: *AFM_1, Peynir, ELISA*

INTRODUCTION

Aflatoxins are toxic metabolites produced by fungi, eg, *Aspergillus flavus* [1], *A. parasiticus* and *A. nomius. A. flavus* produces only B aflatoxin, while the other two species produce both B and G aflatoxins. Aflatoxins contaminate the feeds, especially cereals and oilseeds both in the pre-harvest and postharvest seasons. They present toxigenic, carcinogenic, teratogenic and mutagenic potential [2,3]. Aflatoxin B_1 (AFB_1) and aflatoxin

İletişim (Correspondence)
☎ +90 442 2315533
✉ gulsah@atauni.edu.tr

M_1 (AFM_1) are classified as human carcinogen in group 1 and 2B, respectively [4]. There is a linear relationship between the amount of AFM_1 in milk and AFB_1 in feed consumed by animals. It has been reported that 0.3-6.2% of AFB_1 in animal feed is transformed to AFM_1 and excreted in milk [3]. AFM_1 is comparatively stable during pasteurization, sterilization, preparation, and storage of various dairy products [5-9]. Most of the developed countries have regulated the maximum permissible levels of AFM_1 in milk and milk products, which vary from country to country [10]. The European Commission (EC) has approved a maximum admissible level of 250 ng/kg for AFM_1 in cheese [11]. However, the Turkish Food Codex (TFC), has accepted 500 ng/kg as the action level for AFM_1 [12].

Although there is some information about the occurence of AFM_1 white, kashar and cream cheese there is not enough information about the occurence of AFM_1 in civil cheese in Turkey. For this purpose, this study was designed to determine the presence and levels of AFM_1 in white, kashar, civil and cream cheese that especially sold and consumed in Erzurum province of Turkey, and to compare the results with the legal regulations for AFM_1 legislated by EC and TFC.

MATERIAL and METHODS

Samples

In this study the total 304 cheese samples (85 white cheese, 75 kashar cheese, 62 civil cheese, 82 cream cheese) put up for sale in various places in Erzurum between the dates September 2006 - September 2007 were examined in terms of AFM_1.

Methods

AFM_1 concentrations of the samples were analyzed by competitive ELISA (RIDASCREEN Aflatoxin M_1, R-Biopharm) as procedure described by R-Biopharm GmbH [13]. The samples were evaluated according to the RIDAVIN computer program prepared by R-Biopharm. According to the instructions for use of the RIDASCREEN kit; the lower detection limit was 50 ng/kg. The statistical analysis was performed by SPSS Statistical Program.

RESULTS

In this study a total of 304 cheese samples including 85 white cheese, 75 kashar cheese, 62 civil cheese and 82 cream cheese were analysed for AFM_1 with the competitive ELISA.

The occurrence and the distribution of AFM_1 concentration in various ranges in cheese samples are presented in *Table 1*.

Aflatoxin M_1 was found above measurable level (50 ng/kg) in 82.4% (70/85), 80% (75/65), 19.4% (12/62) and 84.2% (69/82) of white cheese, kashar cheese, civil cheese and cream cheese samples, respectively. Another expression of the results, it was found that 17.7% (15/85) of white cheese, 20% (15/75) of kashar cheese, 80.7% (50/62) of civil cheese and 15.9% (13/82) of cream cheese have no AFM_1 in detectable level by ELISA. According to the European Commission limit (250 ng/kg), 23 (27.1%), 26 (34.7) and 14 (17.1) contaminated white, kashar and cream cheese samples had AFM_1 in concentrations in excess of the maximum tolerance limit, respectively. The sample ratio exceeding the limits regulated by Turkish Food Codex (500 ng/kg) was determined in white cheese, kashar cheese and cream cheese samples as 16.5% (14/85), 14.7% (11/75) and 6.1% (5/82) respectively, any sample exceeding these limits was not met in civil cheese samples. AFM_1 content of positive cheese samples were determined as minimum 51 ng/kg, maximum 860 ng/kg and mean 263.4±198.1 ng/kg.

In other studies made on various cheeses, presence and level of AFM_1 were showed in *Table 2.*

DISCUSSION

Milk and dairy product have an important place in a healthy human diet since they are good sources calcium and proteins. The production and consumption of cheese is widespread in Turkey. AFM_1 has affinity to casein of milk. Therefore AFM_1 concentration is higher than in corresponding milk [14]. For these reasons, cheese could be the most potent source of aflatoxin among dairy products. In some studies made on cheese, AFM_1 was determined in high or low levels in some of them but it was not able to be determined in some cheese samples [15-28] *(Table 2).*

In our study AFM_1 was determined in 216 of the total 304 cheese samples. AFM_1 incidence determined as 71.1% (216/304) in cheese samples is high. It is known that this situation can be sourced from the feeds of animals from which milk is got become contaminated with aflatoxin or *Aspergillus spp.* This information was verified by Bakirci [14] who determined high amounts of AFM_1 in raw milk samples in spring months. While the findings got in our study are in parallel of the studies [15-24] stating that they determined AFM_1 in cheese samples in various levels, they are contradicts with the data [25-28] stating that this substance was not met in determinable

Table 1. *Distribution of aflatoxin M1 contents in various range in white cheese, kashar cheese, civil cheese and cream cheese samples*
Tablo 1. *Beyaz peynir, kaşar peynir, civil peynir ve krem peynir örneklerinin aflatoxin M1 içeriğinin dağılımı*

Kind of Cheese	Samples Tested (n)	Proportion of Positive Samples n (%)	Distribution of Samplesa n (%)							Proportion of Samples Exceeding the > European Commission >250 ng/kg	Proportion of Samples Exceeding the >Turkish Legal Limit >500 ng/kg	Quantity of AFM1 (ng/kg)		
			<50	50-150	151-250	251-450	451-650	651-800	>800			x±Sx	Min.	Max.
White	85	70 (82.4)	15 (17.7)	16 (18.8)	31 (36.5	5 (5.9)	10 (11.8)	7 (8.2)	1 (1.2)	23 (27.1)	14(16.5)	297.6±216.1	58	860
Kashar	75	65 (80)	15 (20)	12 (16)	22 (29.3)	14 (18.7)	5 (6.7)	5 (6.7)	2 (2.7)	26 (34.7)	11(14.7)	309.4±206.7	55	850
Civil Cheese	62	12 (19.4)	50 (80.7)	12 (19.4)	0	0	0	0	0	0	0	66.8±22.6	51	116
Cream Cheese	82	69 (84.2)	13 (15.9)	25 (30.5)	30 (36.6)	9 (11)	2 (2.4)	1 (1.2)	2 (2.4)	14 (17.1)	5(6.1)	222.9±158.5	52	860
Total	304	216 (71.1)	93 (30.6)	65 (21.4)	83 (27.3)	28 (9.2)	17 (5.6)	13 (4.3)	5 (1.6)	63 (20.7)	30(9.9)	263.4±198.1	51	860

x±Sx: *mean±standart deviation,* ***<50:*** *distribution of negative samples,* ***a:*** *ng/kg*
x±Sx: *ortalama±standat sapma,* ***<50:*** *negatif örneklerin dağılımı,* ***a:*** *ng/kg*

Table 2. *Presence and level of AFM_1 in various cheeses*
Tablo2. *Çeşitli peynirlerde AFM_1 varlığı ve seviyeleri*

Country	Cheese Variety	No. of Samples Positive (%)	Range (ng/kg)	Exceed Legal Limit (%)	References	Country	Cheese Variety	No. of Samples Positive (%)	Range (ng/kg)	Exceed Legal Limit (%)	References
Turkey	White	12/25 (48)	51-510	NP	Dagoglu et al.[15]	Iran	Feta	66/80 (82.5)	350-520	48/80 (60.6)	Kamkar [24]
Turkey	Cheese	51/57 (89.47)	40-810	7/57 (12.28)	Oruc & Sonal [16]	Turkey	White	0/30 (0.0)	ND	NP	Demirer [25]
Turkey	White	82/100 (82)	51-800	27/100 (27)	Sarimehmetoglu et al.[17]		Kashar	0/12 (0.0)	ND	NP	
	Kashar	85/100 (85)	51-800	34/100 (34)			Tulum	0/26 (0.0)	ND	NP	
	Tulum	81/100 (81)	51-800	24/100 (24)			Konya Küflü Tulum	0/10 (0.0)	ND	NP	
	Cream	79/100 (79)	51-650	25/100 (25)		Turkey	Konya Küflü Tulum	0/10 (0.0)	ND	NP	Demirer [33]
Turkey	White Cheese	101/110 (91.8)	10-2000	17/110 (15.5)	Seyrek [18]		Diyarbakır Otlu	0/10 (0.0)	ND	NP	
Japan	Cheese	44/303 (14.50)	200-1200	NP	Tabata et al.[19]		Erzincan Küflü Tulum	0/71 (0.0)	ND	NP	
USA	Cheese	80/118 (6.80)	100-1000	NP	Trucksess and Page [20]	Turkey	Tulum	0/4 (0.0)	ND	NP	Coksoyler [26]
Turkey	Van Otlu	52/60 (86.7)	160-7260	12/60 (20)	Tekinsen & Tekinsen [21]		Çökelek	0/9 (0.0)	ND	NP	
	White Brine	31/50 (62.0)	10-5200	30/50 (60)		Turkey	White	0/25 (0.0)	ND	NP	Kivanc [27]
Turkey	White	10/200 (5.0)	100-600	2/200 (1)	Yaroglu et al.[22]		Van otlu	0/25 (0.0)	ND	NP	
	Kashar	24/200 (12.0)	120-800	2/200 (1)		Turkey	White	0/50 (0.0)	ND	NP	Kardes [28]
	Cream	16/200 (8.0)	100-700	2/200 (1)			Kashar	0/50 (0.0)	ND	NP	
Turkey	White Brine	159/193 (82.4)	52- 860	51/193 (26.4)	Ardic et al.[23]						

NP: *Not presented,* ***ND:*** *Not detected*
NP: *Bildirilmemiş,* ***ND:*** *Belirlenememiş*

limits in cheese. It was stated that these variations between the data could be sourced from the difference of AFM1 level in milk used in the production due to seasonal changes [14,17,21,29,30] and from different operation and analysis methods [30-32].

That AFM1 determined in civil cheese in relatively low levels (mean; 66.8±22.6 ng/kg) is in accord with the studies in which AFM1 can not be determined in some civil cheese types such as Konya moldy tulum cheese (its original name is Konya Küflü Tulum) and Erzincan moldy tulum cheese (its original name is Erzincan Küflü Tulum) [25,26,33]. In this study the samples ratio (20.7%) exceeding the EC legal limits is similar to the findings of researchers [17,18,21,23,-25]. The AFM1 levels exceeding the TFC legal limits (500 ng/kg), also were lower than the reported results by Sarimehmetoglu et al.[17], Tekinsen and Tekinsen [21], Ardic et al.[23], Kamkar [24], and were similar to the results reported by Oruç and Sonal [16] and Seyrek [18]. This similarity can be sourced from the highness of AFM1 level regulated by TFC. It was concluded that AFM1 incidence determined in white, kashar and cream cheese were quite higher than legal limits, so it could form an important risk for health. But there isn't a sample in civil cheeses exceeding legal limits, it might be said that civil cheese can carry less risk in terms of AFM1 content.

In conclusion, AFM1 determined in high levels in theree cheese types in the present study and the other researchs made in Turkey show that this subject is still an important public health problem in Turkey. So the public health authorities should train the farmers, dairy companies and dairy product consumers on the potential health results of aflatoxins. Moreover the prevention of aflatoxin formation in feeds is very important. Because the consumption of contaminated feeds by dairy animals causes AFM1 formation in milk. So the easiest and shortest way to deal with this problem is reducing the AFB1 concentration in animal feed by improved processing and storage practices. For this, it is necessary to control well the feeds given to dairy animals and to reduce AFB1 amount permitted to take place in feeds to lower levels. In addition, it is considered that food substances should be produced and kept in convenient conditions to prevent aflatoxin formation.

REFERENCES

1. Doğan A, Bayezit M: Kars yöresinde yemlerde aflatoksin B1 düzeylerinin ELISA yöntemi ile belirlenmesi. *Kafkas Univ Vet Fak Derg,* 5 (1): 63-70, 1999.

2. Sweeney MJ, Dobson ADW: Mycotoxin production by *Aspergillus, Fusarium* and *Penicillium* species. *Int J Food Microbiol,* 43, 141-158, 1998.

3. Creppy EE: Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicol Lett,* 127, 19-28, 2002.

4. IARC (International Agency for Research on Cancer): IARC monographs on the evaluation of carcinogenic risks to humans, some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC Scientific Publication, No. 56, IARC, Lyon, 1993.

5. Stubblefield RD, Shannon GM: Aflatoxin M1: Analysis in dairy products and distribution in dairy foods made from artificially contaminated milk. *J Assoc Off Anal Chem,* 57, 847-851, 1974.

6. Barbieri G, Bergamini C, Ori E, Reska P: Aflatoxin M1 in parmesan cheese: HPLC determination. *J Food Sci,* 59, 1313-1331, 1994.

7. Blanco JL, Dominguez L, Gomez-Lucia E, Garayzabal JFF, Garcia JA, Suarez G: Presence of aflatoxin M1 in commercial ultra-high-temperature-treated milk. *App Environ Microb,* 54, 1622-1623, 1988.

8. Stoloff L: Aflatoxin M1 in perspective. *J Food Protect,* 43, 226-230, 1989.

9. Galvano F, Galofaro V, Galvano G: Occurrence and stability of aflatoxin M1 in milk and milk products. *J Food Protect,* 59, 1079-1090, 1996.

10. Kaniou-Grigoriadou I, Eleftheriadou A, Mouratidou T, Katikou P: Determination of aflatoxin M1 in ewe's milk samples and the produced curd and Feta cheese. *Food Control,* 16, 257-261, 2005.

11. European Commission (EC): No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Off J Eur Union,* 364, 5-24, 2006.

12. Turkish Food Codex (TFC): Gıda Maddelerinde Belirli bulasanların maksimum seviyelerinin belirlenmesi hakkında teblig. *Resmi Gazete,* 17 Mayıs 2008. Sayı: 26879 Basbakanlik Basimevi. Ankara, Turkey, 2008.

13. R-Biopharm GmbH: Enzyme immunoassay for the quantitative analysis of aflatoxins. Ridascreen Aflatoxin M1 Art. No: R-1101. Darmstadt, Germany, 1999.

14. Bakirci I: A study on the occurrence of aflatoxin M1 in milk and milk products produced in Van province of Turkey. *Food Control,* 12, 47-51, 2001.

15. Dagoglu G, Keles O, Yıldırım M: Peynirlerde aflatoksin düzeylerinin ELISA ile araştırılması. *İstanbul Univ Vet Fak Derg,* 21, 313-317, 1995.

16. Oruç HH, Sonal S: Determination of aflatoxin M1 levels in cheese and milk consumed in Bursa, Turkey. *Vet Hum Toxicol,* 43, 292-293, 2001.

17. Sarimehmetoglu B, Kuplulu O, Celik TH: Detection of aflatoxin M1 in cheese samples by ELISA. *Food Control,* 15, 45-49, 2004.

18. Seyrek K: Türk Silahlı Kuvvetleri'ne bağlı birliklerde tüketilen beyaz peynirlerdeki aflatoxin M1 seviyesinin ELISA metodu ile saptanması. *Vet Hek Der Derg,* 72, 55-58, 2001.

19. Tabata S, Kamimura H, Tamura Y, Yasuda K, Ushuyama H, Hasimoto H, Nishijima, M, Nishima T: Investigation of aflatoxins contamination in food and foodstuffs. *J Food Hyg Soc Jpn,* 28, 395-401, 1987.

20. Truckness MV, Page SV: Examination of imported cheese for aflatoxin M1. *J Food Protect,* 49, 632-633, 1986.

21. Tekinsen KK, Tekinsen OC: Aflatoxin M1 in white pickle

and Van otlu (herb) cheeses consumed in southeastern Turkey. *Food Control,* 16, 565-568, 2005.

22. Yaroglu T, Oruc HH, Tayar M: Aflatoxin M_1 levels in cheese samples from some provinces of Turkey. *Food Control,* 16, 883-885, 2005.

23. Ardic M, Karakaya Y, Atasever M, Adiguzel, G: Aflatoxin M_1 levels of Turkish white brined cheese. *Food Control,* 20, 196-199, 2009.

24. Kamkar A: A study on the occurrence of aflatoxin M_1 in raw milk produced in Sarab city of Iran. *Food Control,* 16, 593-599, 2005.

25. Demirer MA: Süt ve süt mamüllerinde aflatoxin M_1 ve B_1 aranması üzerine araştırmalar. *Ankara Univ Vet Fak Derg,* 36, 85-107, 1973.

26. Coksoyler N: Süt ve Süt Mamüllerinde Aflatoxin Oluşumu Üzerine Araştırmalar. *Diploma Sonrası Yüksekokul İhtisas Tezi,* Ankara Univ Ziraat Fak, Ankara, 1977.

27. Kivanc M: Mold growth and presence of aflatoxin in some Turkish cheeses. *J Food Safety,* 10, 287-294, 1990.

28. Kardes E: Türk Silahlı Kuvvetleri'ne bağlı birliklere alınan peynirlerde aflatoxin B_1 ve M_1 varlığının ve seviyelerinin saptanması. *MS Thesis,* Ankara University, 2000.

29. Amra HA: Survey of aflatoxin M_1 in Egyptian raw milk by enzyme-linked immunosorbent assay. *Rev Med Vet,* 149, 695,1998.

30. Tekinsen KK, Ucar G: Aflatoxin M_1 levels in butter and cream cheese consumed in Turkey. *Food Control,* 19, 27-30, 2008.

31. Galvano F, Galofaro V, Ritieni A, Bognanno M, De Angelis, A, Galvano G: Survey of the occurrence of aflatoxin M_1 in dairy products marketed in Italy: Second year of observation. *Food Addit Contam,* 18, 644-646, 2001.

32. Wiseman DW, Marth EH: Behavior of aflatoxin M_1 during manufacturing and storage of queso blanco and bakers cheese. *J Food Protect,* 46, 910-913, 1983.

33. Demirer MA: Bazı peynirlerimizden izole ettiğimiz küfler ve bunların aflatoksin yeteneklerinin araştırılması. *Ankara Univ Vet Fak Derg,* 21, 1-2, 1974.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S93-S97, 2010
DOI:10.9775/kvfd.2010.1744

RESEARCH ARTICLE

Investigations of *Listeria* Species in Milk and Silage Produced in Burdur Province [1]

Fulya TAŞÇI * Hülya TÜRÜTOĞLU ** Hatice ÖĞÜTÇÜ ***

[1] *This study was supported by the Scientific Research Projects Fund of the Akdeniz University (Project No: 2005.01.0108.001)*

* Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, TR-15100 Burdur - TURKEY
** Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Microbiology, TR-15100 Burdur - TURKEY
*** Ahi Evran University, Faculty of Sciences and Arts, Department of Biology, TR-40100 Kırşehir - TURKEY

Makale Kodu (Article Code): KVFD-2010-1744

Summary

The aim of this study was to investigate the presence of *Listeria* species in the milk and silage samples obtained from fifteen different farms in Burdur. A total of 250 samples (silage and cow's milk obtained from animals fed and not fed with silage) were analyzed. *L. monocytogenes* was isolated in 6 (2.4%) out of the 250 samples. Five (6.66%) of the 75 silage samples and 1 (1.17%) of the 85 milk samples obtained from cows fed with silage were contaminated with *L. monocytogenes,* whereas no *Listeria* spp. were isolated from the 90 milk samples from cows not fed with silage. The isolation of *L. monocytogenes* from milk and silage samples in Burdur indicates that these products could create a serious risk to the public health.

Keywords: *Cow's milk, Silage, Listeria spp.*

Burdur Yöresinde Üretilen Süt ve Silajlarda *Listeria* Türlerinin Araştırılması

Özet

Bu çalışmada, Burdur yöresinde onbeş farklı çiftlikte üretilen süt ve silajlarda *Listeria* türlerinin varlığının araştırılması amaçlanmıştır. Toplam 250 örnek (silaj ile beslenen ve beslenmeyen inek sütleri ve silaj) analize alınmıştır. İki yüz elli örneğin 6'sında (%2.4) *L. monocytogenes* izole edilmiştir. Yetmiş beş silajın 5 (%6.66)'inde, 85 silaj verilen inek sütünün 1 (%1.17)'inde *L. monocytogenes* olduğu belirlenmiştir. Silaj ile beslenmeyen 90 inekten alınan sütlerde ise *Listeria* spp. izole edilememiştir. Sonuç olarak, Burdur'da üretilen süt ve silajlarda *L. monocytogenes*'in izole edilmesi bu ürünlerin halk sağlığı açısından bir risk oluşturabileceğini göstermektedir.

Anahtar sözcükler: *İnek sütü, Silaj, Listeria spp.*

INTRODUCTION

Listeria spp. are widely distributed in nature and found in soil, silage, decaying vegetation, animal feces, sewage water, and other environmental sources [1]. *Listeria monocytogenes* may contaminate milk because of mastitis, encephalitis, or abortion related to *Listeria* spp. in animals [1,2]. Listeriosis is a severe and often fatal illness with clinical manifestations such as sepsis or meningitis in immunocompromised patients or neonatal babies and flu-like illness or abortion during pregnancy in women. The major outbreaks of listeriosis have been associated with the consumption of foods of animal origin [3]. The genus *Listeria* contains 6 species: *L.*

İletişim (Correspondence)
☎ +90 248 2344500
✉ fulya_tasci22@hotmail.com

monocytogenes, L. innocua, L. seeligeri, L. welshimeri, L. ivanovii, and *L. grayi* [4]. In addition to *L. monocytogenes, L. seeligeri* and *L. ivanovii* may be pathogenic in humans [1,5]. *L. monocytogenes* can frequently be isolated from unpasteurized milk and milk products [6-10]. Except in modern cheese production plants, raw milk is widely used in cheese production by small and medium domestic and commercial plants in Turkey. Also, isolation rate of *L. monocytogenes* in cheese samples in Turkey has been reported from 2% to 5% [6-8,11].

Silage is produced by harvesting a forage crop with a high moisture content (greater than 50%) and subsequently fermenting. In general, good silage remains stable, with no change in composition or heat, once air is eliminated and the silage has achieved a low pH [12]. *Listeria* spp. are most commonly recovered from improperly fermented silage [13,14]. It has been reported that listeriosis in cattle is mainly feed-borne [1] and *Listeria* spp. have been detected from 1.2% to 60% of the silage samples [15-17]. Also, *Listeria* spp. have been isolated from 2% to 6.1% milk samples from cows fed with silage [17]. In a study by Fenlon et al.[18] has been stated that 29-31% of cattle started to shed *L. monocytogenes* after silage feeding.

The aim of this study was to investigate the presence of *Listeria* species in the milk and silage samples obtained from fifteen different farms in Burdur. The milk obtained from cows fed and not fed with silage were compared in terms of *Listeria*, and its importance in contamination of silage was put forward.

MATERIAL and METHODS

Sampling

Five research centers in Burdur were determined for sampling. Three different farms in every research center were visited every month between December 2007 and May 2008. In fifteen farms, seventy five silage samples, 85 milk samples obtained from cows fed with silage and 90 milk samples obtained from cows not fed with silage were collected. The samples were collected in sterile plastic bags and transported to the laboratory in boxes containing ice.

Isolation and Identification of Listeria spp.

All procedures were applied according to the FDA-Bacteriological Analytical Manual [19]. All media used were obtained from Oxoid (Oxoid Ltd., Hampshire, UK). Each sample (25 g/ml) was taken and placed in a stomacher bag to which 225 ml of sterile *Listeria* Selective Enrichment Broth (Oxoid) was added and homogenized with a stomacher (Masticator, IUL Instruments-Spain) for 1-3 min and incubated at 30°C for 48 h. A loopful of homogenate was surface streaked in duplicate on Palcam agar (Oxoid) and Oxford agar (Oxoid). The Palcam plates were incubated at 37°C for 48 h under microaerophilic conditions and Oxford plates at 35°C for 48 h under aerobic conditions. All colonies surrounded by a brownish green and/or black halo were taken as possible *Listeria* spp. One suspected *Listeria* spp. colony from each plate was chosen and purified on tryptic soy agar (Oxoid CM 131) with 0.6% yeast extract (Oxoid L 21) and incubated at 30°C for 24-48 h for further biochemical characterization. Presumptive *Listeria* isolates were confirmed and identified at the species level based on Gram staining, typical umbrella motility in SIM medium (Oxoid CM 435), H_2S production, indole, urease, catalase, oxidase reaction, β-hemolysis, nitrate reduction, methyl-red/voges-proskauer (Oxoid CM 43), CAMP tests and fermentation of mannitol, L-rhamnose, D-ksilose, sorbitol, dextrose, maltose, esculin, dulcitol and salicin [4,20,21]. Serotyping of isolates was performed with Bacto-*Listeria*-O-antisera types 1 and 4 and poly (Difco Laboratories, Detroit, MI) by the slide agglutination test [4,21].

Measurement of pH Values of the Samples

After the samples were collected for microbiologic analysis, the pH values of the milk samples were measured with an electronic pH meter (Metrohm 704 pH Meter). A 25-g aliquot silage sample was blended with 100 ml of deionized water for 2 min and filtered through four layers of cheesecloth. Then the pH of the extract was measured [22].

Statistical Analysis: The results were analyzed using Minitab-15 with the chi-square analysis.

RESULTS

Overall, *L. monocytogenes* was found in 6 (2.4%) out of 250 samples. Five (6.66%) of the 75 silage samples and 1 (1.17%) of the 85 milk samples obtained from fed with silage were contaminated with *L. monocytogenes,* whereas no *Listeria* spp. were isolated from the 90 milk samples from cows not fed with silage. The differences between isolation rates of *L. monocytogenes* were statistically significant (χ^2=8.02; P=0.018; P<0.05) *(Table 1).* Two selective plating media Palcam and Oxford were compared for isolating *L. monocytogenes* from the samples, and the isolation rates from these media were found to be equal.

In the present study, the pH values of the milk samples varied between 6.6 and 7.1, and the pH values

Table 1. *The isolation rate of L. monocytogenes isolated from milk and silage* [a]

Tablo 1. *Süt ve silajlarda L. monocytogenes izolasyon oranı* [a]

Sample Type and Number (n)	*L. monocytogenes*	
	n	%
Silage (n: 75)	5	6.66
The milk of cows fed with silage (n: 85)	1	1.17
The milk of cows not fed with silage (n: 90)	-	-
Total (n: 250)	6	2.4

[a] *Chi-square statistic is significant, χ^2=8.02 ; P=0.018; P<0.05*

of the silage varied between 4.1 and 8.7. In the silage samples contaminated with *L. monocytogenes,* the pH values varied between 5.1 and 8.3, and the pH value of the milk sample contaminated with *L. monocytogenes* was 6.9.

As the collection period of the milk and silage samples was compared in isolation, the contamination of *L. monocytogenes* was found higher in March (3 silage samples) than in January (2 silage samples) and February (1 milk sample from the cows fed with silage).

In this study, for the serotype determination of 6 isolates defined as *L. monocytogenes* Difco Bacto O Antiserum type 1 and type 4, and type poly were used. The results were as follows: 5 isolates (1 milk and 4 silage samples) type poly and type 4, 1 isolate (1 milk sample) type poly.

DISCUSSION

The isolation rates of *Listeria* spp. in silage has been demonstrated in several studies carried out in Turkey and in other countries [15-17,23,24]. In this study, *L. monocytogenes* was detected in 6.66% of the 75 silage samples. This percentage is lower than the results reported by Oliveira et al.[25] and Grønstøl [26], but similar to the 6.1% obtained by Vilar et al.[17] In Turkey, Aslantaş and Yıldız [23] isolated *L. monocytogenes* from 1 of 11 silage samples. However, Şahin et al.[24] did not isolated *L. monocytogenes* from the silage, but isolated *L. welshimeri* and *L. grayi.* In this study, the low isolation rate of *L. monocytogenes* in silage may be accounted that high-quality silage is produced by mostly producers. However, in our study, silage samples contaminated with *L. monocytogenes* was obtained only from wet silage. In the illumination of this result, we cold say that and rainy weather conditions are the cause of this result.

Many researchers have investigated *L. monocytogenes* contamination of milk [6,9,11,23,27,28] and *Listeria* species have been detected from 0.40% to 10% of milk samples [6,9,11,23,29]. In Turkey, the isolation rates from raw milk samples have been reported 0.45% in İstanbul [7], 0.94% in Ankara [27], 1.20% in Van [6], 3% in West Anatolia [28] and 5% in Ankara [29]. In other countries, the reported isolation rates from bulk tank milk samples were 1.2% in Pennsylvania [30], 4.9% in Ireland [31] and 6.5% in the United States [10]. The sources of *Listeria* spp. in raw milk have been reported to be fecal [32] and environmental contamination during the milking, storage, and transport of infected cows on dairy farms, and poor silage quality [33].

In the present study, *Listeria* species were not found from cow's milk samples not fed silage. But, 1.17% of the milk samples obtained from cows fed with silage were contaminated with *L. monocytogenes.* However, Şahin et al.[24] have reported that *L. monocytogenes* was not isolated from the silage and milk samples of cows fed with silage, but *L. welshimeri* and *L. grayi* were isolated. Vilar et al.[17] detected *Listeria* spp. in 33.7% of silage samples and in 16.3% of milk samples. Donnelly [34] observed that 8 of 44 Holstein cows fed *Listeria*-contaminated silage shed the organism in their milk. Furthermore, milk from these animals was free of *L. monocytogenes* one month after feeding of contaminated silage ceased.

In our study, two selective plating media Palcam and Oxford were compared for isolating of *L. monocytogenes* from the samples, and the isolation rates from these media were found to be equal, which is consistent with the reports by Art and Andre [35], Capita et al.[36] and Uysal and Anğ [7].

L. monocytogenes has thirteen serotypes, but, only three serotypes-4b, 1/2a and 1/2b-are responsible for the majority of veterinary and human listeriosis cases [37]. In this study, for the serotype determination of 6 isolates defined as *L. monocytogenes,* O Antiserum type 1 and type 4, and type poly were used. The results were as follows: 5 isolates (1 milk and 4 silage samples) type poly and type 4, 1 isolate (1 milk sample) type poly. Van Kessel et al.[10] isolated *L. monocytogenes* from 56 (6.5%) of 861 bulk-tank milk samples, and serotyping of these isolates yielded 5 serotypes (1/2a, 1/2b, 3b, 4b, and 4c). Jayarao and Henning [38] reported isolating *L. monocytogenes* in 6 (4.6%) of 131 bulk-tank milk samples and all isolates of *L. monocytogenes* belonged to O antigen type 1.

Multiple studies have reported seasonal variations of *Listeria* spp. isolation, some report that contamination rates increase during the summer months [39], while others [40] reported increased rates during winter. Gaya et

al.[41] found that raw caprine milk contamination by *Listeria* spp. was seasonal; the incidence in the autumn (9.33%) and winter (5.14%) samples was higher than the incidence in the spring (0.85%) and summer (0.85%) samples. Uraz and Yücel [27] isolated 1 of the *L. monocytogenes* in winter whereas the other one was isolated in the spring season. Two factors may explain the increased isolation rate during March in our study: (1) March is usually very rainy in Burdur, and water is moisturised silage. Therefore, the quality silage is changed. (2) Seasonal differences in the incidence of *Listeria* spp. in raw milk may also be related to breeding practices. Dairy cattle typically bear their young in late winter or early spring. During winter gestation, dairy cattle develop a weakened immune system as a direct result of pregnancy, which, in turn, makes these animals more susceptible to listerial infections and abortions [42].

The pH values of the silage samples from which *Listeria* spp. were isolated ranged from 5.1 to 8.3. Different range from those observed in other studies were 3.8 to 5.2 in Rea et al.[31], 5.78 to 5.89 in Ryser et al.[16], and 4.47 to 6.97 in Vilar et al.[17]. A variety of studies have confirmed that *L. monocytogenes* contamination is most frequently associated with poor-quality silage [17]. Poorly fermented silage, which has a pH greater than 5.5, is ideal for *Listeria* growth [16,17]. However, Fensterbank et al.[43] identified *Listeria* spp, including *L. monocytogenes,* in 11 of 31 high-quality silage samples with pHs of 3.6 to 4.0. In our study, the pH value of the milk and silage samples contaminated with *L. monocytogenes* was greater than 6.6. We believe that the contamination sources of *Listeria* spp. are the consumption of bad-quality silage, subjected to inadequate fermentation, with pH values higher than 4.0, which allows the multiplication of *Listeria* spp.

As a conclusion, the isolation of *L. monocytogenes* from milk and silage samples in Burdur indicates that these products could create a serious risk to the public health and could have a potantial risk for animals. Correct practices with respect to silage production and milking are essential for preventing introduction of *Listeria* into the herd, its spread within the herd, and its entry into milk. The risk of contamination of milk by *Listeria* spp. increased when animals were fed low-quality silage, notably silage with pH ≥4.5. Although the contamination ratio is very low in this research, *Listeria* contamination must be obstructed or minimized to achieve standard conditions.

REFERENCES

1. Jay JM: Modern Food Microbiology. 6th ed. Aspen Publishers, Inc, Gaithersburg, 2000.

2. Öcal N, Babür C, Yağcı BB, Macun HC, Çelebi B, Kılıç S, Yağcı İP: Kırıkkale yöresinde süt sığırlarında brusellozis, listeriozis ve toksoplazmozis'in seroprevalansı ve birlikte görülme sıklığı. *Kafkas Univ Vet Fak Derg,* 14 (1): 75-81, 2008.

3. Iida T, Kanzaki M, Nakama A, Kokubo Y, Maruyama T, Kaneuchi C: Detection of *Listeria monocytogenes* in human, animals and foods. *J Vet Med Sci,* 60, 1341-1343, 1998.

4. Anonymous: Bacteriological Analytical Manual Online. FDA, Revision AOAC International, USA 2003. http://www.cfsan.fda.gov/~ebam/bam-10.htm. *Accessed:* 30.04.2009.

5. Wiedmann M: An integrated science based approach to dairy food safety. *Listeria monocytogenes* as a model system. *J Dairy Sci,* 86, 1865-1875, 2003.

6. Sağun E, Sancak YC, İşleyici O, Ekici K: Van ve çevresi süt ve otlu peynirlerinde *Listeria* türlerinin varlığı ve yaygınlığı üzerine bir araştırma. *Turk J Vet Anim Sci,* 25, 15-19, 2001.

7. Uysal HK, Anğ O: Süt ve süt ürünlerinden izole edilen *Listeria* türleri. *Türk Mikrobiyol Cem Derg,* 33, 163-169, 2003.

8. Gülmez M, Güven A: Beyaz ve çeçil peynirlerinde *Campylobacter, Salmonella* ve *Listeria* türlerinin araştırılması. *Kafkas Univ Vet Fak Derg,* 7 (2): 155-161, 2001.

9. Dhanashree B, Otta SK, Karunasagar I, Goebel W, Karunasagar I: Incidence of *Listeria* spp. in clinical and food samples in Mangalore, India. *Food Microbiol,* 20, 447-453, 2003.

10. Van Kessel JS, Karns JS, Gorski L, McCluskey BJ, Perdue ML: Prevalence of Salmonellae, *Listeria monocytogenes,* and fecal coliforms in bulk tank milk on US dairies. *J Dairy Sci,* 87, 2822-2830, 2004.

11. Aygün O, Pehlivanlar S: *Listeria* spp. in the raw milk and dairy products in Antakya, Turkey. *Food Control,* 17 (8): 676-679, 2006.

12. McDonald P, Henderson AR, Heron SJE: The Biochemistry of Silage. Chalcombe Publications, Marlow, England, 1991.

13. Grant MA, Eklund CA, Shields SC: Monitoring dairy silage for five bacterial groups with potential for human pathogenesis. *J Food Protect,* 58, 879-883, 1995.

14. Gazyağcı S, Yıldırım M, Babür C, Kılıç S: Ankara yöresindeki koç ve koyunlarda *Listeria monocytogenes*'e karşı oluşan antikorların varlığının araştırılması. *Kafkas Univ Vet Fak Derg,* 15 (6): 975-977, 2009.

15. Fenlon DR: Rapid quantitative assessment of the distribution of *Listeria* in silage implicated in a suspected outbreak of listeriosis in calves. *Vet Rec,* 118, 240-242, 1986.

16. Ryser E, Arimi SM, Donnelly CW: Effects of pH on distribution of *Listeria* ribotypes in corn, hay, and grass silage. *Appl Environ Microb,* 63 (9): 3695-3697, 1997.

17. Vilar MJ, Yus E, Sanjuán ML, Diéguez JL, Rodríguez-Otero FJ: Prevalence of and risk factors for *Listeria* species on dairy farms. *J Dairy Sci,* 90, 5083-5088, 2007.

18. Fenlon DR, Wilson J, Donachie W: The incidence and level of *Listeria monocytogenes* contamination of food sources at primary production and initial processing. *J Appl* Bacteriol, 81, 641-650, 1996.

19. Hitchens AD: *Listeria monocytogenes.* **In,** FDA Bacteriological Analytical Manual. 8th ed. pp. 10.1-10.13, AOAC International, USA, 1995.

20. Andrews W: Manual of Food Quality Control Microbiological Analysis. Washington, DC, USA: Food and Drug Administration, 1992.

21. Lovett J, Francis DW, Hunt JM: *Listeria monocytogenes* in raw milk: Detection, incidence and pathogenicity. *J Food Protect,* 50, 188-192, 1987.

22. Hart SP, Horn FP: Ensiling characteristics and digestibility of combinations of turnips and wheat straw. *J Anim Sci,* 14, 1790-1800, 1987.

23. Aslantaş O, Yıldız P: Kars ilinde çiğ sütten *Listeria monocytogenes* izolasyonu. *Fırat Univ Sag Bil Vet Derg,* 17 (1): 11-15, 2003.

24. Şahin K, Çerci IH, Güler T, Özcan C, Şahin N: Silaj ve kuru ot katılan rasyonlarla beslenen süt ineklerinin kaba yem ve sütlerinde *Listeria* türlerinin araştırılması. *Fırat Univ Sağlık Bil Vet Derg,* 10, 245-249, 1996.

25. Oliveira M, Guerra M, Bernardo F: Occurrence of *Listeria monocytogenes* in silages assessed by fluorescent in situ hybridization. *Arq Bras Med Vet Zootec,* 60 (1): 267-269, 2008.

26. Grønstøl H: Listeriosis in sheep: Isolation of *Listeria monocytogenes* from grass silage. *Acta Vet Scand,* 20, 492-497, 1979.

27. Uraz G, Yücel N: *Listeria monocytogenes, Yersinia enterocolitica* ve benzerlerinin *(Yersinia kristensenii, Yersinia frederiksenii, Yersinia intermedia)* çig süt örneklerinden izolasyonu. *Turk J Agri Forestry,* 22, 463-468, 1998.

28. Soyutemiz E, Çetinkaya F, Özakın C, Gedikoğlu S: Presence of *Listeria monocytogenes* in raw milk samples from West Anatolia. *Turk J Infect,* 15 (1): 5-9, 2001.

29. Erol İ, Şireli UT: Occurrence and contamination levels of *Listeria* spp. in milk and dairy products in Ankara. *FEMS Symposium on the Versatility of Listeria species* (10-11 October, No. 44, pp. 44-45). İzmir, Turkey: Publication of The Turkish Microbiological Society, 2002.

30. Jayarao BM, Donaldson SC, Straley BA, Sawant AA, Hegde NV, Brown JL: A survey of foodborne pathogens in bulk tank milk and raw milk consumption among farm families in Pennsylvania. *J Dairy Sci,* 89, 2451-2458, 2006.

31. Rea MC, Cogan TM, Tobin: Incidence of pathogenic bacteria in raw milk in Ireland. *J Appl Bacteriol,* 73, 331-336, 1992.

32. Griffiths MW: *Listeria monocytogenes:* Its importance in dairy industry. *J Sci Food Agri,* 47, 133-158, 1989.

33. Bemrah N, Sanaa M, Cassin MH, Griffiths MW, Cerf O: Quantitative risk assessment of human listeriosis from consumption of soft cheese made from raw milk. *Prev Vet Med,* 37, 129-145, 1998.

34. Donnelly CW: Listeriosis and dairy products: Why now and why milk? Hoards Dairyman, 131, 663-687, 1986.

35. Art D, Andre P: Comparison of three selective isolation media for the detection of *L. monocytogenes* in foods. *Zbl Bakteriol,* 275, 79, 1991.

36. Capita R, Alonso Calleja C, Prieto M, Garcia Fernandez MC, Moreno B: Comparison of PALCAM and modified Oxford plating media for isolation of *Listeria* species in poultry meat following UVM II or Fraser secondary enrichment broths. *Food Microbiol,* 18, 555-563, 2001.

37. Wesley IV, Borucki M, Call DR, Larson D, Schroeder-Tucker L: Detection and diagnosis of *Listeria* and listeriosis. **In,** Torrence ME, Issacson RE (Eds): Microbial Food Safety in Animal Agriculture-Current Topics. pp. 233-241, Iowa State Press, Iowa, USA, 2003.

38. Jayarao BM, Henning DR: Prevalence of foodborne pathogens in bulk tank milk. *J Dairy Sci,* 84, 2157-2162, 2001.

39. Farber JM, Sanders GW, Malcom SA: The presence of *Listeria* spp. in raw milk in Ontario. *Can J Microbiol,* 34, 95-100, 1988.

40. Fernández-Garayzábal JF, Domínguez L, Vázquez JA, Gómez-Lucia A, Rodríguez-Ferri ER, Suárez G: Occurence of *Listeria monocytogenes* in raw milk. *Vet Rec,* 120, 258-259, 1987.

41. Gaya P, Saralegui C, Medina M, Nuñez M: Occurrence of *Listeria monocytogenes* and other *Listeria* spp. in raw caprine milk. *J Dairy Sci,* 79, 1936-1941, 1996.

42. Ryser ET: Incidence and behavior of *Listeria monocytogenes* in unfermented dairy products. **In,** Ryser ET, Marth EH (Eds): *Listeria,* Listeriosis, and Food Safety. 3rd ed., Chapter 11, pp. 357-404, CRC Press, Taylor & Francis Group, Boca Raton, 2007.

43. Fensterbank R, Audurier A, Godu J, Guerrault P, Malo N: Study of *Listeria* strains isolated from sick animals and from the silage consumed. *Ann Rech Vet,* 15 (1): 113-118, 1984.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S99-S105, 2010
DOI:10.9775/kvfd.2010.1840

RESEARCH ARTICLE

Effect of Potassium Sorbate on Some Microbiological Properties of Cokelek Stored at Different Temperatures

Gülsüm ÖKSÜZTEPE * O. İrfan İLHAK * Abdullah DİKİCİ **
Mehmet ÇALICIOĞLU * Bahri PATIR *

* Fırat Üniversitesi Veteriner Fakültesi, Besin Hijyeni ve Teknolojisi Bölümü, TR-23119 Elazığ - TÜRKİYE
** Tunceli Üniversitesi Mühendislik Fakültesi, Gıda Mühendisliği, TR-62000 Tunceli - TÜRKİYE

Makale Kodu (Article Code): KVFD-2010-1840

Summary

In this study, the effect of potassium sorbate at 0.01%, 0.05% and 0.1% on the some microbiological (total aerobic mesophilic bacteria-TAMB, coliform bacteria, *Lactobacillus-Leuconostoc-Pediococcus, Stapylococcus-Micrococcus,* enterococcus and yeast-mold) and chemical (pH, acidity) properties of cokelek stored at 4±1°C or 22±1°C was investigated. It was seen that adding potassium sorbate at 0.01%, 0.05% and 0.1% to cokelek did not have an effect on numbers of TAMB, coliform bacteria, *lactobacillus- leuconostoc- pediococcus, staphylococcus-micrococcus* in cokelek stored at both 4±1°C and 22±1°C (P>0.05). However, adding potassium sorbate at 0.05% and 0.1% to cokelek had an effect on numbers of enterococci and yeast-mold in cokelek stored at 4±1°C (P<0.05). It was also not observed significant difference in pH and acidity levels between control group and treatment groups containing potassium sorbate during storage period (P>0.05).

Keywords: *Cokelek, Microbiological quality, Storage temperature, Potassium sorbate*

Potasyum Sorbatın Farklı Sıcaklıklarda Muhafaza Edilen Çökeleğin Bazı Mikrobiyolojik Özellikleri Üzerine Etkisi

Özet

Bu çalışmada, %0.01, 0.05 ve %0.1 oranında potasyum sorbatın 4±1°C ve 22±1°C'de muhafaza edilen çökeleğin bazı mikrobiyolojik (toplam aerobic mezofil bakteri, koliform bakteri, *Lactobacillus-Leuconostoc-Pediococcus, Stapylococcus-Micrococcus,* enterococcus ve maya-küf) parametreleri ile pH ve toplam asidite (laktik asit cinsinden) üzerine etkileri araştırıldı. Hem 4±1°C'de hem de 22±1°C'de muhafaza edilen çökelek örneklerine %0.01, 0.05 ve %0.1 oranında potasyum sorbat ilavesinin toplam aerobik mezofil bakteri, koliform bakteri, *Lactobacillus-Leuconostoc-Pediococcus, Stapylococcus-Micrococcus* sayıları üzerine herhangi bir etkisinin olmadığı görüldü (P>0.05). Bununla birlikte, 4±1°C'de muhafaza edilen çökelek örneklerinde %0.05 ve %0.1 oranında potasyum sorbat ilavesinin enterococcus ve maya-küf sayıları üzerine etkili olduğu bulundu (P<0.05). Çökelek örneklerinin muhafaza süreleri boyunca, kontrol grubu ve potasyum sorbat ihtiva eden gruplar arasında pH ve asidite bakımından bir fark görülmedi (P>0.05).

Anahtar sözcükler: *Çökelek, Mikrobiyolojik kalite, Muhafaza sıcaklığı, Potasyum sorbat*

INTRODUCTION

Cokelek is a popular traditional dairy product in Turkey. Cokelek is produced by diluting yoghurt at 1:1 ratios with potable water and churned for separation of milk fat, and then fat is removed. The remaining portion is boiled until precipitation. The arising precipitate is placed into cloth bags and hanged for overnight for removal of excess fluid. Then, it is kept under pressure for a short time (ca. 1 h) to reduce water content. It is then removed into a large pot and kneaded by hand adding salt at 1 or 2% (w/w). The average values of the chemical parameters of cokelek are 3.8 for pH, 1.25% for acidity (lactic acid), 0.95 for water activity, 1.38% for fat,

İletişim (Correspondence)
☎ +90 424 237000/3962
✉ oiilhak@firat.edu.tr

21.43% for dry substance, and 17.91% for protein [1]. Cokelek is sold without packing under refrigerated or ambient temperature in markets or bazaar. The major problem regarding cokelek is that the shelf life of the product is very short due to traditional production method is used, and the main cause of spoilage is the growth of yeast and molds [2].

Various preservatives have been used against spoilage of dairy products, especially against mold growth. The most frequently used preservative for this purpose is the sorbic acid and its salts [3-5]. Antimicrobial effects of sorbates may vary depending on multiple factors in foods including pH, water activity (a_w), initial microbial flora and other additives used [6]. Potassium sorbate is permitted to be used in dairy products up to 1000 mg/kg concentration according to Turkish Food Codex [7]. There are some studies investigating the effects of potassium sorbate in some dairy products which is popular in Turkey including white cheese, kashar cheese and yoghurt [8,9]. At present, no published study was found on use of sorbates for preservation of cokelek. There is also no standard for cokelek in Turkey.

The aim of this study was to investigate (i) the minimum concentration of potassium sorbate that is needed to limit the growth of yeast and mold in cokelek (ii) the effect of potassium sorbate on microbiological quality, pH and acidity levels of cokelek stored at 4±1 or 22±1°C.

MATERIAL and METHODS

Preparation of Cokelek

Experimental cokelek samples were produced from yoghurt at our laboratory. Briefly, yoghurt was diluted 1/1 ratio with tap water and heated to 90°C. After it was cooled to room temperature, it was transferred to cloth bags and hanged for overnight for removal of excess fluid. Then, it was kept under pressure for a short time (ca. 1 h) to reduce water content. The resulting curd was called as crude cokelek.

Adding Potassium Sorbate

Crude cokelek was divided into 4 equal portions for treatment groups. Treatment groups were called as group A with 2% salt (control), group B with 2% salt + 10 mg/kg potassium sorbate, group C with 2% salt + 100 mg/kg potassium sorbate, and group D with 2% salt + 1000 mg/kg potassium sorbate. A 100 g portion of cokelek samples in each group were placed into styrofoam plates and wrapped with saran film. The resulting packages in each group were further divided into two groups, and then, they were stored at 4±1°C or 22±1°C.

Microbiological Analyses

The samples were taken from crude cokelek, and on days 0, 5, 15, 25, 35, 45, 55, 65 and 75 during storage period of cokelek for microbiological and chemical analyses. A 10 g sample from each package was aseptically transferred to sterile stomacher bag and 90 ml ¼ Ringer solution was added before homogenizing for 2 min using a stomacher (BagMixer® 400, Interscience). Serial dilutions were prepared from the 10^{-1} dilution. Bacterial populations were enumerated using Plate Count Agar (Oxoid) for total aerobic mesophilic bacteria after incubation at 30±1°C for 72 h, Violet Red Bile Agar (Oxoid) for coliforms after incubation at 30±1°C for 24 h, Ragosa Acetate Agar for *Lactobacillus- Leuconostoc- Pediococcus* after incubation at 30±1°C for 5 days, Thallous Acetate Tetrazolium Glucose Agar (TITA) of Barnes for *Enterococcus spp.* after incubation at 45±1°C for 48 h [10], Potato Dextrose Agar (Oxoid) with pH 3.5 (acidified with 10% tartaric acid) for yeast and mold after incubation at 21±1°C for 5 days [11], Mannitol Salt Agar (Oxoid) for *Staphylococcus-Micrococcus* after incubation at 37±1°C for 36-48 h [12].

Chemical Analyses

The pH values of samples were recorded by using a pH meter (pH 2001, Selecta). Titratable acidity as %lactic acid was determined in accordance using method described for yoghurt by Turkish Standards Institute (TS 1330) [13].

Statistical Analyses

The study was composed of three independent replicates. The numbers of bacteria were converted to $\log_{10}$ cfu/g before calculating means and performing statistical analyses. The data were analyzed by analysis of variance (ANOVA) for main (fixed) effects (treatment, storage temperature, and sampling days) and three way interactions between treatments, storage temperature and sampling days by using Statistical Analysis System [14]. The means were separated by using Fisher's Least Significant Differences (LSD) test according to General Linear Model (GLM) procedures. Statistical significant level was accepted as 5% ($P<0.05$).

RESULTS

The data for microbiological analyses of cokelek stored at 4±1°C or 22±1°C were presented in *Table 1* and *2*, respectively. The data for pH level and titratable acidity of cokelek samples during storage at 4±1°C or 22±1°C were showed in *Fig. 1, 2, 3* and *4*, respectively.

Table 1. *Effects of various potassium sorbate levels on viability of some microorganisms in cokelek during storage at 4±1°C (log10 cfu/g)*

Tablo 1. *Çeşitli potasyum sorbat düzeylerinin 4±1°C'de muhafaza edilen çökeleklerde bazı mikroorganizmaların yaşamı üzerine etkisi (log10 cfu/g)*

Microorganisms	Crude Cokelek	Groups	Storage Days							
			0	5	15	25	35	45	55	65
Total Aerobic Mesophilic Bacteria	6.90	A	7.89 ^{az}	6.98 ^{az}	7.94 ^{az}	7.98 ^{az}	8.05 ^{az}	-	-	-
		B	7.27 ^{az}	6.50 ^{az}	6.86 ^{az}	7.14 ^{az}	7.49 ^{az}	8.91 ^{az}	-	-
		C	7.72 ^{az}	6.50 ^{az}	6.64 ^{az}	6.66 ^{az}	7.88 ^{az}	8.02 ^{az}	8.65 ^{az}	-
		D	7.77 ^{az}	6.11 ^{az}	6.54 ^{az}	6.57 ^{az}	7.48 ^{az}	7.71 ^{az}	7.76 ^{az}	8.02 ^{a}
Coliform	1.00	A	1.67 ^{az}	1.49 ^{az}	1.67 ^{az}	1.33 ^{az}	1.00 ^{az}	-	-	-
		B	2.09 ^{az}	1.69 ^{az}	1.63 ^{az}	1.56 ^{az}	1.12 ^{az}	<1.00 ^{az}	-	-
		C	1.00 ^{az}	1.72 ^{az}	1.45 ^{az}	1.30 ^{az}	<1.00 ^{az}	<1.00 ^{az}	<1.00 ^{az}	-
		D	1.00 ^{az}	1.51 ^{az}	1.33 ^{az}	1.20 ^{az}	<1.00 ^{az}	<1.00 ^{az}	<1.00 ^{az}	<1.00 ^{a}
Lactobacillus-Leuconostoc-Pediococcus	5.99	A	7.98 ^{az}	7.28 ^{az}	6.86 ^{az}	7.03 ^{az}	7.04 ^{az}	-	-	-
		B	6.06 ^{az}	7.54 ^{az}	6.43 ^{az}	6.15 ^{az}	6.60 ^{az}	6.15 ^{az}	-	-
		C	6.89 ^{az}	7.18 ^{az}	5.48 ^{az}	5.28 ^{az}	6.04 ^{az}	5.91 ^{az}	6.85 ^{az}	-
		D	7.06 ^{az}	7.04 ^{az}	6.30 ^{az}	5.61 ^{az}	5.64 ^{az}	5.90 ^{az}	6.33 ^{az}	7.03 ^{a}
Staphylococcus-Micrococcus	1.83	A	2.83 ^{az}	2.33 ^{az}	2.02 ^{az}	1.92 ^{az}	2.38 ^{az}	-	-	-
		B	2.10 ^{az}	2.03 ^{az}	1.59 ^{az}	2.10 ^{az}	2.32 ^{az}	2.13 ^{az}	-	-
		C	3.06 ^{az}	1.99 ^{az}	1.62 ^{az}	1.69 ^{az}	2.30 ^{az}	2.02 ^{az}	1.82 ^{az}	-
		D	2.66 ^{az}	2.02 ^{az}	1.65 ^{az}	1.64 ^{az}	2.01 ^{az}	1.49 ^{az}	1.68 ^{az}	1.10 ^{a}
Enterococcus	1.85	A	1.85 ^{azy}	1.51 ^{az}	2.02 ^{az}	2.03 ^{az}	1.32 ^{az}	-	-	-
		B	2.19 ^{az}	1.00 ^{bz}	1.59 ^{az}	1.36 ^{abz}	1.01 ^{bz}	<1.00 ^{bz}	-	-
		C	1.77 ^{azy}	1.00 ^{az}	1.62 ^{az}	1.70 ^{az}	1.43 ^{az}	<1.00 ^{az}	<1.00 ^{az}	-
		D	<1.00 ^{ay}	<1.00 ^{az}	1.65 ^{az}	<1.00 ^{az}	<1.00 ^{az}	<1.00 ^{az}	<1.00 ^{az}	<1.00 ^{a}
Yeast and Mold	5.07	A	5.77 ^{az}	6.86 ^{az}	6.61 ^{az}	7.01 ^{az}	6.89 ^{az}	-	-	-
		B	6.24 ^{az}	6.25 ^{az}	6.25 ^{az}	6.07 ^{azy}	5.92 ^{azy}	5.73 ^{az}	-	-
		C	5.79 ^{az}	5.97 ^{az}	5.68 ^{az}	5.39 ^{azy}	5.20 ^{azy}	4.96 ^{az}	4.81 ^{az}	-
		D	5.38 ^{az}	5.49 ^{az}	5.10 ^{az}	4.83 ^{ay}	4.74 ^{ay}	4.64 ^{az}	4.35 ^{az}	4.19 ^{a}

- : *Not analyzed due to spoilage of the product,* ***a,b:*** *Means with the different superscripts within the same row are significantly different (P<0.05),* ***z,y:*** *Means with the different superscripts within the same column are significantly different (P<0.05)*

Table 2. *Effects of various potassium sorbate levels on viability of some microorganisms in cokelek during storage at 22±1°C (log10 cfu/g)*

Tablo 2. *Çeşitli potasyum sorbat düzeylerinin 22±1°C'de muhafaza edilen çökeleklerde bazı mikroorganizmaların yaşamı üzerine etkisi (log10 cfu/g)*

Microorganisms	Crude Cokelek	Groups	Storage Days			
			0	5	15	25
Total Aerobic Mesophilic Bacteria	6.90	A	7.89 ^{az}	8.62 ^{az}	-	-
		B	7.27 ^{az}	8.04 ^{az}	-	-
		C	7.72 ^{az}	8,26 ^{az}	-	-
		D	7.77 ^{az}	8.66 ^{az}	7.67 ^{a}	8.11 ^{a}
Coliform	1.00	A	1.67 ^{az}	2.32 ^{az}	-	-
		B	2.09 ^{az}	2.24 ^{az}	-	-
		C	1.00 ^{az}	2.41 ^{bz}	-	-
		D	1.00 ^{az}	2.40 ^{bz}	1,62 ^{ab}	1.42 ^{a}
Lactobacillus-Leuconostoc-Pediococcus	5.99	A	7.98 ^{az}	7.52 ^{az}	-	-
		B	6.06 ^{az}	7.00 ^{az}	-	-
		C	6.89 ^{az}	6.91 ^{az}	-	4.94 ^{b}
		D	7.06 ^{az}	6.34 ^{az}	5.25 ^{ab}	
Staphylococcus-Micrococcus	1.83	A	2.83 ^{az}	2.34 ^{az}	-	-
		B	2.10 ^{az}	2.01 ^{az}	-	-
		C	3.06 ^{az}	1.83 ^{az}	-	-
		D	2.66 ^{az}	2.40 ^{az}	1.87 ^{a}	1.47 ^{a}
Enterococcus	1.85	A	1.85 ^{azy}	1.73 ^{az}	-	-
		B	2.19 ^{az}	1.62 ^{az}	-	-
		C	1.77 ^{azy}	1.48 ^{az}	-	-
		D	<1.00 ^{ay}	<1.00 ^{ay}	<1.00 ^{a}	<1.00 ^{a}
Yeast and Mold	5.07	A	5.77 ^{az}	7.46 ^{az}	-	-
		B	6.24 ^{az}	6.78 ^{az}	-	-
		C	5.79 ^{az}	5.67 ^{az}	-	-
		D	5.38 ^{az}	6.50 ^{az}	4.68 ^{a}	5.36 ^{a}

- : *Not analyzed due to spoilage of the product,* ***a,b:*** *Means with the different superscripts within the same row are significantly different (P<0.05),* ***z,y:*** *Means with the different superscripts within the same column are significantly different (P<0.05)*

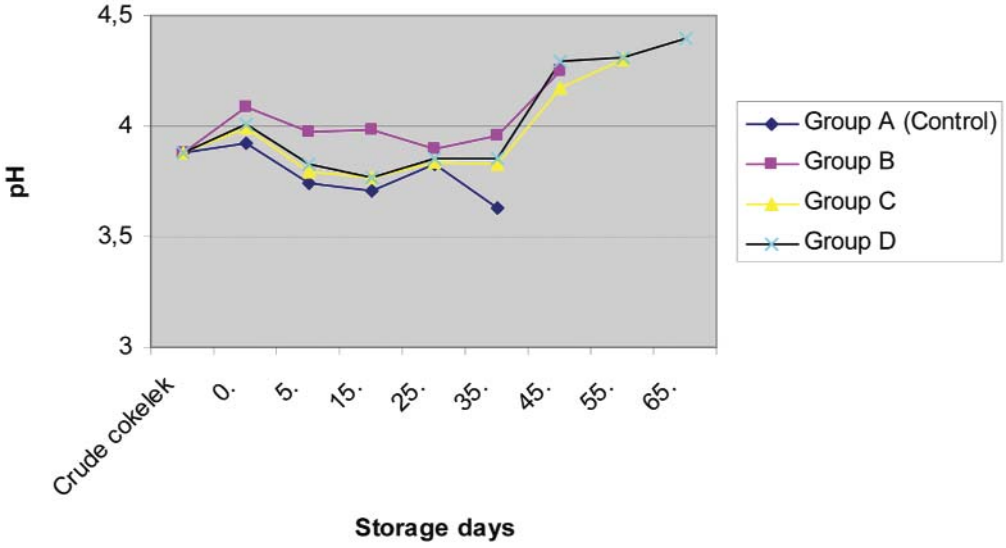

Fig 1. *Effects of various potassium sorbate levels on pH levels in cokelek during storage at 4±1°C*

Şekil 1. *Çeşitli potasyum sorbat düzeylerinin 4±1°C'de muhafaza edilen çökeleklerin pH düzeyleri üzerine etkisi*

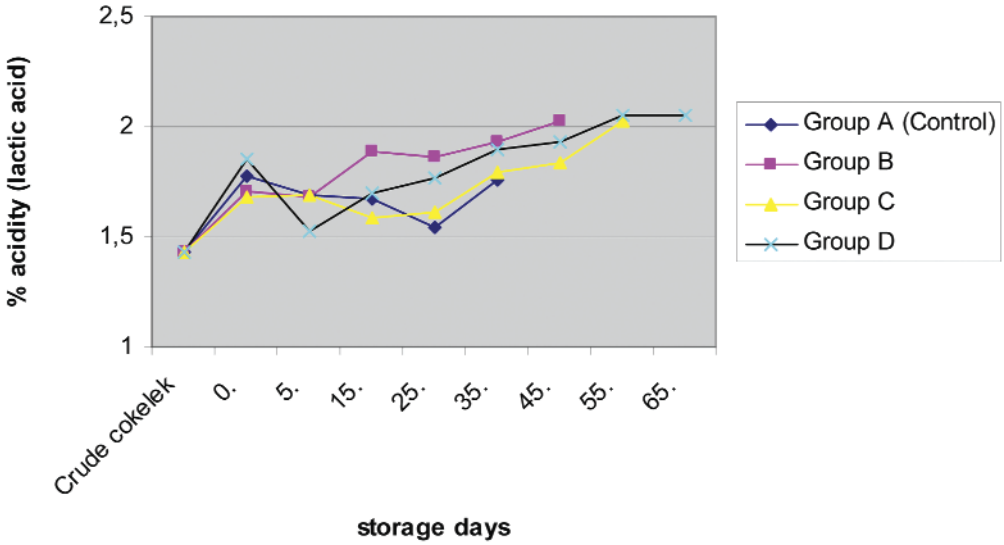

Fig 2. *Effects of various potassium sorbate levels on % acidity levels in cokelek during storage at 4±1°C*

Şekil 2. *Çeşitli potasyum sorbat düzeylerinin 4±1°C'de muhafaza edilen çökeleklerin % asitlik düzeyleri üzerine etkisi (laktik asit cinsinden)*

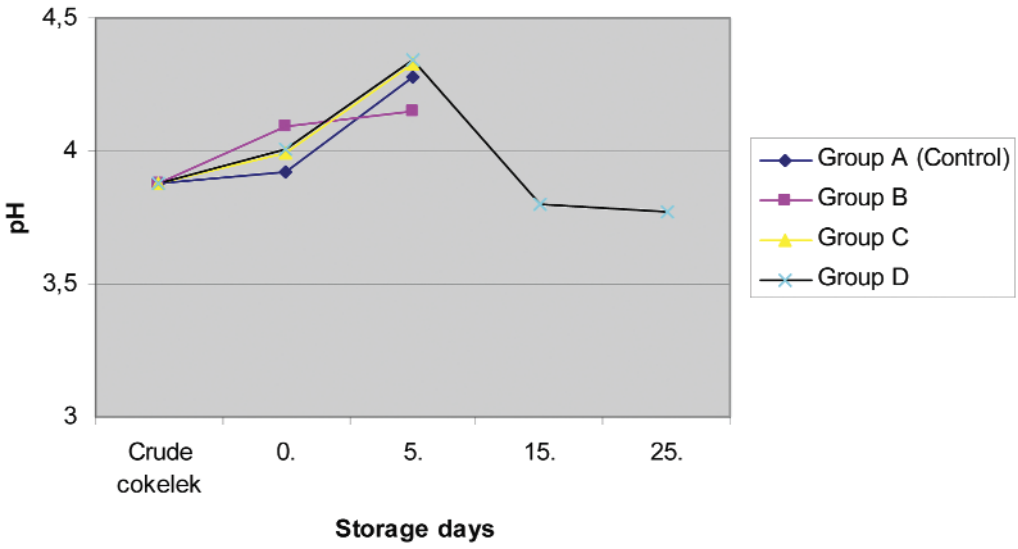

Fig 3. *Effects of various potassium sorbate levels on pH levels in cokelek during storage at 22±1°C*

Şekil 3. *Çeşitli potasyum sorbat düzeylerinin 22±1°C'de muhafaza edilen çökeleklerin pH düzeyleri üzerine etkisi*

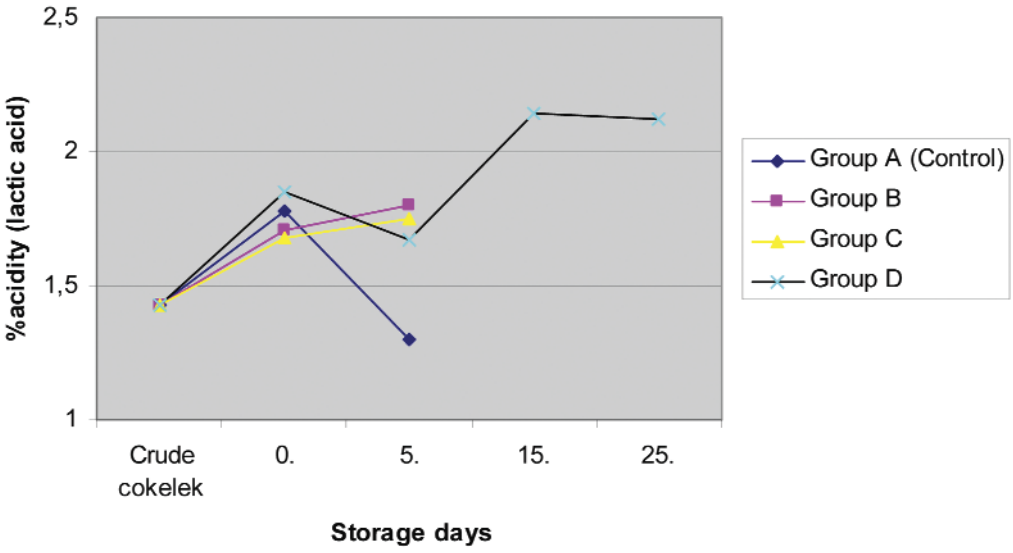

Fig 4. *Effects of various potassium sorbate levels on % acidity levels in cokelek during storage at 22±1°C*

Şekil 4. *Çeşitli potasyum sorbat düzeylerinin 22±1°C'de muhafaza edilen çökeleklerin % asitlik düzeyleri üzerine etkisi (laktik asit cinsinden)*

DISCUSSION

When it was inspected the effects of potassium sorbate with different concentration on counts of total aerobic mesophilic bacteria (TAMB) of cokelek samples within the same storage group (*Table 1* and *2*), it was not seen significant differences during their storage period (P>0.05). After treatment groups were prepared, TAMB increased in all groups on day 0 of storage period. And then, the numbers showed regular increase during the storage period and were found to be 8.05 $\log_{10}$ for control group on day 35, 8.91 $\log_{10}$ for group B on day 45, 8.65 $\log_{10}$ for group C on day 55 and 8.02 $\log_{10}$ for group D on day 65 of storage period at 4±1°C *(Table 1)*. Numbers of TAMB, in all samples stored at 22±1°C, were found to be above 8 $\log_{10}$ on day 5 of storage *(Table 2)*. Lack of significant effect of potassium sorbate on TAMB has been supported by the results of previous studies carried out in white cheese and kashar cheese [8,9,15]. However, it should be emphasized that there are other studies reporting that potassium sorbate had been found to be significant effect on TAMB [16-18]. This discrepancy may be due to the different material and the composition of flora.

Level of coliform bacteria insignificantly increased in group A (control) and group B on day 0, and group C and group D on day 5 of storage period at 4±1°C *(Table 1)*. After day 15 of storage period, coliform numbers continuously decreased in all groups stored at 4±1°C during the remaining period of storage. Their numbers dropped below detection limit on day 35 in group C and D, on day 45 in group B. Numbers of coliforms in samples stored at 22±1°C increased on day 5 of storage. After day 5, coliform numbers in group D continuously decreased and was found to be 1.42 $\log_{10}$ on day 25 of storage *(Table 2)*. In a study [15] carried out on kashar cheese noted that the potassium sorbate at 500 mg/kg concentration in the cheese samples decreased coliform bacteria counts. However, it was not seen statistical analyses on effect of potassium sorbate on coliform bacteria in the study mentioned. In the present study, when the results related to coliform bacteria counts were inspected, it was seen a decrease their counts in all groups during the storage period, not excepting control group. However, no significant differences were observed between treatment groups within the same storage group or the different storage group (P>0.05). There have not been sufficient literatures about the antimicrobial effect of potassium sorbate on growth of coliform bacteria.

The numbers of *Lactobacillus - Leuconostoc - Pediococcus,* in all groups stored at 4±1°C, decreased until on day 25 of storage period, and then continuously increased at the remaining period of storage *(Table 1)*. No significant differences were observed between treatment groups during storage period (P>0.05). These results are in agreement with findings of other researchers [8,15,17] who reported that inhibitory effect of potassium sorbate on this group of microorganisms was very limited (P>0.05). When it was inspected this group of bacteria in samples stored at 22±1°C, however, their numbers continually decreased in group D (1000 mg/kg potassium sorbate) during storage time of 25 days, and it was seen significant difference between days in this group of cokelek *(Table 2)*. This result may be due to more decrease in pH level (pH 3.8) or increase in titratable acidity (2.14% as lactic acid) in this group on day 15 of storage (*Fig. 3* and *4*).

The numbers of *Stapylococcus-Micrococcus* was lower in products treated with higher concentration of potassium sorbate and stored at 4±1°C *(Table 1)*. Statistical analyses showed that the numbers of these bacteria were not significantly different between the treatment groups (P>0.05). Moreover, changes in the numbers of these organisms within individual groups during storage indicates that potassium sorbate was not effective on *Stapylococcus-Micrococcus* (P>0.05) (*Table 1* and *2*). Our results are contradictory to the results reported by Nizamlıoğlu et al [9]. This difference can possibly be explained by the use of higher concentration potassium sorbate (%1, 2%, 3%) in their study.

The numbers of enterococci decreased in all treatment groups until day 5 of the storage. In those products stored at 4±1°C, enterococci numbers increased until day 10, and then showed a decrease during the remaining period of the storage. Moreover, their number dropped below detection limit in groups C and B on day 45. In group D in which potassium sorbate was used at high level, no *enterococcus spp.* was recovered during the storage, even on day 0 *(Table 1)*. In products stored at 22±1°C, numbers of enterococci decreased on day 5 of storage and as expected, no enterococci were recovered in group D during storage period. Numbers of enterococci were lower in cokelek containing potassium sorbate at 0.05% or 0.1% than control groups (P<0.05) (*Table 1* and *2*). This may indicate that potassium sorbate might be effective on survival of this group of bacteria, as previously reported by Doğruer et al [8].

The numbers of yeast and mold was initially 5.07 log cfu/g in crude cokelek, and it was generally lower in groups containing potassium sorbate compared to control group (*Table 1* and *2*). The numbers of yeast and mold was lower in product treated with higher concentration of potassium sorbate (group D) during storage period at 4±1°C. It was also observed that the numbers of yeast and mold continuously decreased in groups containing potassium sorbate as storage periods increased *(Table 1)*. No significant differences were observed between days (P>0.05) during storage period of products at 4±1°C, and there were no significant differences between groups until day 25 of storage. On day 25 and 35 of storage, significant differences were observed between group D and control group depending on continuously decrease of the numbers of yeast and mold in the group D treated with higher level of potassium sorbate during storage period (P<0.05) *(Table 1)*. Our findings are in agreement with findings of other researchers who studied on the effect of potassium sorbate on yeast and mold in different products [5,18,19]. Statistical analyses, however, showed that potassium sorbate was not effective on yeast and mold in cokelek stored at 22±1°C (P>0.05), even though in higher concentration such as 1000 ppm *(Table 2)*. It has been noted that antimicrobial effect of potassium sorbate increases as the storage temperature decrease [17,20]. This information may explain, in the present study, why potassium sorbate had no effect on yeast and mold in cokelek stored at 22±1°C.

The data for pH and acidity were presented in *Fig. 1-4*. pH levels in crude cokelek was 3.9, this level insignificantly decreased to 3.7 until on day 15 of storage, and then increased to 4.2 for group B on day 45, 4.3 for group C on day 55 and 4.4 for group D on day 65 of storage at 4±1°C *(Fig. 1)*. pH level of control group was detected 3.6 on day 35 of storage. Acidity level as lactic acid was 1.43 in crude cokelek. This level increased to 1.76 for control group on day 35, 2.03 for group B and C on day 45 and 55, 2.05 for group D on day 65 of storage at 4±1°C *(Fig. 2)*. As for samples stored at 22±1°C, pH levels of samples increased to 4.2-4.3 for all groups on day 5. After day 5, pH level of group D decreased to 3.7 on day 25 of storage *(Fig. 3)*. Acidity levels of these samples were found to be 1.3% for control, 1.8% for group B and C, 1.7% for group D on day 5. After day 5, acidity level of group D increased to 2.1 on day 25 of storage *(Fig. 4)*. Statistical analyses showed that data obtained from samples were not significantly different (P>0.05) between control and treatment groups depending on temperature and period of storage.

Determining the right concentration of a preservative to be used in a food is not easy. The type of the food, the initial microbiological load, and conditions of processing and storage are important factors that effect the concentration of the preservative to be used [5]. In the present study, all findings showed that application of potassium sorbate at concentration of 100, 500 and 1000 ppm did not have an effect on numbers of total mesophilic aerobic bacteria, coliform bacteria, *lactobacillus- leuconostoc- pediococcus* and *staphylococcus-micrococcus* in cokelek stored at both 4±1°C and 22±1°C. However, potassium sorbate of 500 and 1000 ppm had an effect on numbers of enterococci and yeast-mold in cokelek stored at 4±1°C, not 22±1°C. It was also observed that shelf life of cokelek was depending on the level of potassium sorbate added and storage temperature. However, due to cokelek is produced under unmechanized or artisanal conditions in small dairy plants or on farms; it is difficult to find a standard product with respect to composition and microbiological quality of cokelek. Therefore, it is needed further research on suitable preservatives on cokelek.

REFERENCES

1. Öksüztepe GA, Patır B, Dikici A, Bozkurt Ö, Çalıcıoğlu M: Microbiological and chemical quality of cokelek marketed in Elazig. *Fırat Univ Sağ Bil Derg,* 21 (1): 27-31, 2007.

2. Kıvanç M: Gıda koruyucusu olarak sorbik asit ve tuzları: I. Genel özellikleri. *Gıda,* 14 (5): 315-320, 1989.

3. Marth EH, Constance MC, Hasenzahl L, Jackson HW, Hussong RV: Degradation of potassium sorbate by *Penicillium* species. *J Dairy Sci,* 49 (10): 1197- 1205, 1966.

4. Han JH, Floros JD: Potassium sorbate diffusivity in American processed and Mozzarella cheeses. *J Food Sci,* 63 (3): 435-437, 1998.

5. Mihyar GF, Yamani MI, Al-Sa'ed AK: Resistance of yeast flora of Labaneh to potassium sorbate and sodium benzoate. *J Dairy Sci,* 80 (10): 2304-2309, 1997.

6. Campos CA, Gerchenson LN: Effect of certain additives on sorbate stability. *Food Res Int,* 29 (2): 147-154, 1996.

7. Turkish Food Codex: Gıda katkı maddeleri sıralı EC numaraları kullanım dozu ve özel şartları, Ek 1-13. Ministry of Agriculture and Rural Affairs, Ankara, 2000.

8. Doğruer Y, Gürbüz Ü, Nizamlıoğlu M: Potasyum sorbatın beyaz peynirin kimyasal ve mikrobiyolojik kalitesine etkisi. *Selçuk Univ Vet Bil Derg,* 12 (1): 109-116, 1996.

9. Nizamlıoğlu M, Gürbüz Ü, Doğruer Y: Potasyum sorbatın kaşar peynirin kimyasal ve mikrobiyolojik kalitesine etkisi. *Selçuk Univ Vet Bil Derg,* 12 (2): 23-29, 1996.

10. Harrigan WF: Laboratory methods in food microbiology. 3rd ed. Academic Press, London, 1998.

11. Oxoid: The Oxoid Manuel. 50th ed. Published by Oxoid Ltd, Hampshire, 1982.

12. British Standards Institution: Methods of microbiological examination for dairy purposes. BS 4285. British Standards Institution, London, 1968.

13. Turkish Standards Institute: Yoğurt. TS1330. Ankara, 2006.

14. Statistical Analyses System: SAS, Inst. Inc. Cary. 8. Version, North Caroline, 1999.

15. Ozdemir C, Demirci M: Selected microbiological properties of kashar cheese samples preserved with potassium sorbate. *Int J Food Prop,* 9, 515-521, 2006.

16. Bradley RL, Harmon LG, Stine CM: Effect of potassium sorbate on some organisms associated with cottage cheese spoilage. *J Milk and Food Techn,* 25, 318-323, 1962.

17. Sofos JN, Busta FF: Antimicrobial activity of sorbate. *J Food Protect,* 44 (8): 614-622, 1981.

18. Yılmaz L, Kurdal E: Effect of sorbic acid and potassium sorbate addition to the brine on microbiological and chemical properties of Turkish white cheese during ripening. *Food Sci Technol Res,* 14 (5): 437-444, 2008.

19. Aly ME: Prolongation of the keeping quality of mozzarella cheese by treatment with sorbate. *Nahrung,* 4, 194-200,1996.

20. Gram L: Inhibition of mesophilic spoilage *Aeromonas spp.* on fish by salt, potassium sorbate, liquid smoke and chilling. *J Food Protect,* 54 (6): 436-442, 1991.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S107-S112, 2010
DOI:10.9775/kvfd.2010.2030

RESEARCH ARTICLE

Incidence and Pathogenicity of *Yersinia enterocolitica* Isolates from Foods in Turkey [1]

Abamüslüm GÜVEN * Çiğdem SEZER * Berna DUMAN AYDIN *
Nebahat BİLGE ORAL * Leyla VATANSEVER *

[1] *This Project was supported by the Commission for the Scientific Research Projects of Kafkas Univertsity (The Project number: VF-2003-09)*

* Kafkas University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, TR-36100 Kars - TURKEY

Makale Kodu (Article Code): KVFD-2010-2030

Summary

During a year period, at 150 from each food group, a total of 750 samples including ice cream, raw milk, feta cheese, chicken drumsticks and minced meat collected from markets located in the northeast region of Turkey (Kars, Ardahan and Iğdır) were analyzed for determining of *Yersinia spp.* incidence. Fifty seven of samples (7.6%) were evaluated as positive for *Yersinia spp.* and 18 (2.4% in total) of them, isolated from 6 feta cheese, 4 ice cream, 2 chicken drumsticks, 4 minced meat and 2 raw milk samples, were identified as pathogenic *Y. enterocolitica.* All the 18 pathogenic strains were tested for their antimicrobial susceptibility and some of the isolates were found to be resistant to ticarcillin (n=6), netilmicin (n=5), tetracycline (n=1) streptomycin (n=17), gentamicin (n=12), kanamycin (n=17), furazolidone (n=6), clindamycin (n=18) and cephazolin (n=18) while all of them were susceptible to sulphamethoxazole/trimethoprim, ciprofloxacin, chloramphenicol and imipenem. According to findings, cold enrichment at 4°C for 14 days seems to be more effective for isolation of *Yersinia spp.* than enrichment at 25°C for 24 h.

Keywords: *Yersinia enterocolitica, Pathogenicity, Animal Originated Foods*

Türkiye'de Gıdalardan İzole Edilen *Yersinia entocolitica*'nın Yaygınlığı ve Patojenitesi

Özet

Kars, Ardahan ve Iğdır'da yer alan marketlerden, bir yıl boyunca, her bir gıdadan 150'şer adet olmak üzere, toplam 750 örnek (çiğ süt, dondurma, taze beyaz peynir, tavuk budu ve kıyma) toplanarak *Yersinia spp.* varlığı araştırılmıştır. Örneklerin 57'sinden (%7,6) *Yersinia spp.* izole edilmiş ve bunlar içerisinde 6'sı beyaz peynir, 4'ü dondurma, 2'si tavuk butu, 4'ü kıyma ve 2'si çiğ süt olmak üzere toplam 18 gıdadan (% 2,4) elde edilen izolatlar, patojenik *Yersinia enterocolitica* olarak identifiye edilmiştir. 57 gıda örneğinden %31,57 oranında patojenik *Y. enterocolitica* identifiye edilmiştir. Antibiyotik duyarlılığı yönünden incelenen 18 suşun ticarcillin (n=6), netilmicin (n=5), tetracycline (n=1) streptomycin (n=17), gentamicin (n=12), kanamycin (n=17), furazolidone (n=6), clindamycin (n=18) ve cephazolin'e (n=18) dirençli olduğu görülürken, tümünün sulphamethoxazole/trimethoprim, ciprofloxacin, chloramphenicol ve imipenem'e duyarlı olduğu belirlenmiştir. Elde edilen bulgular, soğuk zenginleştirme (4°C'de 14 gün) yönteminin, 25°C'de 24 saatlik zenginleştirme prosedürüne göre *Yersinia spp.* izolasyonunda daha başarılı olduğuna işaret etmiştir.

Anahtar sözcükler: *Yersinia enterocolitica, Patojenite, Hayvansal gıdalar*

INTRODUCTION

The genus *Yersinia* is a member of the family *Enterobacteriaceae* and includes pathogenic and several non-pathogenic strains [1,2]. The genus *Yersinia* is composed of 11 species, of which three (*Y. pestis, Y. pseudotuberculosis,* and *Y. enterocolitica*) have clearly been shown to cause human disease. The remaining

İletişim (Correspondence)
☎ +90 474 2426807/1184
✉ guvenaba1@hotmail.com

eight species considered as nonpathogen (*Y. frederiksenii, Y. intermedia, Y. kristensenii, Y. bercovieri*, *Y. mollaretii, Y. rohdei, Y. ruckeri* and *Y. aldovae*) have not been studied extensively due to the absence of classical *Yersinia* virulence markers [3]. Currently, *Y. enterocolitica* is represented by six biovars (1A, 1B and 2-5) and more than 50 serovars. The virulence of the pathogenic biovars namely 1B and 2-5 is attributed to the presence of a 70-kb pYV (plasmid for *Yersinia virulence*) and certain chromosomal genes [4]. *Y. enterocolitica* is an important entero-pathogen can cause acute enteritis (especially in children), enterocolitis, mesenteric lymphadenitis, and terminal ileitis. Colonization of the intestinal tract is the primary event of the successful enteric pathogen [5].

The pathogenic bacterium *Y. enterocolitica* has become increasingly important as a food contaminant. Of special significant in food hygiene is the ability of *Y. enterocolitica* to grow in refrigerated foods [6]. The psychrotrophic bacterium *Y. enterocolitica* which is able to grow at temperatures close to 0°C is characterized by temperature-dependent adaptations [7]. Yersiniosis is a typical foodborne disease. Yersinia has been frequently isolated from a variety of foods like untreated milk, chocolate milk, dairy cream and ice cream, vegetables like carrots, tomatoes, lettuce, celery and mushrooms, raw hare, beef and lamb. It has also been isolated from drinking water [8].

Difficulties associated with the isolation of pathogenic *Y. enterocolitica* stem from the small number of pathogenic strains in the samples and the large number of organisms in the background flora, especially in food and environmental samples. However, in order to get epidemiological information, *Y. enterocolitica* isolates are needed. Thus, at least one culture methods has to be used in parallel to PCR method. Selective enrichment is needed, especially when food samples are studied. However, no single procedure is currently available which will recover all bioserotypes [9]. However, the detection, isolation and enumeration of *Y. enterocolitica* remain problematic. The development of isolation procedures which clearly differentiate pathogenic from non-pathogenic variants has been difficult [6,9].

Y. enterocolitica and related species have been isolated from many types of food [8,10-16]. The majority of these food isolates differ in biochemical and serological characteristics from typical clinical strains and are usually mentioned 'non-pathogenic' or 'environmental' Yersinia strains [6,17]. It is important to determine the pathogenic significance of the isolates. *Y. enterocolitica* is thought to be a significant food-borne pathogen although the incidence of the pathogenic isolates in foods is low [6,17-19].

In the northeast region of Turkey, cheese, cream, butter and ice cream are made traditionally using raw milk. Although the consumption rate of unpasteurized dairy products is gradually decreasing in Turkey, little no more information about the incidence of pathogenic *Yersinia spp.* has been documented. In this study, it was aimed to evaluate the presence of pathogenic *Y. enterocolitica* in some foods marketed in a part of the northeast area of Turkey and determine of resistance of isolates to some antibiotics.

MATERIALS and METHODS

Samples

A total of 750 samples including 150 ice cream, 150 raw milk, 150 fresh (feta) cheese, 150 chicken drumsticks and 150 minced meat were collected from markets in three cities (Kars, Ardahan and Iğdır) located at the Northeast of Turkey. Food samples were randomly selected and delivered to the laboratory in an ice box at 4°C within 2 h from collection and tested immediately upon arrival.

Isolation and Identification of Yersinia spp.

A 25 g of sample was aseptically added to 100 ml 0.01 M Phosphate Buffered Saline (PBS, pH 7.6) in a sterile stomacher plastic bag and homogenized for 2 min. The homogenates were incubated at 25°C for 10 min. Different enrichment procedures were applied. One of the methods was adding a 20 ml from homogenate to 80 ml TSB (Trypticase soya broth- Oxoid CM 0129B) and enriching at 25°C for 24 h. Another procedure was adding a 20 ml from homogenate 80 ml PBS (Phosphate Buffered Saline) and enriching at 4°C for 14 days (cold enrichment). The samples were treated with KOH (0.5% KOH in 0.5% saline) to suppress background flora after enrichment. Subculturing on selective CIN agar plates (Yersinia Selective Agar Base -Oxoid CM 0653B) was applied according to the method of the FDA [20]. One to five susceptible colonies of typical “bull’s eye” appearance on the CIN agar plates, if available, were streaked onto Tryptone Soya Agar (Oxoid CM 0131B) plates to create a pure culture. All the isolates from pure cultures were examined for Gram staining, utilization of Simmon’s citrate, Kligler’s Iron agar reaction and urease activity [20].

Confirmation and Biogrouping of Yersinia enterocolitica

All the isolates which were negative for utilization of citrate, positive for urease activity and giving an alkaline slant/acid butt without gas or H_2S on KIA were submitted to further testing. In order to identification

and biogrouping of isolates as *Y. enterocolitica;* activities of oxidase, lysine decarboxylase, ornithine decarboxylase, β-D-glucosidase, lipase and pyrazinamidase, utilization of rhamnose, sucrose, xylose and trehalose, and salicin were evaluated. Further analyses were also conducted applying Esculin hydrolysis, Indole and Voges Proskauer tests to isolates. The reference strain *Y. enterocolitica* O: 3 (serotype 920) used in this study were purchased from culture collection of Refik Saydam Hygiene Center Ankara, Turkey. *Y. enterocolitica* isolates were biotyped according to the revised biogroup scheme of Wauters et al.[21], Schiemann and Wauters [22] and FDA [20].

Testing for Pathogenicity Markers

Y. enterocolitica strains were tested for virulence by Temp-Dependent autoagglutination (25°C-35°C) in Methyl Red-Voges Proskauer broth (Oxoid CM 0043B), occur of small red colonies on CR-MOX agar and Congo red binding/crystal violet binding assays [20,23,24].

Antimicrobial Susceptibility

Determination of antimicrobial susceptibility of *Y. enterocolitica* strains to antibiotics which are used to treat of Yersiniosis was performed according to the National Committee for Clinical Laboratory Standards (NCCLS) [25] using Mueller Hinton agar (Oxoid CM 0337B) and commercially available antimicrobial test discs *(Table 2).* Results were recorded by measuring the inhibition zones and scored as sensitive, intermediate susceptibility and resistant according to the NCCLS [25] recommendations.

RESULTS

Presence of Yersinia spp. in Food Samples

In this study, two different enrichment procedures were applied to each sample as overnight and cold enrichment. All of 18 strains were recovered after cold enrichment for 14 days, but no strain was isolated from the samples enriched at 25°C for 24 h in TSB. Out of the 750 analyzed food samples, 57 samples (7.6%) were evaluated as positive for *Yersinia spp.* and 18 (2.4% in total) of them, isolated from 6 feta cheese, 4 ice cream, 2 chicken drumsticks, 4 minced meat and 2 raw milk samples, were evaluated as pathogenic *Y. enterocolitica.* All the 18 pathogenic strains were tested for their antimicrobial resistance. A total of 31.57% of the 57 food samples including *Yersinia spp.* were contaminated by pathogenic *Y. enterocolitica.* Biotypes 1B, biotype 2, 3 and 4 were identified. Biotype distribution and test applied were documented in *Table 1.*

Antimicrobial Susceptibility Test Results

No strain was resistant to sulphamethoxazole/ trimethoprim, ciprofloxacin, chloramphenicol and imipenem. Six isolates were resistant to ticarcillin, 5 isolates netilmicin, and 1 isolate tetracycline whereas 17 isolates were resistant to streptomycin, 12 isolates gentamicin, 17 isolates kanamycin, and 6 isolates furazolidone. All of strains were resistant to clindamycin and cephazolin *(Table 2).*

Table 1. *Biogrouping results of Y. enterocolitica isolated from different food samples*
Tablo 1. *Değişik gıda örneklerinden elde edilen Y. enterocolitica izolatlarının biogrupları*

Isolates	Feta Cheese						Icecream				Chicken Drumsticks		Minced Meat				Milk	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Indole	+	+	+	+	+	-	-	+	-	+	+	+	-	-	+	+	+	+
Voges Proskauer	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+
Salicin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lysine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-D-Glucosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Esculin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lipase	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CR-MOX *	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Autoagglutination	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pyrazinamidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Biotyping	**1B**	**1B**	**1B**	**1B**	**2**	**3**	**3**	**2**	**4**	**2**	**2**	**2**	**4**	**4**	**2**	**2**	**2**	**2**

** Small red colonies on CR-MOX agar*

Table 2. *Antimicrobial susceptibility test results of pathogenic Y. enterocolitica isolated from food samples*
Tablo 2. *Patojenik Y. enterocolitica izolatlarının antimikrobiyel duyarlılık test sonuçları*

Antimicrobial Agent	Numbers of Isolates (%)		
	Susceptible	Intermediate	Resistant
Amikacin (30 mcg) (Oxoid CTO 107B)	7 (38.8)	3 (16.6)	8 (44.4)
Amoxycillin/Clavulanic acid (30 mcg) (Oxoid CTO 223B)	6 (33.3)	7 (38.8)	5 (27.7)
Ampicillin (10 mcg) (Oxoid CTO 003B)	- (0)	8 (44.4)	10 (55.5)
Cefoperazone (75 mcg) (Oxoid CTO 249B)	1 (5.5)	6 (33.3)	11 (61.1)
Cefotaxime (30 mcg) (Oxoid CTO 166B)	13 (72.2)	5 (27.7)	- (0)
Cephazolin (30 mcg) (Oxoid CTO 011B)	- (0)	- (0)	18 (100)
Ciprofloxacin (5 mcg) (Oxoid CTO 425B)	18 (100)	- (0)	- (0)
Chloramphenicol (30 mcg) (Oxoid CTO 013B)	18 (100)	- (0)	- (0)
Clindamycin (2 mcg) (Oxoid CTO 064B)	- (0)	- (0)	18 (100)
Furazolidone (15 mcg) (Oxoid CTO 448B)	4 (22.2)	- (0)	14 (77.7)
Gentamicin (10 mcg) (Oxoid CTO 024B)	6 (33.3)	- (0)	12 (66.6)
Imipenem (10 mcg) (Oxoid CTO 455B)	18 (100)	- (0)	- (0)
Kanamycin (5 mcg) (Oxoid CTO 025B)	1 (5.5)	- (0)	17 (94.4)
Netilmicin (30 mcg) (Oxoid CTO 225B)	10 (55.5)	3 (16.6)	5 (27.7)
Streptomycin (10 mcg) (Oxoid CTO 047B)	1 (5.5)	- (0)	17 (94.4)
Sulphamethoxazole/trimethoprim (25 mcg) (Oxoid CTO 052B)	18 (100)	- (0)	- (0)
Tetracycline (30 mcg) (Oxoid CTO 041B)	15 (83.3)	2 (11.1)	1 (5.5)
Ticarcillin (75 mcg) (Oxoid CTO 167B)	7 (38.8)	5 (27.7)	6 (33.3)
Ticarcillin/Clavulanic acid (85 mcg) (Oxoid CTO 449B)	17 (94.4)	1 (5.5)	- (0)
Trimethoprim (5 mcg)	11 (61.1)	- (0)	7 (38.8)

All the 18 pathogenic strains were tested for their antimicrobial susceptibility and some of the isolates were found to be resistant to ticarcillin (n=6), netilmicin (n=5), tetracycline (n=1) streptomycin (n=17), gentamicin (n=12), kanamycin (n=17), furazolidone (n=6), clindamycin (n=18) and cephazolin (n=18) while all of them were susceptible to sulphamethoxazole/trimethoprim, ciprofloxacin, chloramphenicol and imipenem.

DISCUSSION

In this study, 750 food samples were analyzed. Pathogenic *Y. enterocolitica* was isolated from 18 samples (2.4%) of all the samples. Two different enrichment procedures were applied to each sample as overnight and cold enrichment. All of 18 strains were recovered after cold enrichment for 14 days, but no strain was isolated from the samples enriched at 25°C for 24 h in TSB. A possible explanation for the very low recovery rate after cold enrichment might be the low number of pathogenic *Y. enterocolitica* strains contaminated in food samples or high background flora on the selective agar. In overnight enrichment at room temperature, endogenous microflora overgrew, suppressing the growth of *Y. enterocolitica.* The psychrotrophic nature of *Y. enterocolitica* is unusual among other *Enterobacteriaceae*; consequently, enrichment in different solutions at 4°C for prolonged periods has been used for isolation of *Yersinia spp.*[9]. However, the time needed for this method is a disadvantage for routine analysis.

The isolation rate of *Y. enterocolitica* observed in this study was close or considerably lower than that of studies which had reported previously *(Table 3)*. This can be explained by differences in isolation and identification methods, false analysis results which might be occurred depend on methods used, different seasons those samples obtained, and diversity of kind, hygienic condition and also competing microflora of samples. In this study, cheese and ice cream samples appeared to be more noticeable samples among others.

Variety of research findings related to antimicrobial susceptibility of *Y. enterocolitica* has been published [33,35,36]. Antibiotic susceptibility data for *Y. enterocolitica*

Table 3. *Isolation of Y. enterocolitica from foods (literature data)*
Tablo 3. *Gıdalardan Y. enterocolitica izolasyon oranları (literatür bilgisi)*

Foods	Country	Incidence (%)	Reference
Raw milk	USA	6.1	26
Raw milk	Turkey	20	16
Raw milk	Iran	1.6	27
Raw milk	Normandy	36	28
Cheese	Argentina	0	29
Cheese	Turkey	35.7	15
Cheese	Morocco	4	30
Ice cream	India	40.3	14
Ice cream	India	0	31
Chicken meat	Argentina	4.3	32
Chicken meat	Spain	50	10
Chicken meat	Austria	44.9	33
Minced meat	German	0.5	34
Meat	Spain	0	13

has been somewhat inconsistent and has validated between concordance and nonconcordance among different serogroups and biotypes [2]. Aarestrup et al.[37] reported that some of their *Y. enterocolitica* strains were intermediate resistant against ampicillin although it had been previously noted as naturally resistant. Indeed, eight years before that study, Kwaga and Iversen [38] showed that all or most of the strains were resistant against ampicillin, clindamycin, cephazolin and amoxicillin-clavulanic acid while 100% of them were susceptible to sulfamethoxazole-trimethoprim, imipenem and ticarcillin-clavulanic acid. We also found 8 (44.4%) isolates evaluated as intermediate resistant against ampicillin in addition to 10 (55.5%) resistant strains. Those results are important for their role warning about ampicillin resistance. In another study, Lyons et al.[39] reported that 100% of strains were resistant against tetracycline and trimethoprim. In this study no strain was resistant against trimethoprim and tetracycline. In another study, Pham et al.[40] assessed the antibiotic susceptibility profile of 100 clinical isolates of *Y. enterocolitica.* According to their results, all the 100 isolates were uniformly susceptible to chloramphenicol, ciprofloxacin, gentamicin, tetracycline and trimethoprim. Our findings representing susceptibility of all the strains investigated in this study were parallel to those of Pham et al.[40]. In an early study, all *Y. enterocolitica* isolates were subjected to resistance against tetracycline, gentamicin, kanamycin, trimethoprim and chloramphenicol [33]. In this study, antimicrobial susceptibility results of *Y. enterocolitica* isolates were generally similar to that of previous ones. However, it is difficult to predict warning signals about gaining resistance ability of that Yersinia strain in time, investigating just 18 isolates.

Consequently, our results showed that raw and ready to eat animal originated foods tested in this study were contaminated with pathogenic biotype *Y. enterocolitica* even though in low percentage and thereby represented a risk to the consumers in regard to yersiniosis.

REFERENCES

1. Bercovier H, Mollaret HH: Yersinia. **In,** Krieg NR, Holt JG (Eds): Bergey's Manual of Systematic Bacteriology Vol 1. pp. 498-506, Williams and Wilkins, Baltimore, 1984.

2. Bottone EJ: *Yersinia enterocolitica*: Overview and epidemiological correlates. *Microbes Infec,* 1, 323-333, 1999.

3. Sulakvelidze A: Yersiniae other than *Y. enterocolitica, Y. pseudotuberculosis* and *Y. pestis:* The ignored species. *Microbes Infec,* 2, 497-513, 2000.

4. Bhagat N, Virdi JS: Distribution of virulence-associated genes in *Yersinia enterocolitica* biovar 1A correlates with clonal groups and not the source of isolation. *FEMS Microbiol Letters,* 266, 177-183, 2007.

5. Bottone EJ: *Yersinia enterocolitica:* The charisma continues. *Clin Microbiol Rev,* 10, 257-276, 1997.

6. Kapperud G: *Yersinia enterocolitica* in food hygiene. *Int J Food Microbiol,* 12, 53-66, 1991.

7. Goverde RL, Huis in't Veld JH, Kuster JG, Mooi FR: The psychrotrophic bacterium Yersinia enterocolitica requires expression of pnp, the gene for polynucleotide phosphoralase, for growth at low temperature (5 degrees C). *Mol Microbiol,* 28, 555-569, 1998.

8. Filetici E, Anastasio MP, Pourshaban M, Fantasia M: Genotypic and phenotypic characteristics of *Yersinia spp.* isolates from food and man. *Food Microbiol,* 17, 261-267, 2000.

9. Fredriksson Ahomaa M, Korkeala H: Low occurrence of pathogenic *Yersinia enterocolitica* in clinical, food and environmental samples: A methodological problem. *Clin Microbiol Rev,* 16, 220-229, 2003.

10. Capita R, Alonso-Calleja C, Prieto M, Garcia-Fernandez MC, Moreno B: Incidence and pathogenicity of *Yersinia spp.* Isolates from poultry in Spain. *Food Microbiol,* 19, 295-301, 2002.

11. Hudson JA, King NJ, Cornelius AJ, Bigwood T, Thom K, Monson S: Detection, isolation and enumeration of *Yersinia enterocolitica* from raw pork. *Int J Food Microbiol,* 123, 25-31, 2008.

12. Çetinkaya A, Güven A: Kars ilinde tüketime sunulan çiğ süt ve beyaz peynirlerde *Yersinia enterocolitica* türlerinin araştırılması. *Kafkas Univ Vet Fak Derg,* 7 (2): 135-142, 2001.

13. Soriano JM, Rico H, Molto JC, Manes J: Incidence of microbial flora in lettuce, meat and Spanish potato omelette from restaurants. *Food Microbiol,* 18, 159-163, 2001.

14. Warke R, Kamat A, Kamat M, Thomas P: Incidence of pathogenic psychotrophs in ice creams sold in some retail outlets in Mumbai. India. *Food Cont,* 11, 77-83, 2000.

15. Yucel N, Ulusoy H: A Turkey survey of hygiene indicator bacteria and *Yersinia enterocolitica* in raw ilk and cheese samples. *Food Cont,* 17, 383-388, 2006.

16. Soyutemiz E, Çetinkaya F, Özakın C, Gedikoğlu S: Çiğ sütlerde *Yersinia enterocolitica* varlığının araştırılması. *Türk Mikrobiyol Cem Derg,* 30, 30-34, 2000.

17. De Boer E: Isolation of *Yersinia enterocolitica* from foods. *Int J Food Microbiol,* 17, 75-84, 1992.

18. Fredriksson Ahomaa M, Korte T, Korkeala H: Contamination of carcasses, offals and the environmental with yadA-positive *Yersinia enterocolitica* in a pig slaughterhouse. *J Food Prot,* 63, 31-35, 2000.

19. Fredriksson Ahomaa M, Lyhs U, Korte T, Korkeala H: Prevalence of pathogenic *Yersinia enterocolitica* in food samples at retail level in Finland. *Arch Lebensmittelhyg,* 52, 66-68, 2001.

20. FDA/CFSAN/BAM: *Yersinia enterocolitica.* Bacteriological Analytical Manual Online Chp. 8. US Food and Drug Administration Center for Food Safety and Applied Nutrition. Weagant SD and Feng P, http://www.cfsan.fda.gov/~ebam/bam-8.html, 2001, Update 2007.

21. Wauters G, Kandolo K, Janssens M: Revised biogrouping scheme of Yersinia enterocolitica. *Contr Microbiol Immunol,* 9, 13-21, 1987.

22. Schiemann DA, Wauters G: Yersinia. **In,** Vanderzant C, Splittstoesser F (Eds): Compendium of Methods for the Microbiological Examination of Foods, 3rd Washington, DC, American Public Health Association, pp. 433-450, 1992.

23. Laird W, Cavanough DC: Correlation of autoagglutination and virulence of Yersinia. *J Clin Microbiol,* 11, 430-432, 1980.

24. Farmer JJ, Carter GP, Miller VL, Falkow S, Wachmuth IK: Pyrazinamidase, CR-MOX agar, salicin fermentation-esculin hydrolysis, and D-xylose fermentation for identifying pathogenic serotypes of Yersinia enterocolitica. *J Clin Microbiol,* 30, 2589-2594, 1992.

25. National Committee for Clinical Laboratory Standards (NCCLS): Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard - 9th ed. NCLSI document M2-A9. CLSI, Wayne, PA. Vol 26, Number 1, 2006.

26. Jayarao BM, Henning DR: Prevalence of foodborne pathogens in bulk tank milk. *J Dairy Sci,* 84, 2157-2162, 2001.

27. Soltan-Dallah MM, Tabarraie A, MoezArdalan K: Comparison of four methods for isolation of *Yersinia enterocolitica* from raw and pasteurized milk from northern Iran. *Int J Food Microbiol,* 94, 87-91, 2004.

28. Desmasures N, Bazin F, Gueguen M: Microbiological composition of raw milk from selected farms in Camembert region of Normandy. *J Appl Microbiol,* 83, 53-58, 1997.

29. Tamagnini LM, Sousa GB, Gonzalez RD, Budde CE: Microbiological characteristics of Crottin goat cheese made in different seasons. *Small Rumin Res*, 66, 175-180, 2006.

30. Hamama A, el Marrakchi A, el Othmani F: Occurrence of *Yersinia enterocolitica* in milk and dairy products in Morocco. *Int J Food Microbiol,* 16, 69-77, 1992.

31. Kamat A, Warke R, Kamat M, Thomas P: Low-dose irradiation as a measure to improve microbial quality of ice cream. *Int J Food Microbiol,* 62, 27-35, 2000.

32. Floccari ME, Carranza MM, Parada JL: *Yersinia enterocolitica* biogroup 1A, serotype O:5 in chicken carcasses. *J Food Prot,* 63, 1591-1593, 2000.

33. Mayrhofer S, Paulsen P, Smulders FJM, Hilbert F: Antimicrobial resistance profile of five major food-borne pathogens isolated from beef, pork and poultry. *Int J Food Microbiol,* 97, 23-29, 2004.

34. Arnold T, Neubauer H, Nikolaou K, Roesler U, Hensel A: Identification of Yersinia enterocolitica in minced meat: A comparative analysis of API 20E, Yersinia identification kit and a 16S rRNA-based PCR method. *J Vet Med,* 51, 23-27, 2004.

35. Stock I, Wiedemann B: An *in-vitro* study of the antimicrobial susceptibilities of *Yersinia enterocolitica* and the definition of a database. *J Anti Chem,* 43, 37-45, 1999.

36. Baumgartner A, Küffer M, Suter D, Jemmi T, Rohner P: Antimicrobial resistance of *Yersinia enterocolitica* strains from human patients, pigs and retail pork in Switzerland. *Int J Food Microbiol,* 115, 110-114, 2007.

37. Aarestrup FM, Bager F, Jensen NE, Madsen M, Meyling A, Wegener HC: Resistance to antimicrobial agents used for animal therapy in pathogenic, zoonotic and indicator bacteria isolated from different food animals in Denmark: A baseline study for the Danish Integrated Antimicrobial Resistance Monitoring Programme (DANMAP). APMIS. 106, 745-770, 1998.

38. Kwaga J, Iversen JO: *In vitro* antimicrobial susceptibility of *Y. enterocolitica* and related species isolated from slaughtered pigs and pork products. *Antimicrob Agents Chemother,* 34, 2423-2425, 1990.

39. Lyons MM, Prentice MB, Cope D, Swann RA: Antimicrobial susceptibility of pathogenic Yersinia enterocolitica strains in the British Isles. *Contrib Microbiol Immunol,* 12, 251-254, 1991.

40. Pham JN, Bell SM, Lanzarone JYM: Biotype and antibiotic sensitivity of 100 clinical isolates of Yersinia enterocolitica. *J Antimicrob Chemother,* 28, 13-18, 1991.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S113-S118, 2010
DOI:10.9775/kvfd.2010.2119

RESEARCH ARTICLE

Observation of Lactic Acid Bacteria and Yeast Populations During Fermentation and Cold Storage in Cow's, Ewe's and Goat's Milk Kefirs

Hilmi YAMAN * Mehmet ELMALI ** Ufuk KAMBER ***

* Afyonkocatepe Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı, TR-03100 Afyon - TÜRKİYE
** Mustafa Kemal Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı, TR-31100 Hatay - TÜRKİYE
*** Kafkas Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabili Dalı, TR-36100 Kars - TÜRKİYE

Makale Kodu (Article Code): KVFD-2010-2119

Summary

Changes in the population numbers of *Lactobacillus* spp, *Lactococcus* + *Leuconostoc* spp. and yeasts in the kefirs made of cows, ewes and goats milk were determined in this study. The highest count of *Lactobacillus* spp. was in ewe's milk. There was a slight or no difference in the goats and cow's milk. At 24 h, one $\log_{10}$ cfu/ml decrease was observed for *Lactobacillus* spp. in cows and ewes milk. *Lactobacillus* spp. were more steady in goat milk with a half $\log_{10}$ cfu/ml decrease. *Lactococcus* + *Leuconostoc* spp. similarly reached their maximum populations in cow, ewe and goat milk at the time period of 15-24 h. *Lactococcus* + *Leuconostoc* spp. in all milks were in parallel with the maximum populations of *Lactobacillus* spp. in ewe milk at the time period of 12-15 h. Yeasts had similar numbers in cow, ewe and goat milk at the time period of 15-21 h. Some decreases were observed in the numbers of *Lactobacillus* spp, *Lactococcus* + *Leuconostoc* spp, and yeasts of the kefirs kept at 4ºC for 7 days. The decreases of *Lactobacillus* spp. in cows' and goats' milk kefirs were slight and similar, while one and half $\log_{10}$ cfu/ml decrease was counted in ewes' milk kefir. The decrease in the numbers of *Lactococcus* + *Leuconostoc* spp. was more pronounced than those of *Lactobacillus* spp. and yeasts. pH decreased during the fermentation and all remained constant over the storage period of 7 days. The type of milk had an influence on the population development of kefirs. Ewe's milk supported the growth of *Lactobacillus* spp. and *Lactococcus* + *Leuconostoc* spp. better. This indicates that different milks may influence the population development of kefir microflora which may affect the quality of kefir.

Keywords: *Kefir, Milk, Lactic acid bacteria*

İnek, Koyun ve Keçi Sütünden Yapılan Kefirlerde Fermentasyon Süresince ve Soğukta Muhafazada Laktik Asit Bakteri ve Maya Populasyonunun Gözlemlenmesi

Özet

Bu çalışmada, inek, koyun ve keçi sütünden yapılan kefirlerde *Lactobacillus* spp, *Lactococcus* + *Leuconostoc* spp. ve mayaların popülasyon düzeyindeki değişim belirlendi. En yüksek *Lactobacillus* spp. düzeyi koyun sütünde saptandı. Keçi ve inek sütünde ise çok az veya hiç fark yoktu. Yirmi dördüncü saatte inek ve koyun sütünde *Lactobacillus* spp. için bir $\log_{10}$ cfu/ml azalma gözlemlendi. *Lactobacillus* spp. keçi sütünde yarım $\log_{10}$ cfu/ml azalma ile daha istikrarlıydı. *Lactococcus* + *Leuconostoc* spp. benzer olarak maksimum popülasyonlarına inek, koyun ve keçi sütünde 15-24 saatlik zaman periyodunda ulaştı. *Lactococcus* + *Leuconostoc* spp. bütün sütlerde, *Lactobacillus* spp.'nin koyun sütünde 12-15 saatlik zaman periyoundaki maksimum popülasyonu ile parelellik içersindeydi. Mayalar inek, koyun ve keçi sütünde 15-21 saatlik zaman periyodunda benzer düzeylere sahipti. 4ºC'de 7 gün muhafaza edilen kefirlerde *Lactobacillus* spp., *Lactococcus* + *Leuconostoc* spp. ve maya düzeylerinde azalmalar gözlemlendi. *Lactobacillus* spp.'de koyun sütü kefirinde bir buçuk $\log_{10}$ cfu/ml azalma saptanırken, inek ve keçi sütü kefirlerindeki azalmalar az veya paraleldi. *Lactococcus* + *Leuconostoc* spp. düzeyindeki azalma, *Lactobacillus* spp. ve maya'nın düzeyine göre daha belirgindi. pH fermentasyon sürecinde düşerken, 7 günlük depo süresi boyunca değişmeden kaldı. Sütün çeşidinin kefirdeki popülasyon gelişmesine etkisi oldu. Koyun sütünün *Lactobacillus* spp. ve *Lactococcus* + *Leuconostoc* spp.'nin üremesi için daha uygun olduğu görüldü. Bu durum farklı sütlerin kefir mikroflorasının popülasyon gelişimini etkileyebileceğine diğer bir ifadeyle kefirin kalitesini etkileyebileceğine işaret etmektedir.

Anahtar sözcükler: *Kefir, Süt, Laktik asit bakterisi*

İletişim (Correspondence)
☎ +90 326 2455706
✉ elmali25erz@gmail.com

INTRODUCTION

Milk has an essential role in most diets world-wide. Consumption of milk and fermented milk products is widespread and yet increasing. In developed countries where there is an established dairy industry, new products are being developed to sustain consumer interest.

In recent years, there has been more interest in different fermented milk products known only to particular countries with a view to adapting them for commercial large-scale production in other parts of world. Among the best known are yoghurt, acidophilus milk, kefir, koumiss and yakult. In Turkey, kefir is made traditionally at home but interest is growing and there has been a promotion for its large-scale production due to its health benefits [1]. Recently, kefir has been produced on a commercial scale by a few dairy companies in Turkey and available in big supermarkets. Few people, however, recognize that these products are prepared by bacterial and/or yeast action, and the characteristic flavours and textures of these products are results of these fermentations. In natural fermentations lactic acid bacteria implement competitive characteristics which allow them to produce good quality milk fermentations.

For the manufacture of kefir, the traditional starter cultures are in the form of grains of variable sizes, which resembles cauliflower florets in shape and colour [2,3] but also sheet-like structures [4] and a globular, sagy structure resembling small pouches are exist [5]. These grains contain a wide and varying microflora, such as lactic acid bacteria, yeasts, acetic acid bacteria and moulds [6-13], but the culture is dominated by a *Lactobacillus*/yeast population. The evidence suggest that population development in kefir fermentation appears to be controlled and there is a pattern of microbial succession [14] and compared to other milk fermentations (e.g. yoghurt and cheese) the microbiology of kefir grain is less well understood [15].

The majority of fermented milk products are made from cow milk, but sheep, goat, buffalo, camel, and horse milk can be also used [16]. Kefir is mainly produced from cow milk and little information is available on kefir made from different mammalian milk [17]. Therefore, this paper deals with the changes in the *Lactobacillus*/yeast flora during the kefir fermentations in cow's, ewe's and goat's milk.

MATERIAL and METHODS

This study was performed at Kafkas University Animal Research Center (HAUM) between May and June in 2006. Milk samples of cow, ewe, and goat were obtained from local farms in Kars city, Turkey. Kefir grains were purchased from the Department of Dairy Technology of Agriculture Faculty, Ege University in Izmir, Turkey, in the sterile 0.9% NaCl solution, and were propagated at the Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Kafkas University in Kars, Turkey, by daily transfer into UHT milk at room temperature.

The milk samples were heated at 85°C for 30 min and cooled to 25°C in a water bath before inoculation. A liter of each sample was inoculated with 30 g active kefir grains. Fermentation was carried out at 25°C for 21 h. Subsequently all the fermentate was transferred to the cold store at 4°C. Microbiological analysis for total *Lactobacillus* spp., *Lactococcus + Leuconostoc* spp., and yeasts were performed. *Lactobacillus* spp. was grown on MRS agar at 30°C for 48 h in an anaerobic jar (Gas-pack anaerobic system, BBL). Total number of *Lactococcus + Leuconostoc* spp. was enumerated on M17 agar medium (Oxoid). Yeasts were grown on Potato Dextrose Agar (PDA, Oxoid).

The pH was determined electrometrically (Orion Model 420A) and acidity by titration with N/10 NaOH in the presence of phenolphthalein. Acidity was expressed as per cent lactic acid (LA).

Chemical analyses were performed in accordance with the inditacions by Oysun [18].

RESULTS

The chemical composition of each type of milk is shown in *Table 1*. The pH and acidity values of cows', ewes' and goats' milk kefirs during 21 h (fermentation process) at 25°C, and 7 days storage-ripening at 4°C are given in *Table 2* and *Table 3*, respectively. The population numbers of *Lactobacillus* spp., *Lactococcus + Leuconostoc* spp. and yeasts in the kefirs made from ewe, cow and goat milk during 21 h at 25°C fermentation, and 7 days storage-ripening at 4°C are given in *Tables 4-6* and *Tables 7-9*, respectively.

Table 1. *The chemical composition of cow, ewe, and goat milk*
Tablo 1. *İnek, koyun ve keçi sütünün kimyasal kompozisyonu*

Parameter	Cow Milk	Ewe Milk	Goat Milk
Total solid (%)	12.0	19.3	13.0
Protein (%)	3.3	6.0	3.6
Carbohydrates (%)	4.7	5.4	4.5
Ash (%)	0.7	0.9	0.8

Table 2. *Changes in pH and acidity of each milk sample during kefir fermentations at 25ºC for 21 h*
Tablo 2. *Yirmi bir saat süreyle 25ºC'de kefir fermentasyonu süresince her bir süt örneğindeki asit ve pH değişimi*

Time (h)	pH Cows' Milk	Acidity Cows' Milk	pH Ewes' Milk	Acidity Ewes' Milk	pH Goats' Milk	Acidity Goats' Milk
0	7.02	0.17	7.03	0.24	7.01	0.17
3	6.62	0.2	6.82	0.27	6.72	0.22
6	6.36	0.24	6.63	0.31	6.52	0.25
9	6.04	0.28	6.29	0.38	6.17	0.35
12	5.70	0.38	5.26	0.56	5.8	0.43
15	4.81	0.55	5.26	0.72	5.3	0.64
18	4.56	0.68	5.06	0.94	5	0.89
21	4.54	1.2	4.52	1.21	4.6	0.96

Table 3. *Changes in pH and acidity of each kefir sample during storage at 4ºC for 7 days*
Tablo 3. *Yedi gün 4ºC'de depolama süresince her bir kefir örneğindeki asit ve pH değişimi*

Day	pH of Kefir Made from Cows' Milk	Acidity of Kefir Made from Cows' Milk	pH of Kefir Made from Ewes' Milk	Acidity of Kefir Made from Ewes' Milk	pH of Kefir Made from Goats' Milk	Acidity of Kefir Made from Goats' Milk
1	4.41	1.34	4.49	1.66	4.51	1.21
2	4.28	0.78	4.42	1.17	4.33	1.04
3	4.24	0.72	4.37	1.19	4.27	1
4	4.22	0.85	4.34	1.13	4.22	1.01
5	4.12	0.83	4.31	1.12	4.2	0.99
6	4.08	0.82	4.3	1.12	4.15	0.98
7	4.02	0.81	4.3	1.12	4.03	0.97

Table 4. *The counts (cfu/ml) of Lactobacillus spp. in cow's, goat's and ewe's milk during kefir fermentation at 25ºC for 21 h*
Tablo 4. *Yirmi bir saat süreyle 25ºC'de kefir fermentasyonu sürecinde inek, keçi ve koyun sütünde Lactobacillus spp. miktarı (cfu/ml)*

Time (h)	Cow's Milk	Ewe's Milk	Goat's Milk
0	4.11	4.30	4.04
3	5.04	5.47	5.48
6	6.08	6.41	5.84
9	6.90	7.38	6.38
12	7.25	9.54	7.69
15	8.69	9.68	8.50
18	8.78	8.69	8.00
21	7.57	8.79	8.00

Table 5. *The counts (cfu/ml) of Lactococcus+Leuconostoc spp. populations in cow's, goat's and ewe's milk during kefir fermentation at 25ºC for 21 h*
Tablo 5. *Yirmi bir saat süreyle 25ºC'de kefir fermentasyonu sürecinde inek, keçi ve koyun sütünde Lactococcus + Leuconostoc spp. miktarı (cfu/ml)*

Time (h)	Cow's Milk	Ewe's Milk	Goat's Milk
0	4.36	4.14	4.62
3	4.78	5.77	4.95
6	6.08	5.95	5.95
9	6.60	7.28	6.69
12	7.28	8.61	8.25
15	8.69	9.78	9.17
18	8.95	9.45	9.36
21	9.28	9.32	8.11

Table 6. *The counts (cfu/ml) of yeast populations in cow's, goat's and ewe's milk during kefir fermentation at 25ºC for 21 h*
Tablo 6. *Yirmi bir saat süreyle 25ºC'de kefir fermentasyonu sürecinde inek, keçi ve koyun sütünde maya miktarı*

Time (h)	Cow's Milk	Ewe's Milk	Goat's Milk
0	3.60	3.30	3.36
3	4.39	5.17	4.30
6	4.90	5.34	4.60
9	4.60	5.15	4.84
12	5.20	5.39	5.00
15	5.30	5.38	5.41
18	5.67	5.47	5.58
21	5.72	5.86	5.20

Table 7. *The counts (cfu/ml) of Lactobacillus spp. populations in kefir samples made from cow's, goat's and ewe's milk during 7 days storage at 4ºC*
Tablo 7. *Dört santigrat derecede 7 gün süreyle depolamada inek, keçi ve koyun sütünden yapılan kefir örneklerinde Lactobacillus spp. miktarı (cfu/ml)*

Day	Cow's Milk	Ewe's Milk	Goat's Milk
1	7.48	8.84	8.00
2	7.41	8.15	7.95
3	7.30	7.76	7.47
4	7.47	7.47	7.69
5	7.17	7.23	7.60
6	7.04	7.28	7.54
7	7.00	7.23	7.51

Table 8. *The counts (cfu/ml) of Lactococcus + Leuconostoc spp. populations in kefir samples made from cow's, goat's and ewe's milk during 7 days storage at 4°C*

Tablo 8. *Dört santigrat derecede 7 gün süreyle depolamada inek, keçi ve koyun sütünden yapılan kefir örneklerinde Lactococcus + Leuconostoc spp. miktarı (cfu/ml)*

Day	Cow's Milk	Ewe's Milk	Goat's Milk
1	9.68	9.00	8.95
2	9.55	9.11	8.20
3	9.56	8.95	8.30
4	8.60	8.30	8.38
5	7.60	8.20	8.00
6	7.30	8.00	7.90
7	7.25	8.00	7.72

Table 9. *The counts (cfu/ml) of Yeast populations in kefir samples made from cow's, goat's and ewe's milk during 7 days storage at 4°C*

Tablo 9. *Dört santigrat derecede 7 gün süreyle depolamada inek, keçi ve koyun sütünden yapılan kefir örneklerinde maya miktarı (cfu/ml)*

Day	Cow's Milk	Ewe's Milk	Goat's Milk
1	5.81	5.84	5.69
2	5.90	5.60	5.47
3	5.50	5.58	5.44
4	5.48	5.47	5.30
5	5.60	5.47	5.30
6	5.47	5.47	5.25
7	5.47	5.44	5.00

DISCUSSION

During the fermentation a sharpe decrease of around 2 pH units was observed but pH did not vary during storage. This may be due to the presence of yeasts since Collar [19] reported that lactic acid bacteria multiply and produce lactic and acetic acids more slowly in mixture with yeasts than in pure culture. pH values at 21 h fermentation were in agreement with the average samples pH at 22 h fermentation as reported by Guzel-Seydim et al.[20] and Cais-Sokolinska et al.[21]. Maximum acidity was observed between the time period of 21 h and the first day of storage. This showed similarity with the results of Fontan et al.[22] but was slightly higher than the results of Beshkova et al.[23].

The lactic acid bacteria and yeasts are the pre-dominating microflora in kefir grains [24]. Considering the population counts of viable *Lactococcus + Leuconostoc* spp., our results are in agreement with those of Wszolek et al.[17], Simova et al.[24] and also Ninane et al.[25] who reported that *Lactococcus* showed by far the greatest variability comparing to *Lactobacillus* spp. and yeasts.

During the fermentation for 21 h, increases were observed in the numbers of *Lactobacillus* spp., *Lactococcus + Leuconostoc* spp. and yeasts. Maximum populations of *Lactobacillus* spp., *Lactococcus + Leuconostoc* spp. and yeasts were reached at different time periods, and showed variations in different milks *(Table 4-6)*. The highest counts of *Lactobacillus* spp. were observed in ewe's milk between 12 h and 15 h time period *(Table 4)*. It was 2 $\log_{10}$ cfu/ml higher than the numbers observed for *Lactobacillus* spp. in cow's and goat's milk at 12 h. This may be due to variations in composition between different types of milks (cow, ewe and goat) as ewe milk has a higher content of protein and fat than cow and goat milk [26]. Furthermore, it contains higher levels of vitamins, such as folic acid, pantothenic acid, riboflavin and niacin which are considered to be essential for the growth of some *Lactobacillus* spp. strains [27], providing perhaps the best substrate for the manufacture of a fermented milk product [28]. As far as the goat and cow milk were concerned, it was interesting to mention that there was only a slight or no difference in the population numbers of *Lactobacillus* spp. After 21 h fermentation, there was almost one $\log_{10}$ cfu/ml decrease in the population numbers of *Lactobacillus* spp. in cow and ewe milk, as compared to the maximum population numbers of *Lactobacillus* spp. at 15 h in the same milks. The numbers of *Lactobacillus* spp. were steadier in goat milk with a half $\log_{10}$ cfu/ml decrease comparing to the maximum population number at 15 h in goat milk. The maximum numbers of *Lactobacillus* spp. at 21 h and in following 2 days storage at 4°C were in parallel with the results of Irigoyen et al.[16], Wszolek et al.[17], Guzel-Seydim et al.[20], Fontan et al.[22], Witthuhn et al.[29], and Witthuhn et al.[30].

Unlike *Lactobacillus* spp., *Lactococcus + Leuconostoc* spp. reached their maximum populations which were similar in numbers in cow, ewe and goat milk but at the different time periods of 18-21 h, 15-21 h and 15-18 h, respectively *(Table 5)*. The maximum population numbers of *Lactococcus + Leuconostoc* spp. in cow, ewe and goat milk were in parallel with the maximum populations of *Lactobacillus* spp. in ewe milk at the time period of 12-15 h. Likewise, yeasts had similar maximum population numbers in cow, ewe and goat milk at the time periods of 18-21 h, 18-21 h and 15-18 h, respectively *(Table 6)* and these values were slightly lower than the values of Guzel-Seydim et al.[20] at 22 h fermentation time but our 7^{th} day storage values showed similarity with the 7^{th} day values of Guzel-Seydim et al.[20].

The storage and ripening of cows', ewes' and goats' milk kefirs at 4°C for 7 days resulted in decreases of the population numbers of *Lactobacillus* spp., *Lactococcus +*

Leuconostoc spp. and yeasts *(Table 7-9).* The decrease of *Lactobacillus* spp. numbers in cows' and goats' milk kefirs were slight and similar, while one and half $\log_{10}$ cfu/ml decrease was counted in ewes' milk *(Table 7).* Likewise, the decrease in the population numbers of *Lactococcus + Leuconostoc* spp. was more pronounced rather than in the populations of *Lactobacillus* spp. and yeasts, and there were 2.43, 1.23 and 1 $\log_{10}$ cfu/ml difference in cows', goats' and ewes' milk kefirs, respectively *(Table 8).* For the yeasts, a slight decrease was also observed in goats' cows' and ewes' milk kefirs *(Table 9).* These results were in parallel with the study of Irigoyen et al.[16] and Oner et al.[31]. As expected, lactic acid bacteria and yeasts were the predominant flora in fresh (21 h) and stored (7 days) kefirs but some strains decreased by 1 to 2.43 $\log_{10}$ cfu/ml during the storage period. *Lactobacillus* spp. reached the highest population numbers in ewe's milk while *Lactococcus + Leuconostoc* spp. reached the highest population numbers in ewe's and cow's milk. Yeast population numbers were not affected largely by the type of milk used. Since these population numbers may indicate that ewe's milk supports the growth of the *Lactobacillus* spp. and *Lactococcus+ Leuconostoc* spp. better, which may lead to the thought of ewe's milk being better choice, as compared to cow's and goat's milk. Likewise, Wszolek et al.[17] reported that the firmness of the product was also influenced by the type of milk used stating the order of ovine>bovine >caprine. Furthermore Wojtowski et al.[32] claimed that kefir produced from sheep milk can have a considerably more advantageous effect on the health of the consumers than kefirs produced from goat or cow milk, however the ratings for acceptability of kefirs were ranked in the order of kefir made from cow milk>ewe milk>goat milk. On the other hand, Sahan [33] concluded that organoleptic qualities of kefirs made from cow's, ewe's and goat's milk indicated that the best kefir could also be produced from goat's milk as well as the cow's milk. Likewise, Kaptan and Gursel [34] also stated that the best quality kefir can be made from goat milk.

In conclusion, this study showed that the type of milk has an influence on the population development of the kefir flora. Although the differences in the population development may influence the quality of kefir, considering the previous studies [12,21] it can be presumed that the type of milk has a greater influence than the starter cultures and their population development on the sensory profile.

REFERENCES

1. Karagozoglu C: Kefir. *Yüksek Lisans Tezi.* Ege Univ Fen Bil Enst, Bornova, Izmir, Türkiye, 1990.

2. Koroleva NS: Products prepared with lactic acid bacteria and yeasts. **In,** Robinson RK (Ed): Therapeutic Properties of Fermented Milks. pp. 159-169. Elvesier Applied Science, London, UK, 1991.

3. Tamime A, Marshall VME: Microbiology and technology of fermented milks. **In,** Law BA (Ed): Microbiology and Biochemistry of Cheese and Fermented Milk. pp. 57-152. Blackie Academic Professional, London, UK, 1997.

4. Marshall VM, Cole WM, Brooker BE: Observations on the structure of kefir grains and the distribution of the microflora. *J Appl Bacteriol,* 57, 491-497, 1984.

5. Yaman H, Elmali M, Karadagoglu G, Cetinkaya A: Observations of kefir grains and structure from different geographical regions: Turkey and Germany. *Atatürk Univ Vet Bil Derg,* 1 (1-2): 11-15, 2006.

6. Rasic JL: Other products. Milk-Vital Force, XXII International Dairy Congress. pp. 673-683. D. Reidel Publishing, Dordrecht, 1978.

7. Libudzisz Z, Piatkiewicz A: Kefir production in Poland. *Dairy Indust Int,* 55 (7): 31-32, 1990.

8. Thompson JK, Johnston DE, Murphy RJ, Collins MA: Characteristics of a milk fermentation from rural Northern Ireland which resembles kefir. *Irish J Food Sci Technol,* 14, 35-49, 1990.

9. Angulo L, Lopez E, Lema C: Microflora present in kefir grains of the Galician Region (North-west of Spain). *J Dairy Res,* 60, 263-267, 1993.

10. Pintado ME, De Silva JAL, Fernandes PB, Malcata FX, Hogg AT: Microbiological and rheological studies on Portuguese kefir grains. *Int J Food Sci Technol,* 31, 15-26, 1996.

11. Rea MC, Lennartsson T, Dillon P, Drinan FD, Reville WJ, Heapes M, Cogan TM: Irish kefir-like grains: Their structure, microbial composition and fermentation kinetics. *J Appl Bacteriol,* 81, 83-94, 1996.

12. Garrote GL, Abraham AG, De Antoni GL: Preservation of kefir grains, a comparative study. *Lebensm Wiss Technol,* 30, 77-84, 1997.

13. Kuo CY, Lin CW: Taiwanese kefir grains: Their growth, microbial and chemical composition of fermented milk. *Austr J Dairy Technol,* 54, 19-23, 1999.

14. Yaman H: Partial characterization of Lactobacilli isolated from commercial kefir grain. *PhD Thesis,* Huddersfield University, Huddersfield, UK, October 2000.

15. Yaman H: Isolation of Lactobacilli from a commercial Polish kefir grain. *Kafkas Univ Vet Fak Derg,* 10 (1): 99-102, 2004.

16. Irigoyen A, Arana I, Castiella M, Torre P, Ibanez FC: Microbiological, physicochemical and sensory characteristics of kefir during storage. *Food Chem,* 90, 613-620, 2005.

17. Wszolek M, Tamime AY, Muir DD, Barcly MNI: Properties of kefir made in Scotland and Poland using bovine, caprine and ovine milk with different startercultures. *Lebensm Wiss Technol,* 34, 251-261, 2001.

18. Oysun G: Süt Ürünlerinde Analiz Yöntemleri. I. Basım. Ege Universitesi Yayınları No: 504. Ofset Basımevi, Bornova, İzmir. 1991.

19. Collar C: Biochemical and technological assessments of

the metabolism of pure and mixed cultures of yeast and lactic acid bacteria in breadmaking application's. *Food Sci Techno Int,* 2, 349-367, 1996.

20. Guzel-Seydim Z, Twyffels J, Seydim AC, Greene AK: Turkish kefir and kefir grains: Microbial enumeration and electron microscobic observation. *Int J Dairy Techno,* 58 (1): 25-29, 2005.

21. Cais-Sokolinska D, Dankow R, Pikul J: Physicochemical and sensory characteristics of sheep kefir during storage. *Acta Sci Pol Techno Aliment,* 7 (2): 63-73, 2008.

22. Fontan MCG, Martinez S, Franco I, Carballo J: Microbiological and chemical changes during the manufacture of Kefir made from cows' milk, using a commercial starter culture. *Int Dairy J,* 16, 762-767, 2006.

23. Beshkova DM, Simova ED, Simov ZI, Frengova GI, Spasov ZN: Pure cultures for making kefir. *Food Microbiol,* 19, 537-544, 2002.

24. Simova E, Beshkova D, Angelov A, Hristozova T, Frengova G, Spasov Z: Lactic acid bacteria and yeasts in kefir grains and kefir made from them. *J Indust Microbiol Biotechnol,* 28, 1-6, 2002.

25. Ninane V, Berben G, Romnee JM, Oger R: Variability of the microbial abundance of a kefir grain starter cultivated in partially controlled conditions. *Biotechnol Argon Soc Environ,* 9 (3): 191-194, 2005.

26. Kalantzopoulos G: Cheeses from ewe's and goat's milk. **In,** Fox PF (Ed): Cheese Chemistry Physics and Microbiology. Vol. 2, pp. 507-543. Chapman Hall, London, UK, 1996.

27. Gomes AMP, Malcata FX: *Bifidobacteria* spp. and *Lactobacillus acidophilus:* Biological, biochemical, technological and therapeutical properties relevant for use as probiotics. *Trends Food Sci Techno,* 10, 139-157, 1999.

28. Drakoularakou AP, Kehagias C, Karakanas PN, Koulouris S, Timbis D: A study of the growth of *Lactobacillus acidophilus* in bovine, ovine and caprine milk. *Int J Dairy Technol,* 56 (1): 59-61, 2003.

29. Witthuhn RC, Schoeman T, Britz TJ: Isolation and characterization of the microbial population of different South African kefir grains. *Int J Dairy Technol,* 57 (1): 33-37, 2004.

30. Witthuhn RC, Schoeman T, Britz TJ: Characterisation of the microbial population at different stages of kefir production and kefir grain mass cultivation. *Int Dairy J,* 15, 383-389, 2005.

31. Oner Z, Karahan AG, Cakmakci ML: Effect of different milk types and starter cultures on kefir. *GIDA,* (In publication), http://www.gidadernegi.org. *Erişim tarihi:* 02.01.2010.

32. Wojtowski J, Dankow R, Skrzypek R, Fahr RD: The fatty acid profile in kefirs from sheep, goat and cow milk. *Milchwissensch,* 58 (11-12): 633-636, 2003.

33. Sahan N: An investigation on the properties of kefirs made from bovine, ovine and caprine milk and the effect of storage period on these properties. *Yüksek Lisans Tezi.* Çukurova Univ Fen Bilim Enst, Adana, Türkiye, 1987.

34. Kaptan N, Gursel A: Studies on some properties of kefir made under laboratory and home conditions. Ankara Univ Zir Fak Yıllığı, 33, 68-75, 1983.

Kafkas Univ Vet Fak Derg
16 (Suppl A): S119-S122, 2010
DOI:10.9775/kvfd.2010.2135

RESEARCH ARTICLE

Occurrence of Aflatoxin M_1 in UHT Milk in Erzurum-Turkey

Meryem AYDEMİR ATASEVER * Gülşah ADIGÜZEL * Mustafa ATASEVER *
Hayrunnisa ÖZLÜ * Korhan ÖZTURAN **

* Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Ataturk University, TR-25240 Erzurum - TURKEY
** Nineth Army Cops "A" Type Food Control Detachment Commandery, TR-25200 Erzurum - TURKEY

Makale Kodu (Article Code): KVFD-2010-2135

Summary

In this study, 150 UHT milk samples were analyzed for aflatoxin M_1. They were obtained from supermarkets in Erzurum city. The occurrence and concentration range of AFM_1 in the samples were investigated by ELISA method. Fifty-nine percent of the UHT milk samples contained AFM_1. AFM_1 levels in 16 (10.7%) UHT milk samples exceeded the maximum tolerable limit of the European Community and the Turkish Food Codex. It was concluded that high AFM1 level is an important problem threatening the public health in Turkey.

Keywords: *Aflatoxin M1, UHT milk, ELISA*

Türkiye (Erzurum)'de UHT Sütlerde Aflatoxin M_1 Oluşumu

Özet

Bu çalışmada toplam 150 UHT süt örneği AFM_1 yönünden incelendi. Örnekler Erzurum şehir merkezindeki marketlerden temin edildi. Örneklerin AFM_1 içeriği ve konsantrasyonu kompetitiv ELISA metoduyla araştırıldı. UHT süt örneklerinin %59'unun AFM_1 içerdiği belirlendi. 16 numunenin (10.7%) AFM_1 yönünden Türk Gıda Kodeksi ve Avrupa Birliği tarafından düzenlenen yasal limitleri aştığı belirlendi. Sonuç olarak yüksek AFM_1 düzeyi Türkiye'de halk sağlığını tehdit eden önemli bir problemdir.

Anahtar sözcükler: *Aflatoksin M1, UHT süt, ELISA*

INTRODUCTION

Aflatoxin is common contaminant of foods. This toxins is produced by fungal action during production, harvest, storage, and food processing. The toxin is produced as secondary metabolites by *Aspergillus flavus* and *A. parasiticus* and the rare *A. nomius* fungi [1,2]. *A. flavus* produces only B aflatoxins, while the other two species produce both B and G aflatoxins. Aflatoxins M_1 and M_1 are the hydroxylated metabolites of aflatoxin B_1 and B_1. International Agency for Research and Cancer (IARC) [3] of WHO included AFB1 as primary and AFM_1 as secondary groups of carcinogenic compounds [4,5].

The residues of AFM_1 remain stable when milk is processed by heat or is fermented. There is no evidence that cold storage, freezing, heat-treating, fermenting, concentrating or drying of the contaminated milk changes the level of AFM_1 [6-8]. AFM_1 is mainly soluble in the aqueous phase of milk or adsorbed to casein particles; data of several studies show that a small proportion of AFM1 in milk is carried-over to cream, and yet a smaller proportion to butter. The remainder of AFM_1 in milk, however, remains in skim milk and buttermilk [9].

Milk is a major nutrient for infants, children, convalescents and old people. However, milk and milk products are the most potent source of aflatoxin among foods. To protect consumers several countries have established legislation to regulate the levels of AFB_1 in

İletişim (Correspondence)
☎ +90 442 2315533
✉ gulsah@atauni.edu.tr

feeds and AFM_1 in milk and dairy products [10-12].

The European Community (EC) and the Turkish Food Codex (TFC) legal limit for AFM_1 in milk is 50 ng/kg [13,14]. Despite lots of studies on AFM_1 in cheese varieties [11,15-18] only a few are concerned with UHT milk. The aim of this study was to investigate the occurrence of AFM_1 in UHT milk and to compare the results with the maximum AFM_1 tolerance limits which are accepted by the EC and TFC.

MATERIAL and METHODS

Samples: A total of 150 samples of UHT milk (whole milk) samples were obtained randomly from supermarkets between September 2006 and September 2007 in Erzurum city. The samples were transported to the laboratory in an insulated container at about 4°C and analyzed upon arrival. All samples were analyzed before their expiry date.

Methods: AFM_1 concentrations of the samples were analyzed by competitive ELISA (RIDASCREEN Aflatoxin M_1, R-Biopharm). The samples were evaluated according to the RIDAVIN computer program prepared by R-Biopharm [19]. According to the instructions for use of the RIDASCREEN kit; the lower detection limit was 5 ng/kg.

Calculation of extrapolated values of AFB_1 concentration in cattle feeding stuffs based on AFM_1 in UHT milk samples

It has been suggested that only 1.6% of ingested AFB_1 is converted to AFM_1 by the dairy cattle [10,20-22].

Hence, the values of AFB_1 contamination in feeding stuffs were back calculated by using the formula:

$$AFB_1\ (\mu g/kg) = \frac{AFM_1\ (ng/kg) \times 100}{1.6 \times 1000}$$

RESULTS

In this study a total of 150 UHT milk (whole milk) samples were analyzed for AFM_1 with the competitive ELISA. The occurrence and the distribution of AFM_1 concentration in various ranges in UHT milk samples are presented in *Table 1*.

As shown in *Table 1*, AFM_1 was found above the detectable level in 59% (89/150) in UHT milk samples. AFM_1 levels in 16/150 (10.7%) UHT milk samples were found to be higher than the maximum acceptable limits (milk; 50 ng/kg) of the EC [13] and TFC [14]. AFM_1 content of positive samples were determined in UHT milk samples as minimum 5 ng/kg, maximum 185 ng/kg, and mean 36±38 ng/kg.

Earlier studies have shown that contamination of AFM_1 in milk and dairy products is a result of exposure of AFB_1 to dairy cattle through feedstuffs [23]. Further, investigators have suggested that on an average 1.6% of AFB_1 fed to the lactating cattle is excreted in milk as AFM_1 [20,21]. Using this factor, the content of AFB_1 in the dairy cattle feeding stuffs was extrapolated from AFM_1 contamination in the UHT milk samples *(Table 2)*.

Table 1. *Occurrence and distribution of AFM_1 in UHT milk samples*
Tablo 1. *UHT süt örneklerinin AFM_1 içeriği ve dağılımı*

Kind of Samples	Samples Tested (n)	Proportion of Positive Samples n (%)	Distribution of Samples [a] n (%)					Proportion of Samples Exceeding the EC and TFC Legal Limit >50 ng/kg	Quantity of AFM_1 (ng/kg)		
			<5*	5-25	26-50	51-100	>100		x±Sx	Min.	Max.
UHT Milk	150	89/150 (59)	61/150 (40.0)	42/150 (28)	31/150 (20.7)	10/150 (6.7)	6/150 (4)	16/150 (10.7)	36±38	5	185

** distribution of negative samples, **a:** ng/kg, **EC:** European Community, **TFC:** Turkish Food Codex, **( ):** indicates percent,*
x±Sx: *mean±standart deviation*
** negatif örneklerin dağılımı, **a:** ng/kg, **EC:** Avrupa Birliği, **TFC:** Türk Gıda Kodeksi, **( ):** yüzde ifadesi, **x±Sx:** ortalama±standat sapma*

Table 2. *Extrapolated AFB_1 concentration in cattle feedstuffs based on AFM_1 contamination in UHT milk samples*
Tablo 2. *UHT süt örneklerin AFM_1 kontaminasyonuna dayanılarak tahmin edilen AFB_1 konsantrasyonları*

Kind of Samples	Samples Tested (n)	Level of Positive Samples n (%)	Range (µ/kg)	Exceeding EC and TFC (5 µg/kg)	Positive Samples (µ/kg)		
					x±Sx	Min.	Max.
UHT Milk	150	89/150 (59)	0-11.6	6/150 (4)	2.3±2.4	0.3	11.6

x±Sx: *mean±standart deviation, **EC:** European Community, **TFC:** Turkish Food Codex, **( ):** indicates percent*
x±Sx: *ortalama±standat sapma, **EC:** Avrupa Birliği, **TFC:** Türk Gıda Kodeksi, **( ):** yüzde ifadesi*

It can be seen from the results that the contamination of feed with AFB_1 in cattle feed may range of 0-11.6, with an average of 2.3±2.4 µg/kg. Moreover, 4% (6/150) of the samples exceeded the limits recommended by EC and TFC regulations.

In some studies made on UHT milk, presence and level of AFM_1 were showed in *Table 3*.

Table 3. *AFM1 contents of UHT milk reported in previous studies*
Tablo 3. *Önceki çalışmalarda UHT sütlerde bildirilen AFM1 içerikleri*

Sample	Country	No. of Samples Positive	Range of Samples Positive (ng/kg)	Exceed Legal Limit *	References
UHT Milk	Turkey	75/129 (58.1)	10-543.6	61/129 (47)	Unusan [22]
	Portugal	60/70 (85.7)	5-61	20/70 (2.9)	Martin and Martin [27]
	Turkey	14/24 (58.3)	10-50.5	1/24(4.2)	Gurbay et al.[24]
	Turkey	67/100 (67)	10-630	31/100 (31)	Tekinsen and Eken [25]

** Turkish Food Codex limits in UHT milk is 50 ng/kg,* ***( ):*** *indicates percent*
** Türk Gıda Kodeksi UHT süt limiti 50 ng/kg,* ***( ):*** *yüzde ifadesi*

DISCUSSION

In our study, AFM_1 was determined in 59% of the UHT milk samples. These results are in parallel with the findings of some previous reports [22,24,25] which pointed out the presence of AFM_1 in all or most of the UHT milk samples in Turkey *(Table 3)*.

These reports and the present findings suggest that the milk which is processed in to dairy products may contain a high concentration of AFM_1 and/or be contaminated with *Aspergillus spp.* The results confirm the findings of Bakirci [26] who reported high concentrations of the AFM_1 in raw milk during spring. The AFM_1 levels (as incidence) also were lover than the reported results by Martin and Martin [27], Oliveira and Ferraz [28]. In addition, the AFM_1 level in the milk was significantly affected by the geographical region and the country [16,29]. Moreover, differences in the hygiene and storage conditions at the dairies and retail points are other key factors on the variations of the results [30,31].

Some of the AFM_1 amounts in the UHT milk samples were at the risk level for human health because the AFM_1 in 16 UHT milk samples (10.7%) exceeded the EC and TFC legal limits of 50 ng/kg. In this study AFM_1 incidence of exceeding legal limit in UHT milk samples was lower than the reported results by Unusan [22], Tekinsen and Eken [25], Oliveira and Ferraz [28] and were higher than the results reported by Gurbay et al.[24], Martin and Martin [27]. Very high AFM_1 levels (51 - >100 ng/kg) in 10.7% of the UHT milk samples are of great importance. Also it should be kept in mind that, total daily aflatoxin intake from other foods could be an important risk factor for people as well. The weighted mean concentration of AFM_1 in milk is 0.023 µg/kg in the European-type diet, 0.022 µg/kg in the Latin American diet, 0.36 µg/kg in the Far Eastern diet, 0.005 µg/kg in the Middle Eastern diet and 0.002 µg/kg in the African diet. These mean concentrations are based on a large number of milk samples analyzed. The intake of AFM_1 from milk is calculated to be 6.8 ng/person per day for the European diet, 3.5 ng/person per day for the Latin American diet, 12 ng/person per day for the Far Eastern diet, 0.7 ng/person per day for the Middle Eastern diet and 0.1 ng/person per day for the African diet [2,22].

The content of AFB_1 in the dairy cattle feeding stuffs was extrapolated from AFM_1 contamination in the UHT milk samples *(Table 2)*. In a previous study, contamination of AFB_1 in Turkey cattle feedstuffs was found to be in the range of 0-33.98 µg/kg [22]. At the present study, AFB_1 incidence of exceeding legal limit in UHT milk samples was lower than the reported results by Unusan [22].

In conclusion, AFM_1 is common contaminant UHT milk can be considered to be a main concern for public health. So the public health authorities should take necessary measures and the producers should be informed. Moreover the prevention of aflatoxin formation in feeds is very important. Because the consumption of contaminated feeds by dairy animals causes AFM_1 formation in milk. So the easiest and shortest way of reducing AFM_1 amount forming in milk to minimum focuses on the prevention of AFB_1 formation in feeds. For this, it is necessary to control well the feeds given to dairy animals and to reduce AFB_1 amount permitted to take place in feeds to lower levels. In addition, it is considered that food substances should be produced and kept in convenient conditions to prevent aflatoxin formation.

REFERENCES

1. Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D: Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences,

and interventions 1-3. *Am Soc Clinical Nutr,* 80, 1106-1022, 2004.

2. Creppy EE: Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicol Lett,* 127, 19-28, 2002.

3. IARC (International Agency for Research on Cancer): IARC monographs on the evaluation of carcinogenic risks to humans, some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC Scientific Publication, No. 56, IARC, Lyon, 1993.

4. Cathey CG, Nuang, ZG, Sarr AB, Clement BA, Phililips TD: Development and evaluation of a minicolumn assay fro the detection of aflatoxin M1 in milk. *J Dairy Sci,* 77, 1223-1231, 1994.

5. Dragacci S, Gleizes E, Fremi JM Candlish AAG: Use of immunoaffinity chromatography as a purification step for the determination of aflatoxin M1 in cheeses. *Food Addit Contam,* 12, 59-65,1995.

6. Yousef AE, Marth EH: Degradation of aflatoxin M1 in milk by ultraviolet energy. *J Food Protect,* 48, 697-698, 1985.

7. Barbieri G, Bergamini C, Ori E, Reska P: Aflatoxin M1 in parmesan cheese: HPLC determination. *J Food Sci,* 59, 1313-1331, 1994.

8. Blanco JL, Dominguez L, Gomez-Lucia E, Garayzabal JFF, Garcia JA, Suarez, G: Presence of aflatoxin M1 in commercial ultra-high-temperature-treated milk. *Appl Environ Microb,* 54, 1622-1623, 1988.

9. Prandini A, Tansini G, Sigolo S, Filippi L, Laporta M, Piva G: On the occurrence of aflatoxin M1 in milk and dairy products. *Food Chem Toxicol,* 47, 984-991, 2008.

10. Rastogi S, Dwivedi DP, Khanna KS, Das M: Detection of Aflatoxin M1 contamination in milk and infant milk products from Indian Markets by ELISA. *Food Control,* 15, 287-290, 2004.

11. Sarimehmetoglu B, Kuplulu O, Celik TH: Detection of aflatoxin M1 in cheese samples by ELISA. *Food Control,* 15, 45-49, 2004.

12. Aycicek H, Aksoy A, Saygi S: Determination of aflatoxin levels in some dairy and food products which consumed in Ankara, Turkey. *Food Control,* 16, 263-266, 2005.

13. European Community (EC): No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of European Union,* L 364, pp. 5-24, 2006.

14. Turkish Food Codex (TFC): Gıda Maddelerinde Belirli bulaşanların maksimum seviyelerinin belirlenmesi hakkında tebliğ. *Resmi Gazete,* 23 Eylül 2002. Sayı 24885. Başbakanlık Basımevi. Ankara, Turkey, 2002.

15. Atasever Aydemir M, Adiguzel G, Atasever M, Ozturan K: Determination of Aflatoxin M1 levels in some cheese types consumed in Erzurum-Turkey. *Kafkas Univ Vet Fak Derg,* 2010 (In press).

16. Ardic M, Karakaya Y, Atasever M, Adiguzel G: Aflatoxin M1 levels of Turkish white brined cheese. *Food Control,* 20, 196-199, 2009.

17. Yaroglu T, Oruc HH, Tayar M: Aflatoxin M1 levels in cheese samples from some provinces of Turkey. *Food Control,* 16, 883-885, 2005.

18. Tekinsen KK, Tekinsen OC: Aflatoxin M1 in white pickle and Van otlu (herb) cheeses consumed in southeastern Turkey. *Food Control,* 16, 565-568, 2005.

19. R-Biopharm GmbH: Enzyme immunoassay for the quantitative analysis of aflatoxins. Ridascreen Aflatoxin M1 Art. No.:R-1101. Darmstadt, Germany, 1999.

20. Forbisch RA, Bradley BD, Wagner DD, Long-Bradley PE, Hariston H: Aflatoxin residues in milk of dairy cows after ingestion of naturally contaminated grain. *J Food Protect,* 49, 781-785, 1986.

21. Price RL, Paulson JH, Lough OG, Ginng C, Kurtz AG: Aflatoxin conversion by dairy cattle consuming naturally contaminated whole cottonseed. *J Food Protect,* 48, 11-15, 1985.

22. Unusan N: Occurrence of aflatoxin M1 in UHT milk in Turkey. *Food Chem Toxicol,* 44, 1897-1900, 2006.

23. Applebaum RS, Brackett RE, Wiseman DW, Marth EH: Aflatoxin: Toxicity in dairy cattle and occurrence in milk and dairy products-a review. *J Food Protect,* 45, 752-777, 1982.

24. Gurbay A, Aydin S, Girgin G, Engin AB, Sahin G: Assessment of aflatoxin M1 levels in milk in Ankara, Turkey. *Food Control,* 17, 1-4, 2006.

25. Tekinsen KK, Eken S: Aflatoxin M1 levels in UHT milk and kashar cheese consumed in Turkey. *Food Chem Toxicol,* 46, 3287-3289, 2008.

26. Bakirci I: A study on the occurrence of aflatoxin M1 in milk and milk products produced in Van province of Turkey. *Food Control,* 12, 47-51, 2001.

27. Martin ML, Martin HM: Aflatoxin M1 in raw and UHT treated milk commercialised in Portugal. *Food Addit Contam,* 17, 871-874, 2000.

28. Oliveira CAF, Ferraz JCO: Occurrence of aflatoxin in pasteurised, UHT milk and milk powder from goat origin. *Food Control,* 18, 375-378, 2007.

29. Galvano F, Galofaro V, Galvano G: Occurrence and stability of aflatoxin M1 in milk and milk products. *J Food Protect,* 59, 1079-1090, 1996.

30. Tekinsen KK, Ucar G: Aflatoxin M1 levels in butter and cream cheese consumed in Turkey. *Food Control,* 19, 27-30, 2008.

31. Wiseman DW, Marth EH: Behavior of aflatoxin M1 during manufacturing and storage of queso blanco and bakers cheese. *J Food Protect,* 46, 910-913, 1983.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S123-S127, 2010
DOI:10.9775/kvfd.2010.2398

RESEARCH ARTICLE

Mısır Silajında Aflatoksin B_1 Varlığının ve Süte Geçme Durumunun Araştırılması [1] [2]

Yakup KARAKAYA * Mustafa ATASEVER **

[1] *Bu araştırma Atatürk Üniversitesi Araştırma Fonu tarafından desteklenmiştir (Proje No 2006/88)*
[2] *Bu makale Yakup Karakaya'nın Yüksek Lisans tezinden derlenmiştir*

* Directorates of Agriculture, Köprüköy, TR-25320, Erzurum - TURKEY
** Department of Food Science and Technology, Faculty of Veterinary Science, Ataturk University, TR-25240, Erzurum - TURKEY

Makale Kodu (Article Code): KVFD-2010-2398

Özet

Bu çalısmada, Erzurum ili Pasinler ilçe merkezi ve köylerindeki süt sığırcılığı işletmelerinden alınan mısır silajlarının aflatoksin B_1 ve bu yemi tüketen hayvanların sütlerinin aflatoksin M_1 içeriği ile aflatoksinin yemden süte geçiş durumu araştırıldı. İncelenen yem örneklerinde aflatoksin B1 miktarı ortalama 361.12±94.76 ppt ve süt örneklerindeki aflatoksin M_1 miktarı ortalama 3.85±3.71 ppt olarak belirlendi. Tüketilen yemdeki aflatoksin B_1'in %1.07'sinin süte aflatoksin M_1 olarak geçtiği saptandı. Süt numunelerinin 6 adedinde (%8.33) ölçülebilir aflatoksin M_1 tespit edilemedi. 66 örnekte (%91.67) ise Türk Gıda Kodeksi'ne göre kabul edilebilir sınırların altında aflatoksin M_1 belirlendi. İncelenen 72 yem örneğinin 3 adedinde (%4.16) aflatoksin B_1 saptanmadı. Diğer örneklerde ise standartlarda belirtilen sınırın altında aflatoksin B_1 saptandı. İncelenen yem örneklerinde saptanan aflatoksin B_1 miktarı ile süt örneklerindeki aflatoksin M_1 miktarı arasında çok önemli ilişki ($P<0.01$) ve pozitif korelasyon (+0.329) olduğu belirlendi. Süt ve ürünlerindeki aflatoksin M_1 miktarının minimum düzeyde tutulabilmesi için, modern üretim teknikleri uygulanmalı, süt hayvanlarına verilen yemlerin depolanma koşulları uygun hale getirilmeli, gerekli kontroller yapılmalı ve süt üreticileri bu konuda bilinçlendirilmelidir. Gıda maddeleri ve yemler her aşamada aflatoksin yönünden analiz edilmeli ve kabul edilen sınırlardan fazla içerenlerin tüketimine izin verilmemelidir. Yem ve besinlerde küf bulaşmasını ve dolayısıyla aflatoksin oluşumunu önlemek için etkili, ekonomik ve uygulanabilir çalışmaların yapılması gereklidir.

Anahtar sözcükler: *Aflatoksin B_1, Aflatoksin M_1, Süt, Silaj, Yem, ELISA*

Aflatoxin B_1 in Corn Silage and Its Probability Passing in Milk

Summary

The aim of this study was undertaken to determine the levels of aflatoxin B_1 contamination in corn silage samples and aflatoxin M_1 in milk samples collected from cow dairy farms in Pasinler (Erzurum, Turkey) region. Average amount of aflatoxin B_1 in feed and aflatoxin M_1 in milk were found 361.12±94.76 ppt and 3.85±3.71 ppt respectively. It was found that, 1.07% aflatoxin B_1 passed to milk as a aflatoxin M_1. Aflatoxin M_1 was not found in 6 samples (8.33%) of the milk. Aflatoxin M_1 was found in 66 samples (91.67%) of the examined milk samples. None of the samples had aflatoxin M_1 greater than the maximum tolerance limit accepted by Turkish Food Codex. Aflatoxin B_1 was not found in 3 samples (4.16%) of the silage. Aflatoxin B1 was found in 69 samples (95.84%) of the examined silage samples. None of the samples had aflatoxin B_1 greater than the maximum tolerance limit accepted by Turkish Standard. In the statistical analysis, there was positive correlation ($P<0.01$) between aflatoxin M_1 in the silage samples and aflatoxin M_1 in the examined milk samples. To achieve minimum levels of aflatoksin M_1 in milk and milk products, modern production techniques should be employed, the animal feed storage conditions should be well- maintained and inspected, milk producers should be well-informed. Food and feed items should be analyzed for the presence of aflatoxin at every level the consumption of the ones that contain aflatoxin in amounts higher than the tolerable limits valid in our country should not be permitted. In order to prevent mould growth, and consequently, forming of aflatoxin on animal feed and food, effective, economical and applicable chemical materials should be researched and field investigations should be conducted for confirmation of the applications.

Keywords: *Aflatoxin B_1, Aflatoxin M_1, Milk, Silage, Feed, ELISA*

İletişim (Correspondence)
☎ +90 442 2360880
✉ atasever@atauni.edu.tr

GİRİŞ

En yaygın bilinen mikotoksin aflatoksindir. Aflatoksinler, *Aspergillus flavus, Aspergillus paraciticus, Aspergillus nomius* ile bazı Penicillium ve Rhizopus türleri tarafından sentezlenen, insan ve hayvanlarda akut ve kronik zehirlenmelere neden olan metabolitler olup, aflatoksin B_1, B_2, G_1, G_2, M_1 ve M_2 olmak üzere başlıca altı ana bileşikten oluşur [1-4].

Aflatoksinler, karsinojenik, mutajenik ve teratojenik etkileri yanında, ısıl işlemlere de dirençli olmaları nedeniyle sağlık açısından önem taşırlar [5-7]. Aflatoksinler, küflü gıdalarda görülmesine karşın doğrudan insan tüketimine sunulan gıdalarda da oluşabilir. Bu toksinlerin çeşitli işleme yöntemleri ile tamamen ortadan kaldırılamadığı ve hayvan yeminde bulunabilecek aflatoksinlerin çok az bir oranda da olsa et, süt ve yumurta gibi gıdalara geçerek insan sağlığı açısından risk oluşturabileceği ifade edilmektedir [8,9]. Aflatoksin M_1 ve M_2 toksinleri aflatoksijenik küfler tarafından direkt olarak sentezlenmemetedir. Bu toksinler, aflatoksin B_1 ve B_2 içeren yemlerle beslenen hayvanların bunları metabolize ederek sütlerine geçirmeleri sonucunda oluşmakta ve sütten izole edilmeleri nedeniyle de 'M' harfiyle simgelendirilmektedir [10].

Aflatoksin sentezleyen Aspergillus türleri bütün dünyada yaygın bir şekilde bulunur ve her türlü iklim koşulunda kolayca üreyebilir. Çevre sıcaklığının 24-45˚C olduğu ortamda ürün çeşidine göre %9-14 arası veya daha yüksek oranlarda rutubet içeren besinler 3-4 günde bile ağır bir şekilde küflenebilir. Aspergillus türü küfler birçok besin maddesinde (örn, mısır, pamuk tohumu küspesi, ayçiçeği küspesi, soya fasulyesi unu, fındık, yerfıstığı, ceviz, yağlı tohumlar ile balık ve etkemik unu, karma yem hazırlanmasında kullanılan hammaddeler) çok yaygın bir şekilde ve tehlikeli düzeylerde küflenme oluşturduğu bilinmektedir.

Dünyada çeşitli ülkeler aflatoksinler için izin verilebilecek maksimum limitleri belirlemişlerdir. Türkiye'de ise Türk Gıda Kodeksi; çiğ süt, ısıl işlem görmüs süt, süt bazlı ürünlerin üretiminde kullanılan süt için kabul edilebilir maksimum limiti 50 ppt olarak kabul etmiştir [11]. Yemlerdeki kabul edilebilir maksimum aflatoksin B_1 miktarı ise 20.000 ppt'dir [12].

Küfler, tarımsal ürünlerin üretimi, işlenmesi, depolanması ve tüketimi sırasında ürünleri kontamine etmek sureti ile bozulmalara sebep olmaktadır. Süt işletmelerinde küflere sıklıkla rastlanabilmektedir. Aspergillus türü küfler hayvan yemlerine bulaşabilmekte ve daha sonra da süt ve ürünlerine geçebilmektedir. Son yıllarda Erzurum ve çevresinde silaj tüketiminin artması nedeniyle yemlerde ve sütte aflatoksinin daha fazla bulunabileceği düşünülmektedir. Bu nedenle bu çalışma ile hayvan yemlerinde ve bu yemleri tüketen hayvanların sütlerinde aflatoksin içeriğinin belirlenmesi amaçlanmıştır.

MATERYAL ve METOT

Materyal

Bu araştırmada Erzurum ili Pasinler ilçe merkezi ve köylerindeki 72 adet süt sığırcılığı işletmesinden alınan mısır silajları (72 adet) ve bu yemi tüketen hayvanların sütleri (72 adet) numune olarak kullanılmıştır.

Metot

Örneklerin Toplanması

Erzurum'da mısır silajı üretiminin yoğun yapıldığı Pasinler ilçe merkezi ve köylerinden 72 adet 250 g mısır silajı örneği uygun poşetlere ve 72 adet 250 ml süt örneği de steril kavanozlara alındı. Alınan bu örnekler soğuk muhafaza altında laboratuara getirilmiş ve analiz yapılıncaya kadar 4±1˚C'de muhafaza edildi.

Aflatoksin Analizi

Bu çalışmada aflatoksin M_1 ve B_1 analizi için ELISA yöntemi kullanılmıştır [13] (r-biofarm RIDASCREEN aflatoksin M_1 test kitleri kullanıldı).

- Aflatoksin M_1 Analizinin Yapılışı

Süt Örneklerinin Hazırlanması: Süt örnekleri, yağın uzaklaştırılması için 3500 devir/20 d/10˚C'de santrifüj edildi. Santrifüj işleminden sonra, üstteki krema tabakası pastör pipeti ile çekilerek alındı. Ayrılmış süt testte direkt olarak kullanıldı (her kuyucuğa 100 µl).

Analizin Test Prosedürü: Standartlar ve örnekler için yeterli sayıda kuyucuk pleyte yerleştirildi. Standart ve örnek pozisyonları bir kenara not edildi. Standart solüsyonlardan ya da hazırlanmış örnekten 100 µl alıp kuyucuklara ilave edildi. Oda ısısında, karanlık ortamda 60 dak. inkubasyona bırakıldı. Kuyucuklardaki sıvı dışarı boşaltıldı. Kuyucuklar 250 µl distile su ile yıkanarak tekrar boşaltıldı ve aynı işlem bir kez daha tekrar edildi. Kuyucuklara 100 µl dilüe edilmiş enzim konjugat ilave edildi ve oda ısısında tekrar 60 dak. inkubasyona bırakıldı. İnkubasyondan sonra kuyucuklardaki sıvı boşaltılarak tekrar 2 kez yıkama işlemi yapıldı. Daha sonra her bir kuyucuğa 50 µl substrat ve 50 µl kromojen ilave edilerek iyice karıştırıldı ve 30 dak. oda ısısında karanlıkta inkubasyona bırakıldı. İnkubasyon sonunda her bir kuyucuğa 100 µl stop solüsyonu koyularak iyice karıştırıldı ve absorbansı 450 nm'de ELISA okuyucu ile okundu.

Sonuçlar hesaplanırken kalibrasyon eğrisi üzerinden elde edilen konsantrasyonlar dilusyon faktörleri ile çarpıldı (Dilusyon Faktörü: 1).

- Aflatoksin B1 Analizinin Yapılışı

Yem Örneklerinin Hazırlanması: 5 g yem numunesine 25 ml %70 lik metanol eklendi. Yaklaşık 3 dak. karıştırıldı. Whatman No:1 filtreden süzüldü. Temiz cam tüpe 1 ml filtrat alındı. Üzerine 1 ml distile su ilave edildi. Hazırlanan bu karışımdan her kuyucuğa 50 µl testte direkt olarak kullanıldı.

Analizin Test Prosedürü: Standartlar ve örnekler için yeterli sayıda mikrotitler kuyucuk pleyte yerleştirildi. Standart ve örnek pozisyonları bir kenara not edildi. Standart solüsyonlardan ve hazırlanmış örneklerden 50 µl alıp kuyucuklara ilave edildi. Kuyucuklara 50 µl dilüe edilmiş enzim konjugat ilave edildi ve oda ısısında 30 dak. inkubasyona bırakıldı. İnkubasyondan sonra kuyucuklardaki sıvı boşatılarak yıkama işlemi yapıldı. Daha sonra her bir kuyucuğa 100 µl substrat/kromojen ilave edilerek iyice karıştırıldı ve 15 dak. oda ısısında karanlıkta inkubasyona bırakıldı. İnkubasyon sonunda her bir kuyucuğa 100 µl stop solüsyonu koyularak iyice karıştırıldı ve absorbansı 450 nm'de ELISA okuyucu ile okundu. Sonuçlar hesaplanırken kalibrasyon eğrisi üzerinden elde edilen konsantrasyonlar dilusyon faktörleri ile çarpıldı (Dilusyon Faktörü: 10).

İstatistik Analizleri

Çalışmanın istatistiki analizleri SPSS 10.0 paket istatistik programı ile korelasyon analizi yapıldı.

BULGULAR

Yapılan çalışmada, toplam 72 adet yem örneği aflatoksin B_1 yönünden ve 72 adet süt örneği de aflatoksin M_1 yönünden incelendi.

İncelenen yem örneklerinde aflatoksin B_1 miktarı ortalama 361.12±94.76 ppt ve süt örneklerindeki aflatoksin M_1 miktarı da ortalama 3.85±3.71 ppt olarak belirlendi. Tüketilen yemdeki aflatoksin B_1'in %1.07'sinin süte aflatoksin M_1 olarak geçtiği saptandı.

Yem örneklerinde tespit edilen aflatoksin B_1 miktarlarının dağılımı *Tablo 1*'de ve süt örneklerinde tespit edilen aflatoksin M_1 miktarlarının dağılımı da *Tablo 2*'de gösterilmiştir.

İncelenen 72 yem örneğinin 3 adedinde (%4.16) aflatoksin B_1 saptanmadı. Diğer örneklerin tümünün aflatoksin B_1 içeriği yemler için izin verilen yasal limitlerin [12] altında bulundu.

Tablo 1. *Yem örneklerinin AFB_1 içeriği ve dağılımı*
Table 1. *Occurrence and distribution of AFB_1 in corn silage samples*

Örneklerde Tespit Edilen Aflatoksin B1*					
Saptanamadı		< 20		> 20 [a]	
Sayı	Oran	Sayı	Oran	Sayı	Oran
3	%4.17	69	%95.83	0	%0

** ppb, [a] Yem yönetmeliğinde [12] belirtilen yemler için en üst limit aflatoksin B1 değeri (20 ppb=20.000 ppt)*
** ppb, [a] The maximum tolerance limit accepted by Turkish Standard [12] (20 ppb=20.000 ppt)*

Tablo 2. *Süt örneklerinin AFM_1 içeriği ve dağılımı*
Table 2. *Occurrence and distribution of AFM_1 in milk samples*

Örneklerde Tespit Edilen Aflatoksin M1*									
Saptanamadı		< 5		5-10		10-50		> 50 [a]	
Sayı	Oran (%)	Sayı	Oran (%)	Sayı	Oran (%)	Sayı	Oran (%)	Sayı	Oran (%)
6	8.33	44	61.11	15	12.83	7	9.72	-	0

** ppb, [a] Türk Gıda Kodeksinde11 sütler için en üst limit aflatoksin M_1 miktarı (0.05 ppb=50 ppt)*
** ppb, [a] Proportion of samples exceeding the Turkish Food Codex Legal limit 11 (0.05 ppb=50 ppt)*

Yapılan çalışmada, 72 süt örneğinden hiç birisinde aflatoksin M_1 miktarının Türk Gıda Kodeksine [11] göre kabul edilebilir sınırların üzerinde (50 ppt) olmadığı saptandı.

Süt numunelerin 6 adedinde (%8.33) ölçülebilir aflatoksin M_1 tespit edilemedi. 66 örnekte (%91.67) ise Türk Gıda Kodeksine [11] göre kabul edilebilir sınırların altında aflatoksin M_1 belirlendi. İncelenen yem örneklerinde saptanan aflatoksin B_1 miktarı ile süt örneklerindeki aflatoksin M_1 miktarı arasında çok önemli ilişki ($P<0.01$) ve pozitif korrelasyon (+0.329) olduğu belirlendi.

TARTIŞMA ve SONUÇ

Bu çalışmada, Erzurum ili Pasinler ilçe merkezi ve köylerindeki 72 adet süt sığırcılığı işletmesinden alınan mısır silajlarının aflatoksin B_1 ve bu yemi tüketen hayvanların sütlerinin aflatoksin M_1 içeriği yönünden incelendi ve aflatoksinin yemden süte geçiş durumu araştırıldı. Küfler tarım ürünlerinin üretimi, işlenmesi, depolanması ve tüketimi sırasında ürünleri kontamine etmek sureti ile bozulmalara sebep olmaktadır. Son yıllarda Erzurum ve çevresinde silaj tüketiminin artması nedeniyle yemlerde ve sütte aflatoksin daha fazla bulunabileceği düşünülmektedir. Bu nedenle çalışmada hayvan yemleri ile ve bu yemleri tüketen hayvanların süt-

lerinde aflatoksin içeriğinin belirlenmesi amaçlanmıştır.

Aflatoksin M_1, süt ve ürünlerinde besin hijyeni açısından ciddi sorunlar oluşturmaktadır. Sütten kremanın ayrılması işlemi sırasında aflatoksin M_1'in bir kısmı kremaya geçer, geri kalan kısmı kazeine bağlanma özelliğinden dolayı sütte kalır [14-16].

Yemlerde aflatoksin miktarını belirlemeye yönelik yapılan çalışmalar mevcuttur. Tayland'daki mısırların %35'inde, Filipinler'deki mısırların %97'sinde ve Uganda'daki mısırların %40'ında Aflatoksin B_1 tespit edilmiştir [17]. Amerika Birleşik Devletleri'nde 644 mısır örneği aflatoksin yönünden analiz edilmiş ve sonuçta örneklerin %1.6'sında ortalama 300 ppb düzeyinde aflatoksin tespit edilmiştir [18].

Bu çalışmada ise incelenen 72 yem örneğinin 3 adedinde (%4.16) aflatoksin B_1 saptanmamıştır. Diğer örneklerde ise Yem Yönetmeliği'nde [12] belirtilen sınırın altında aflatoksin B_1 saptanmıştır. Bu araştırmada yemlerde belirlenen aflatoksin B_1 düzeyi bazı araştırmaların [16-18] bulgularıyla benzerlik arz etmektedir.

Bu çalışmada tüketilen yemdeki aflatoksin B_1'in %1.07'si aflatoksin M_1 olarak süte geçtiği saptandı. Bu durum bazı çalışmalar [18-21] ile uyumlu bir sonuçtur. Süt hayvanlarının aflatoksin B_1 içeren yemlerle beslenmeleri sonucunda sütlerinde aflatoksin M_1'e rastlanabilir. Aflatoksin B_1'in metaboliti olan aflatoksin M_1 süt hayvanlarının karaciğerlerinde metabolize olarak süt bezleri ile süte salgılanır [14]. Süt veren hayvanların yemlerle beraber aldıkları aflatoksin B_1'in hangi oranlarda aflatoksin M_1'e dönüştüğünü saptamak amacıyla yapılmış çeşitli çalışmalar bulunmaktadır. Bu çalışmaların birçoğunda genel bir yargı olarak tüketilen yemdeki aflatoksin B_1'in %0.35-5'i arasında değişen oranlarda sütte aflatoksin M_1 olarak görüldüğü belirtilmektedir [7,18,20]. Yapılan bu çalışmalarda, aflatoksin B_1'in aflatoksin M_1'e dönüşüm oranının hayvandan hayvana, günden güne, bir süt verme döneminden diğerine, sağım zamanına hatta sağım aralığına ve hayvanın süt verim düzeyine göre değiştiği rapor edilmiştir. Ayrıca yemlerle beraber alınan aflatoksin B_1 düzeyinin de dönüşüm oranına etkili olabileceği bildirilmektedir [18].

Yapılan çalışmada incelenen 72 süt örneğinden hiç birisinde aflatoksin M_1 miktarının Türk Gıda Kodeksine [11] göre kabul edilebilir sınırların üzerinde olmadığı saptandı. Altmışaltı süt örneğinde (%91.67) aflatoksin M_1 belirlenirken 6 adedinde (%8.33) ölçülebilir aflatoksin M_1 tespit edilemedi. Bu araştırmada sütlerde belirlenen aflatoksin M_1 düzeyi birçok araştırmacının [14,22-24] bulgularıyla benzerlik göstermektedir.

Çalışmada yem ve sütlerde aflatoksin bulunması, Erzurum çevresinde tüketilen et, süt ve ürünlerinin kronik sağlık sorunlarına neden olabileceğini düşündürmektedir. Yemin, özellikle silajın, üretim ve depolama esnasında şartlar uygun olduğu takdirde toksijenik küf kontaminasyonu, dolayısıyla aflatoksin bulaşma riski her zaman gözönünde tutulmalıdır. Bu nedenle silajın üretiminden tüketimine kadar tüm işlemler, küf gelişimini en aza indirecek şekilde gerçekleştirilmelidir. Bu amaçla modern üretim teknikleri uygulanmalı, uygun biçimde depolanmalı ve sürekli olarak kontrol edilmelidir. Aflatoksin oluşumuna uygun şartlar oluşturulmamalıdır. Uygun şartlar altında biçimi yapılan silajlık materyalin silolama işlemi yapılırken; yemlerin arasında fazla oksijen kalmayacak şekilde silolara doldurulmalıdır. Siloya doldurulan materyalde oksijen yaklaşık 6 saat içerisinde kullanılarak anaerobik ortam oluşur. Bu ortamda laktik asit bakterileri üreyerek laktik asit miktarını artırır. Eğer anaerobik şartlar iyi bir şekilde devam ettirilirse, pH 4 civarında kalır ve önemli bir küf gelişimi gözlenmez. Fakat herhangi bir şekilde anaerobik şartlarda değişiklik olması durumunda (örn. silo içerisine hava ve su girmesi), silajda küfler üreyerek aflatoksin oluşumuna yolaçabilirler.

Yemlerde aflatoksin B_1 ve sütlerde de aflatoksin M_1'in kontrolü ve detoksifikasyonu göz önünde bulundurulması gereken bir olgu olarak ortaya çıkmaktadır. Yem ve sütlere periyodik kontrol programları uygulanarak kontrol altında tutulmalı ve yüksek düzeyde aflatoksin içeren ürünlerin kullanılmaları önlenmelidir. Ayrıca, silaj üretiminde hijyene ve teknolojiye özen gösterilmesinin gerekliliği de ortaya çıkmıştır.

Aflatoksin B_1'in yaklaşık %1'nin süte aflatoksin M_1 olarak geçtiği dikkate alındığında yemlerde yasal olarak izin verilen aflatoksin B_1 limitinin (20.000 ppt) yüksek olduğu kanısına varılmaktadır. Bu nedenle yasal miktarın azaltılması gerektiği düşünülmektedir.

Araştırma, Pasinler ilçesindeki yem ve sütlerde aflatoksin bulunduğunu ortaya koymuştur. Bu durum, Erzurum çevresinde aflatoksinden kaynaklanan kronik sağlık sorunlarının olabileceğini göstermektedir.

KAYNAKLAR

1. Steyn PS: Mycotoxins, general view, chemistry and structure. *Toxicol Lett,* 82-83, 843-851, 1995.

2. Mello JPF, Macdonald AMC: Mycotoxins. *Anim Feed Sci Technol,* 69, 155-166, 1997.

3. Marth EH: Aflatoxin in milk, cheese and other dairy products, *Marschall Italian & Specialty Cheese Seminars,* 1979.

4. Sibanda L, De Saeger S, Van Peteghem C: Development of portable field immunoassay for the detection of aflatoxin M1

in milk. *Int Food Microbiol,* 48, 203-209, 1999.

5. Govaris A, Roussi V, Koidis PA, Botsoglou NA: Distribution and stability of aflatoxin M1 during processing, ripening and storage of Telemes cheese. *Food Addit Contam,* 5, 437-443, 2001.

6. Srivastava VP, Bu-Abbas A, Alaa-Basuny, Al-Johar W, Al-Mufti S and Siddiqui MKJ: Aflatoxin M1 contamination in commercial samples of milk and dairy products in Kuwait. *Food Addit Contam,* 11, 993-997, 2001.

7. Veldman A, Meijst JAC, Borggreve GJ, Heeres-van der Tol JJ: Carry-over of aflatoxin from cow's food to milk. *Anim Prod,* 55, 163-168, 1992.

8. Bullerman LB: Significance of mycotoxins to food safety human health. *J Food Protect,* 42, 65-86, 1979.

9. Çoksöyler N: İçel Yöresinde Yetiştirilmekte Olan Yerfıstıklarında Aflatoksin Oluşumu Üzerine Araştırmalar. Tarım Orman ve Köyişleri Bakanlığı Ankara İl Kontrol Laboratuvarı Müdürlüğü. Yayın No: 6, Ankara, 28, 1987.

10. Tunail N: Aflatoksinlerin detoksifikasyonu. **In,** Gıda Mikrobiyolojisi ve Uygulamaları. Sim Matbaacılık, Ankara, 522, 2000.

11. Tarım ve Köyişleri Bakanlığı: Türk Gıda Kodeksi Gıda maddelerinde belirli bulaşanların maksimum seviyelerinin belirlenmesi hakkında tebliğ. Tebliğ No: 2008/26, *Resmi Gazete,* Sayı: 26879, Tarih: 17.05.2008.

12. Tarım ve Köyişleri Bakanlığı: Yemlerde istenmeyen maddeler hakkında tebliğ. Tebliğ No: 2005/3, *Resmi Gazete,* Sayı: 25718, Tarih: 05.02.2005.

13. R-Biopharm GmbH: Enzyme immunoassey for the quantitative analysis of aflatoxin M1. Art. No: R. 1101. R-Biopharm GmbH, Darmstadt. Germany, 1999.

14. Galvano F, Galofaro V, De Angelis A, Galvano M, Bognanno M, Galvano G: Survey of the occurrence of aflatoxin M1 in dairy products marketed in Italy. *J Food Protect,* 61, 738-741, 1998.

15. Barbieri G, Bergamini C, Ori E, Reska P: Aflatoxin M1 in parmesan cheese: HPLC determination, *J Food Sci,* 56, 1313-1331, 1994.

16. Pittet A: Natural occurance of mycotoxins in foods and feeds-an updated review. *Rev Med Vet,* 149, 479-492, 1998.

17. Concon JM: Contaminants and additives. Food Toxicology, Part-B, pp. 667-743, Marcel Dekker Inc, New York, 1988.

18. Price WD, Lovell RA, Mc Chesney DG: Naturally occurring toxins in feedstuffs: Center for Veterinary Medicine Perspective. *J Anim Sci,* 71, 2556-2562, 1993.

19. Van Egmond HP: Aflatoxin M1: Occurence, toxicity, regulation. **In,** Van Egmond HP (Ed): Mycotoxins in Dairy Products. pp. 11-49, Elsevier, London, 1989.

20. Scott PM: Methods for determination of aflatoxin M1 in milk and milk products review of performance characteristics. *Food Addit Contam,* 6, 283-305, 1989.

21. Oruç HH: Süt ve süt ürünlerinde aflatoksin M1 ve Türkiye'deki durumu. *Uludağ Univ Vet Fak Derg,* 22, 121-125, 2003.

22. Blanco JL, Dominguez L, Gomez-Lucia E, Arayzabal JFF, Garcia JA, Suarez G: Presence of aflatoxin M1 in commercial Ultra-High-Temperature-Treated milk. *Appl Environ Microb,* 54, 1622-1623, 1988.

23. Domagala BJ, Kisza J, Blüthgen A, Heeschen W: Contamination of milk with aflatoxin M1 in Poland. *Milchwissenschaft,* 52, 631-633, 1997.

24. Meerarani S, Ramadass P, Padmanaban CD, Nachimuthu K: Incidence of aflatoxin M1 in milk samples Chennai (Madras) city. *J Food Sci Tech,* 34, 506-508, 1997.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S129-S133, 2010
DOI:10.9775/kvfd.2010.2479

RESEARCH ARTICLE

Comparison of Virulence Gene Profiles of *Enterococcus faecium* and *Enterococcus faecalis* Chicken Neck Skin and Faeces Isolates

Aylin KASIMOĞLU DOĞRU * Yılmaz Emre GENÇAY * Naim Deniz AYAZ *

* Kırıkkale Üniversitesi Veteriner Fakültesi, Besin Hijyeni ve Teknolojisi Anabilim Dalı, TR-71450 Kırıkkale - TÜRKİYE

Makale Kodu (Article Code): KVFD-2010-2479

Summary

The objective of this study was to find out the distribution of major virulence determinants *asa1*, *gelE*, *cylA*, *esp*, and *hyl* by multiplex PCR in 132 *Enterococcus faecium* and 67 *Enterococcus faecalis* isolates originated from chicken neck skin samples at slaughterhouse and faeces samples from intensive broiler enterprises and rural poultry establishments. In the study, 31.2% (62/199) of the enterococcal strains harbored at least one virulence determinant. The *gelE* gene was the predominant (30.2%) virulence trait among the enterococci investigated followed by *asa1* (15.6%). Both *gelE* and *asa1* genes were significantly higher in *E. faecalis* than *E. faecium.* The *hyl*, *esp* and *cylA* genes were detected with percentages of 1.5%, 1.5% and 0.8% in *E. faecium* isolates. None of the *E. faecalis* strains harbored *cylA*, *esp* and *hyl* genes. The results indicate that a clear difference was observed in the kind of virulence factor present in strains between faecal samples and skin samples. Also, *E. faecium* strains isolated from both chicken skin samples and faeces presented lower pathogenicity potential than did *E. faecalis.*

Keywords: *Chicken, Enterococcus faecalis, E. faecium, multiplex PCR, Virulence genes*

Enterococcus faecium ve *Enterococcus faecalis* Tavuk Boyun Derisi ve Dışkı İzolatlarının Virülens Gen Profillerinin Karşılaştırılması

Özet

Bu çalışmada, mezbahada tavuk boyun derisinden, entansif broyler çiftlikleri ve köylerde aile işletmelerindeki tavukların dışkı örneklerinden izole edilen 132 *Enterococcus faecium* ve 67 *Enterococcus faecalis*'in başlıca virülens genleri olan *asa1*, *gelE*, *cylA*, *esp* ve *hyl* genlerinin multipleks PCR ile tespiti amaçlanmıştır. Enterokok izolatlarının %31.2'sinin en az bir virülens genine sahip olduğu belirlenmiştir. *gelE* geninin dominant (%30.2) virülens faktörü olduğu ve bunu %15.6 ile *asa1*'in takip ettiği saptanmıştır. Hem *gelE* hem de *asa1* genlerinin *E. faecalis*'te *E. faecium*'a oranla önemli ölçüde yüksek olduğu tespit edilmiştir. *E. faecium* izolatlarının %1.5, %1.5 ve %0.8'inde sırasıyla *hyl*, *esp* ve *cylA* genleri belirlenmiştir. *E. faecalis* izolatlarının hiçbirinde *cylA*, *esp* ve *hyl* genleri belirlenememiştir. Çalışma bulguları, tavuk dışkı örneklerinden izole edilen enterokoklar ile boyun derisi örneklerinden izole edilenler arasında virülens faktörleri açısından önemli bir fark olduğunu ifade etmektedir. Ayrıca, hem tavuk boyun derisinden hem de dışkısından elde edilen *E. faecium* izolatlarının *E. faecalis*'e oranla daha düşük patojenite potansiyeline sahip oldukları ortaya konulmuştur.

Anahtar sözcükler: *Tavuk, Enterococcus faecalis, E. faecium, multiplex PCR, Virulence genleri*

INTRODUCTION

Genetical similarities between animal and human originated enterococci have been reported and role of natural transmission of enterococci from food animals and contaminated foods to human tract can not be ruled out [1]. Enterococci can cause food intoxication through production of biogenic amins and worrisome opportunistic infections because of the virulence traits [2].

Some strains are resistant to many antibiotics, but antibiotic resistance alone cannot explain the virulence of enterococci [1]. The differentiation of apparently safe and non-safe enterococcal strains is not simple, especially because of effective horizantal gene transfer mechanisms [3,7]. *Enterococcus faecalis* and *E. faecium* are the most relevant species of *Enterococcus* genus with

İletişim (Correspondence)
☎ +90 318 3573301/3161
✉ akasimoglu@superonline.com

regard to clinical aspects. Also, virulence of *Enterococcus spp.* may be linked to these species [4].

A number of genes encoding for virulence factors including *asa1, esp, hyl, gelE,* and *cyl* in *E. faecalis* and *E. faecium* have been described and their effects have been shown in human and animal studies [2,5]. Aggregation substance (AS), a surface protein adhesin encoded by the gene *asa1* has a contribution to virulence together with cytolysin [6], facilitates the aggregation of the donor and recipient bacteria for efficient transfer of transmissible conjugative plasmids [4,7]. Another enterococcal adhesin is the "enterococcal surface protein" (ESP), encoded by *esp* gene that plays a role in biofilm formation and adherence to abiotic surfaces [1]. Hyaluronidase, which is expressed by the *hyl* gene, acts on hyaluronic acid and increases bacterial invasion [8]. The *gelE* gene encodes for an extracellular Zn-metalloendopeptidase that is capable of hydrolysing gelatin, collagen, casein, hemoglobin and other biological peptides [9]. The cytolysin (Cyl) is a cellular toxin and capable of lysing a range of prokaryotic and eukaryotic cells [10].

Although chicken meat consumption is estimated around a million tone [11], there is a lack of information on virulence genes of enterococci from poultry in Turkey. Therefore, the objective of this study was to investigate and compare the distribution of major virulence determinants *cylA, hyl, asa1, esp,* and *gelE* in *E. faecalis* and *E. faecium* strains isolated from neck skin samples and faeces of chicken.

MATERIAL and METHODS

Bacterial Strains

A total of 199 *Enterococcus* including 132 *E. faecium* and 67 *E. faecalis* strains were investigated. Faecal strains consisted of 36 *E. faecium* and 41 *E. faecalis* from intensive broiler enterprises, and 56 *E. faecium* and 10 *E. faecalis* from rural poultry establishments in Kirikkale district [12]. Additionally, previously PCR verified 40 *E. faecium* and 16 *E. faecalis* strains that were isolated from chicken neck skin samples at slaughter in Ankara [13] were included.

Reference strains that were used in multiplex PCR assays were *E. faecalis* MMH594 (*gelE*$^+$, *asa1*$^+$, *esp*$^+$ and *cylA*$^+$), *E. faecalis* ATCC 29212 (*gelE*$^+$ and *asa1*$^+$), *E. faecium* C68 (*hyl*$^+$ and *esp*$^+$), *E. faecium* C38 (*esp*$^+$) and *E. faecalis* 217 (*gelE*$^-$, *asa1*$^-$, *esp*$^-$ and *cylA*$^-$). *Enterococcus faecalis* MMH594, *E. faecalis* 217, *E. faecium* C68, and *E. faecium* C38 were kindly provided from Vanessa Vankerckhoven from University of Antwerp, Vaccine and Infectious Disease Institute Medical Microbiology, Antwerp, Belgium.

Species Verification of Faecal E. faecium and E. faecalis Strains By Multiplex PCR Assay

Faecal *E. faecium* and *E. faecalis* strains previously isolated from intensive broiler enterprises and rural poultry establisments were verified by multiplex PCR. The extraction of DNA from the isolates was done with Chelex-100 (Bio-Rad, Hercules, CA, USA) resin based technique [13]. Resulting supernatant was used as template DNA for amplification procedures in the multiplex PCR assays. For the verification of *E. faecium* and *E. faecalis,* primer pairs (Alpha DNA, Montreal, Canada) and multiplex PCR protocol of Kariyama et al.[14] was used *(Table 1)*.

Detection of Virulence Genes By Multiplex PCR

The extraction of DNA was done as mentioned

Table 1. *Primer sequences used in this study for verification of Enterococcus species and virulence determinants*
Tablo 1. *Enterokok türlerinin doğrulanmasında ve virülens genlerinin tespitinde kullanılan primer dizileri*

Target	Oligonucleotide Sequence (5'-3')	Product Size	Reference
ddl E. faecalis	ddlE1- ATCAAGTACAGTTAGTCTTTATTAG ddlE2- ACGATTCAAAGCTAACTGAATCAGT	941 bp	14
ddl E. faecium	ddlF1- TTGAGGCAGACCAGATTGACG ddlF2- TATGACAGCGACTCCGATTCC	658 bp	15
asa1	ASA11- GCACGCTATTACGAACTATGA ASA12- TAAGAAAGAACATCACCACGA	375 bp	8
gelE	GEL 11- TATGACAATGCTTTTTGGGAT GEL 12- AGATGCACCCGAAATAATATA	213 bp	8
cylA	CYT I- ACTCGGGGATTGATAGGC CYT IIb- GCTGCTAAAGCTGCGCTT	688 bp	16
esp	ESP 14F- AGATTTCATCTTTGATTCTTGG ESP 12R- AATTGATTCTTTAGCATCTGG	510 bp	17
hyl	HYL n1- ACAGAAGAGCTGCAGGAAATG HYL n2- GACTGACGTCCAAGTTTCCAA	276 bp	8

above. Virulence genes specific primers (Alpha DNA) *(Table 1)* were used in the multiplex PCR according to the Vankerckhoven et al.[8]. However, different from Vankerckhoven et al.[8]'s protocol, 2.5 U Taq polymerase (Bioron GmbH, Ludwigshafen, Germany) and 1 x PCR Buffer [10 mmol l^{-1} Tris-HCl (pH 8.3), 50 mmol l^{-1} KCl, 0.01% Tween-20] (Bioron) were used and initial denaturation time was decreased from 15 to 5 min. In every multiplex PCR analysis positive controls were used in order to eliminate false negative results.

Electrophoresis of the Multiplex PCR Products

A 20 µl aliquot of each PCR products stained with 6x loading dye (Promega, Madison, USA) were analyzed by agarose gel (1.5% Agarose-Basica LE, Prona, Spain) electrophoresis (CSL MSMixi-Duo, Corston, UK), stained with 0.1 µg ml^{-1} ethidium bromide (BioChemica GmbH, Darmstadt, Germany), at 85 V for 1.5 h and visualized by a gel documentation and analysis system (Sygene Ingenius, Cambridge, UK).

Statistical Analysis

Comparison between *E. faecalis* and *E. faecium* isolates from the incidence of virulence genes including *asa1, gelE, cylA, esp* and *hyl* were analyzed with Fisher Exact statistical analysis [18].

RESULTS

A total of 199 *Enterococcus* including 132 *E. faecium* and 67 *E. faecalis* originated from intensive broiler enterprises, rural poultry establishments and chicken neck skin samples at slaughter level were analyzed for the presence of virulence genes *cylA, hyl, asa1, esp* and *gelE (Fig. 1)*. Virulence gene distributions of *E. faecium* and *E. faecalis* strains from intensive broiler enterprises, rural poultry establishments and chicken neck skin samples are shown in *Table 2*.

In the present study, the percentage of enterococci harboring at least one virulence determinant was 31.2% (62/199) and was significantly (P<0.0001) high in *E. faecalis* (33/67, 49.3%) than *E. faecium* (29/132, 22.0%). Statistically, the *E. faecium* strains of intensive broilers (18/36, 50.0%) were significantly more virulent than the *E. faecium* strains of either rural establishments (9/56, 16.1%) (P<0.0001) or slaughter level (2/40, 5.0%) (P<0.0001). No significant difference was observed between the virulent strain percentages of *E. faecium* strains isolated from rural establishments and neck skin samples. *E. faecalis* strains of intensive broiler origin (15/41, 36.6%) were significantly (P<0.0001) less virulent than *E. faecalis* strains originated from neck skin samples (16/16, 100.0%) while, were significantly (P=0.0079) more virulent than strains of rural establishments (2/10, 20.0%). A significant (P<0.0001) difference was observed between the virulence gene distributions of *E. faecalis* (16/16, 100.0%) and *E. faecium* (2/40, 5.0%) strains from neck skin samples. Additionally, no significant difference was found in virulence gene prevalences between the strains of *E. faecalis* and *E. faecium* isolated from rural establishments (P=0.4624) and intensive broilers (P=0.0946).

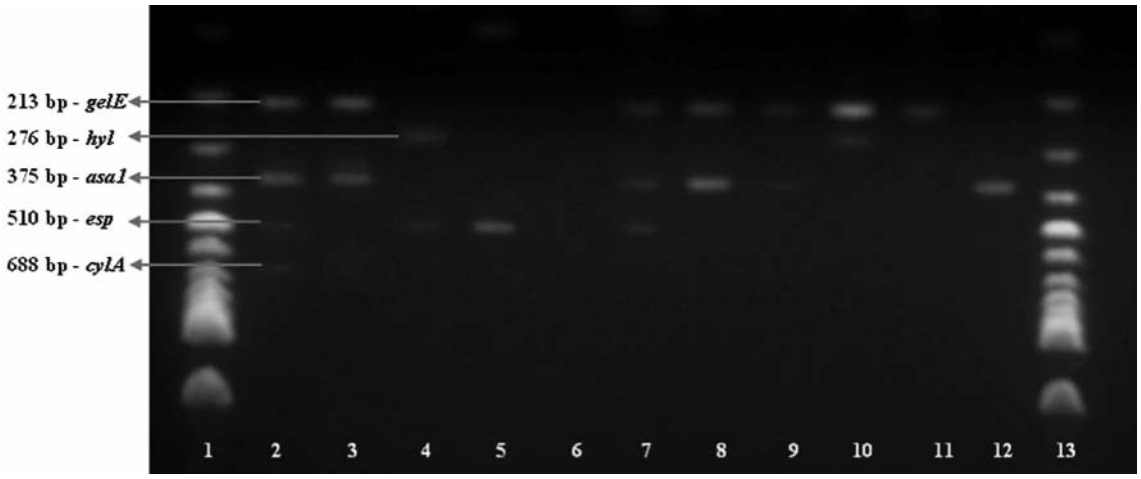

Fig 1. Major virulence genes of *E. faecium* and *E. faecalis*

(Lanes **1** and **13:** 100 bp DNA marker; **2:** *E. faecalis* MMH 594; **3:** *E. faecalis* ATCC 29212; **4:** *E. faecium* C68; **5:** *E. faecium* C38; **6:** negative control; **7:** *gelE, asa1* and *esp* positive E. faecium isolate; **8:** *gelE* and *asa1* positive *E. faecalis* isolate, **9:** *gelE* and *asa1* positive *E. faecium* isolate; **10:** *gelE* and *hyl* positive *E. faecium* isolate; **11:** *gelE* positive *E. faecalis* isolate; **12:** asa1 positive *E. faecalis* isolate)

Şekil 1. *E. faecium* ve *E. faecalis*'in başlıca virülens genleri

(Sıra **1** ve **13:** 100 bp DNA cetveli; **2:** *E. faecalis* MMH 594; **3:** *E. faecalis* ATCC 29212; **4:** *E. faecium* C68; **5:** *E. faecium* C38; **6:** negatif kontrol; **7:** *gelE, asa1* ve *esp* pozitif *E. faecium* izolatı; **8:** *gelE* ve *asa1* pozitif *E. faecalis* izolatı, **9:** *gelE* ve *asa1* pozitif *E. faecium* izolatı; **10:** *gelE* ve *hyl* pozitif *E. faecium* izolatı; **11:** *gelE* pozitif *E. faecalis* izolatı; **12:** *asa1* pozitif *E. faecalis* izolatı)

Table 2. *Sources and virulence gene distributions of E. faecium and E. faecalis*
Tablo 2. *E. faecium ve E. faecalis'lerin orijinleri ve virülens gen dağılımları*

Enterococcus faecium			***Enterococcus faecalis***		
Origin of Strains *	**n of Strains**	**Virulence gene Profile (n) (%)**	**Origin of Strains ***	**n of Strains**	**Virulence gene Profile (n) (%)**
I	36	*gelE* (4) (11.1) *gelE* + *asa1* (11) (30.6) *gelE* + *hyl* (2) (5.6) *gelE* + *asa1* + *esp* + *cylA* (1) (2.8)	I	41	*gelE* (9) (22.0) *gelE* + *asa1* (6) (14.6)
R	56	*gelE* (4) (7.1) *gelE* + *asa1* (4) (7.1) *gelE* + *asa1* + *esp* (1) (1.8)	R	10	*gelE* (1) (10.0) *gelE* + *asa1* (1) (10.0)
CNS	40	*gelE* (2) (5.0)	CNS	16	*gelE* (9) (56.3) *asa1* (2) (12.5) *gelE* + *asa1* (5) (31.3)

**** I:*** *intensive broiler enterprise;* ***R:*** *rural poultry establishment;* ***CNS:*** *chicken neck skin samples*

The *gelE* gene was the predominant (60/199, 30.2%) virulence trait among the enterococci investigated followed by *asa1* (31/199, 15.6%). Both *gelE* (P<0.0001) and *asa1* (P=0.0498) genes were significantly higher in *E. faecalis* (31/67, 46.3% for *gelE*; 14/67, 20.9% for *asa1*) than *E. faecium* (29/132, 22.0% for *gelE*; 17/132, 12.9% for *asa1*).

While none of the *E. faecalis* strains harbored *cylA, esp* and *hyl* genes, *E. faecium* strains harbored the *hyl, esp* and *cylA* genes as 1.5% (2/132), 1.5% (2/132) and 0.8% (1/132), respectively.

DISCUSSION

Since chicken meat and products are highly consumed and influx of virulence genes from enterococci of chicken origin to human intestinal tract is a possible route, this study has an impact on understanding the distribution of major virulence genes among *E. faecium* and *E. faecalis* of chicken origin.

According to the results of present study, *E. faecium* strains isolated from both chicken neck skin samples and faeces have lower potential pathogenicity than *E. faecalis*. However, virulence genes in *E. faecium* isolates presented more variable genotypes than did *E. faecalis* strains, as none of the *hyl, esp* or *cylA* genes were detected in *E. faecalis* isolates. On the other hand, the gene *gelE* and *asa1* were present in both analyzed species. A clear difference was observed in the kind of virulence factor present in strains between faecal samples and neck skin samples. Franz et al.[19] previously reported that the presence of virulence factors is a strain specific character. In the present study, *gelE* gene was determined as the predominant (30.2%) virulence trait among all of the enterococcal strains, and especially in *E. faecalis*. Similarly, the high distribution of the *gelE* gene in *E. faecalis* reported by Franz et al.[19] and Poeta et al.[20,21] for the faecal poultry samples. Also, results of the present study show that the prevalence of *gelE* and *asa1* genes were higher in *E. faecalis* than *E. faecium*. Similarly some researchers stated that *gelE* appear to be relatively frequent among *E. faecalis* strains coming from various sources [3, 19].

In the present study, the *hyl, esp* and *cylA* genes were detected with percentages of 1.5% (2/132), 1.5% (2/132) and 0.8% (1/132) in *E. faecium* strains, respectively. Moreover, all strains of *E. faecium* harboring *hyl* were also harboring *gelE*. Also, none of the *E. faecalis* strains harbor the *hyl, esp* and *cylA* genes. Poeta et al.[21] reported 30% *cylA* positivity for *E. faecalis* and *E. faecium* strains of poultry origin. The results of the present study for the *esp* negativity in *E. faecalis* is in compliance with previous reports [20,22,23]. According to the literature review, we could not find any previous report about *esp* gene in *E. faecium* strains of poultry faeces origin.

Consequently, the results indicate that, a clear difference was observed in the kind of virulence factor present in strains between faecal samples and neck skin samples. Also, *E. faecium* strains isolated from both chicken neck skin samples and faeces have lower pathogenicity potential than *E. faecalis*. Therefore, *E. faecium* strains of poultry origin may play no or only a minor role in this increasing virulence trend.

ACKNOWLEDGEMENTS

The authors would like to thank Vanessa Vankerckhoven (University of Antwerp, Vaccine and Infectious Disease Institute Medical Microbiology, Antwerp, Belgium) for providing the reference strains *E. faecalis* MMH594, *E. faecalis* 217, *E. faecium* C68 and *E. faecium* C38 and Mehmet Gülşen (Diagen Co. Inc.) for supplying primers.

REFERENCES

1. Franz CMAP, Stiles ME, Schleifer KH, Holzapfel WH: Enterococci in food-a conundrum for food safety. *Int J Food Microbiol,* 88, 105-122, 2003.

2. Giraffa G: Enterococci from foods. *FEMS Microbiol Rev,* 26, 163-171, 2002.

3. Eaton TJ, Gasson MJ: Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Appl Environ Microbiol,* 67, 1628-1635, 2001.

4. Mundy LM, Sahm DF, Gilmore M: Relationship between enterococcal virulence and antimicrobial resistance. *Clin Microbiol Rev,* 13, 513-522, 2000.

5. Shankar V, Baghdayan A, Huycke M, Lindahl G, Gilmore M: Infection derived *Enterococcus faecalis* strains are enriched in esp, a gene encoding a novel surface protein. *Infect Immun,* 67, 193-200, 1999.

6. Chow JW, Thal LA, Perri MB, Vazquez JA, Donabedian SM, Clewell DB, Zervos MJ: Plasmid-associated hemolysin and aggregation substance production contribute to virulence in experimental enterococcal endocarditis. *Antimicrob Agents Chemother,* 37, 2474-2477, 1993.

7. Pillar CM, Gilmore MS: Enterococcal virulence-pathogenicity island of *E. faecalis. Front Biosci,* 9, 2335-2346, 2004.

8. Vankerckhoven V, Autgaerden TV, Vael C, Lammens C, Chapelle S, Rossi R, Jabes D, Goossens H: Development of a multiplex PCR for the detection of *asa1, gelE, cylA, esp* and *hyl* genes in enterococci and survey for virulence determinants among European hospital isolates of *Enterococcus faecium. J Clin Microbiol,* 42, 4473-4479, 2004.

9. Jett BD, Huycke MM, Gilmore MS: Virulence of enterococci. *Clin Microbiol Rev,* 7, 462-478, 1994.

10. Shankar N, Coburn P, Pillar C, Haas W, Gilmore M: Enterococcal cytolysins: Activities and association with other virulence traits in a pathogenicity island. *Int J Med Microbiol,* 293, 609-618, 2004.

11. TURKSTAT: The Results of Animal Production Statistics. http://www.turkstat.gov.tr. *Accessed:* 14.03.2010.

12. Kasimoglu A, Istanbulluoglu E, Cay Z: Studies on phenotype and genotype characteristics and profiles of *Enterococcus spp.* isolated from intensive broiler farms and rural poultry establishments. Project No: K.U. BAP 2005/11, 2005.

13. Kasimoglu-Dogru A, Gencay YE, Ayaz ND: Prevalence and antibiotic resistance profiles of *Enterococcus* species in chicken at slaughter level; absence of *vanA* and *vanB* genes in *E. faecalis* and *E. faecium. Res Vet Sci,* DOI: 10.1016/ j.rvsc.2010.02.005, 2010.

14.Kariyama R, Mitsuhata R, Chow JW, Clewell DB, Kumon H: Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. *J Clin Microbiol,* 38, 3092-3095, 2000.

15. Cheng S, McCleskey FK, Gress MJ, Petroziello JM, Liu R, Namdari H, Beninga K, Salmen A, DelVecchio VG: A PCR assay for identification of *Enterococcus faecium. J Clin Microbiol,* 35, 1248-1250, 1997.

16. Coque TM, Patterson JE, Steckelberg JM, Murray BE: Incidence of hemolysin, gelatinase, and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from faeces of hospitalized and community-based persons. *J Inf Dis,* 171, 1223-1229, 1995.

17. Willems RJ, Homan W, Top J, Santen-Verheuvel M, Tribe D, Manzioros X, Gaillard C, Vandenbroucke-Grauls CM, Mascini EM, van Kregten E, van Embden JD, Bonten MJ: Variant esp gene as a marker of a distinct genetic lineage of vancomycin-resistant *Enterococcus faecium* spreading in hospitals. *Lancet,* 357, 853-855, 2001.

18. Anon: SPSS, Statistical analysis package program. Version 11.5. Ref. No: 9024147, 2007.

19. Franz CMAP, Silberhorn ABM, Yousif NMK, Vancanneyt M, Swings J, Holzapfel WH: Incidence of virulence factors and antibiotic resistance among enterococci isolated from food. *Appl Environ Microbiol,* 67, 4385-4389, 2001.

20. Poeta P, Costa D, Rodrigues J, Torres C: Detection of genes encoding virulence factors and bacteriocins in fecal enterococci of poultry in Portugal. *Avian Dis,* 50, 64-68, 2006.

21. Poeta P, Costa D, Klibi N, Rodrigues J, Torres C: Phenotypic and genotypic study of gelatinase and β-haemolysis activities in faecal enterococci of poultry in Portugal. *J Vet Med,* 53, 203-208, 2006.

22. Hammerum AM, Jensen LB: Prevalence of esp, encoding the enterococcal surface protein, in *Enterococcus faecalis* and *Enterococcus faecium* isolates from hospital patients, poultry, and pigs in Denmark. *J Clin Microbiol,* 40, 4396, 2002.

23. Khan SA, Nawaz MS, Khan AA, Hopper SL, Jones RA, Cerniglia CE: Molecular characterization of multi-drug resistant *Enterococcus spp.* from poultry and dairy farms: detection of virulence and vancomycin resistance gene markers by PCR. *Mol Cell Probe,* 19, 27-34, 2005.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S135-S139, 2010
DOI:10.9775/kvfd.2010.2509

RESEARCH ARTICLE

Distribution of Intestinal Parasites for Age and Gender in the 13 to 18 Years Age Group at the Niğde Orphanage

Birol ÖZKALP * Battal ÇELİK * Muhammet Güzel KURTOĞLU ** Recep KEŞLİ **

* Department of Medicinal Laboratory, Vocational School of Health Services of Selçuk University, TR-42031 Konya - TURKEY

** Konya Educational and Research Hospital, Microbiology and Clinical Microbiology Laboratory, TR-42031 Konya - TURKEY

Makale Kodu (Article Code): KVFD-2010-2509

Summary

The present study was aimed at the determination of the rate of infection with intestinal parasites in children cared for at the Niğde Orphanage, and the demonstration of the distribution of infection in these children for age and gender. Faecal samples collected from 110 children, aged 13 to 18 years, were examined by the native method using physiological saline. Of the 110 faecal samples examined, 41 (37.3%) contained intestinal parasites, and of the samples containing parasites, 25 (61%) contained helminths and 16 (39%) contained protozoa. The number of faecal samples identified to contain a single parasite species was 39 (95%), while 2 (5%) samples contained more than one parasite species. As regards the distribution of the intestinal parasites for gender, it was ascertained that of the 75 females 30 (40%) individuals and of the 35 males 11 (31.5%) individuals were infected with intestinal parasites. The difference between the two sexes was considered to be insignificant. Faecal examination revealed the presence of the following parasite species at the indicated rates: *Ascaris lumbricoides* (34.1%), *Entamoeba histolytica* (29.3%), *Taenia saginata* (17.1%), *Giardia intestinalis* (9.8%), *Enterobius vermicularis* (7.3%) *Trichuris trichiura* (2.4%). The present study demonstrated that the environment of the orphanage was favourable for autoinfection and the spread of infection by direct contact. Furthermore, it was determined that the rate of parasitic infection was higher in the age group of 13 to 14 years, compared to the age group of 15 to 18 years.

Keywords: *Intestinal parasites, Children, Orphanage, Niğde*

Niğde Yetiştirme Yurdunda 13-18 Yaş Grubunda Görülen Barsak Parazitlerinin Yaşa ve Cinsiyete Göre Dağılımı

Özet

Bu çalışmada yurt çocuklarında barsak parazitlerinin yol açtığı infeksiyon oranlarının ve bu oranların çocuklarda yaş ve cinsiyete göre dağılımın belirlenmesi amaçlanmıştır. Niğde Yetiştirme Yurdunda yaşları 13-18 arasında değişen 110 çocuktan alınan dışkı örnekleri, serum fizyolojik (nativ - salin) yöntemi kullanılarak incelenmiştir. İncelenen 110 dışkı örneğinden 41' inde (%37.3) barsak parazitleri belirlenmiş, bunlardan 25' inin (%61) helmint, 16'sının (%39) protozoon olduğu görülmüştür. Tek tür parazit görülenler 39 (%95), birden çok parazit görülen 2 (% 5) olarak tespit edilmiştir. Parazit görülenlerin cinsiyet dağılımı, 75 kızdan 30'unda (%40), 35 erkekte ise 11(%31.5), olmuştur. Cinsiyetler arasındaki fark, önemsiz olarak kabul edilmiştir. Parazit türleri ise sırasıyla; *Ascaris lumbricoides* (%34.1), *Entamoeba histolytica* (%29.3), *Taenia saginata* (%17.1), *Giardia intestinalis* (%9.8) *Enterobius vermicularis* (%7.3) *Trichuris trichiura* (%2.4)olarak belirlenmiştir. Bu çalışma ile yaşanılan yurt ortamının oto-enfeksiyon ve direkt temasla bulaşmaya elverişli olup parazitlenmenin 13-14 yaş grubunda, 15-18 yaş grubuna oranla daha fazla olduğu tespit edilmiştir.

Anahtar sözcükler: *Barsak parazitleri, Çocuk, Yetiştirme yurdu, Niğde*

İletişim (Correspondence)
☎ +90 332 2231064
✉ ozkalpbirol@hotmail.com

INTRODUCTION

Various factors, including the temperate climate of the country, unfavourable environmental conditions, inadequate sanitary services and education, and insanitation result in predisposition to the establishment and spread of intestinal parasites [1].

In humans, infection with intestinal parasites is a major phenomenon in regions characterized by unfavourable nutritional and climatic conditions, where hygiene is poor and the socioeconomic level is low [2-6]. Furthermore, inadequate sanitation and health checks in environments of collective residence or education, such as student dormitories, lead to the spread of infection [7-11].

Parasitic infections of the gastrointestinal tract are very common across the world, and gastrointestinal parasites are reported as major causes of morbidity and mortality [12,13].

Intestinal parasitosis, which still constitute a major problem in developing countries, are known to cause physical and mental retardation, difficulty in adaptation to the environment, insomnia, fatigue, anaemia and psychological disorders in children [14].

The prevalence of intestinal parasites in a community is a phenomenon closely related to public health, and which is considered to be an indicator of the development level of the community [15]. Parasitic diseases have a significant share in the causes of death in children under the age of five throughout the year across the world. Similarly, in Turkey, intestinal parasites are a major health problem, and it is reported by various researchers that intestinal parasitosis are very common in nurseries, infant schools and schools, where children reside collectively [16-18].

In a multitude of studies conducted in different regions of Turkey, the prevalence (proportion of number of individuals in a population having disease) and incidence (number of new cases of a disease occurring during a certain time period) of parasitic infections, and thereby, the significance of these infections, have been demonstrated [19-25].

The present study was aimed at the determination of the rate of infection with intestinal parasites in certain age groups in children cared for at the Niğde Orphanage, and the demonstration of the distribution of the determined infection rates for age and gender.

MATERIAL and METHODS

Faecal samples collected from 110 children, cared for at the Niğde Orphanage, constituted the material of the study. The age and gender of the children were recorded on the container of each faecal sample. For the collection of faecal samples, sterile, capped containers, which were identified with numbers, were given to the children. The samples collected were transferred to the laboratory for examination. The samples were subjected to both macroscopic and microscopic examination. For this purpose, after native examination using Lugol's solution, the formol-ethyl acetate concentration technique was applied. The specimens prepared for native examination with Lugol's solution were examined with standard light microscopy under x40 magnification, while the specimens prepared for the formol-ethyl acetate concentration technique were examined with standard light microscopy under x10 magnification. Faecal samples were recorded as positive when one or more parasites were observed with light microscopy under x40 magnification [26,27].

For the detection of *E. vermicularis,* the children were applied the cellophane tape method, and the slides were examined with standard light microscopy under x10 magnification. Another method used for diagnosis was the preparation of faecal smears and their staining with trichrome. These smears, which were stained with permanent stains, were examined with standard light microscopy under x100 magnification [27]. Statistical analyses were performed using the Pearson chi-square test.

RESULTS

Of the 110 faecal samples examined with the native method using Lugol's solution, 41 (37.4%) contained intestinal parasites. Of the 41 intestinal parasites detected, 25 (61%) were identified as helminths and 16 (39%) were identified to be protozoa. Two students were concurrently infected with two helminth species. The results of faecal examination and distribution for gender are given in *Table 1,* while the results for the distribution of the parasite species identified are presented in *Table 2*. The distribution of children diagnosed with intestinal parasites for gender is given in *Table 3,* and the

Table 1. *The results of faecal examination and distribution for gender*

Tablo 1. *Dışkı incelemesi sonuçları ve cinsiyet dağılımı*

Variable Gender	Parasites Were Found	Parasites Were Not Established	Total	X^2	P
Girl	30	45	75		
Boy	11	24	35	0.750	0.258
Total	41	69	110		

Table 2. *The distribution of the parasite species*
Tablo 2. *Parazitlerin türlerine göre dağılımı*

Parasite Species	Number	%
Ascaris lumbricoides	14	34.1
Entamoeba histolytica	12	29.3
Taenia saginata	7	17.1
Giardia intestinalis	4	9.8
Enterobius vermicularis	3	7.3
Trichuris trichiuraü	1	2.4
Total	41	100

distribution of parasites for age groups is presented in *Table 4*. Of the children diagnosed with parasitic infection, 30 (73.2%) were female and 11 (26.8%) were male. Of the children determined to be infected with intestinal parasites, 10 (24.3%) were aged 13 years, while 13 (31.8%) were aged 14 years, and 8 (19.5%) were aged 15 years. Furthermore, 5 (12.2%) were 16 years old, 3 (7.3%) were 17 years old, and finally, 2 (4.9%) were of the age 18.

Table 3. *The distribution of diagnosed parasites for gender*
Tablo 3. *Parazit tespit edilenlerin cinsiyete göre dağılımı*

Gender	Number	%
Girl	30	73.2
Boy	11	26.8
Total	41	100

Table 4 . *The distribution of parasites for age groups*
Tablo 4. *Yaş gruplarına göre parazitlerin dağılımı*

The Group of Ages	Number	%
13	10	24.3
14	13	31.8
15	8	19.5
16	5	12.2
17	3	7.3
18	2	4.9
Total	41	100

DISCUSSION

In cases of intestinal parasitosis, factors directly affecting the incidence of infection are level of education, food habits, traditions and customs, and socioeconomic and medicosocial living standards. In addition, geography, climate, meteorology and infrastructure are other factors influential on parasitosis. The above mentioned factors are highly variable in Turkey, and thereby, naturally, cases of parasitosis vary between different regions of the country [3] . It has been reported that the prevalence of intestinal parasitosis ranges from 10% to 96% in the community, and that the socioeconomic level of provinces and parasite incidence are inversely proportional to each other [8,9]. Similar to reports on the insignificance of the difference between females and males in parasite distribution for gender in foreign countries, no statistical significance has been reported in Turkey for parasitic infections in females and males, yet, it has been pointed out to the rates of infection being close to each other in the two sexes [27]. The present study investigated and demonstrated the distribution of intestinal parasites for gender, yet, it was determined that no statistically significant difference existed between females and males.

Previously conducted research has shown that the prevalence of intestinal parasites in children in Turkey ranges from 13% to 66% [1]. Furthermore, surveys conducted for parasitosis in primary school children in Turkey has demonstrated that the incidence ranges from 11.1% to 98.8% [11].

In a study, in which 607 primary school students, 307 (48.5%) of which were girls and 300 (51.5%) were boys, were applied the cellophane tape method, it was determined that 16.14% were infected with *E. vermicularis* [28].

In another study conducted in patients, who were referred to the clinics of the Faculty of Medicine of Cumhuriyet University with diarrhoea, the prevalence of intestinal protozoa was determined as 26% [29]. Furthermore, of 2351 faecal samples examined at the Parasitology Laboratory of the Faculty of Medicine of Kocaeli University, 257 (10.93%) contained parasites [30].

In a study conducted in Burdur province for the investigation of the species, prevalence and socio-demographic and behavioural patterns of intestinal parasites in primary school children, the researcher collected 172 answers to a questionnaire, cellophane tape specimens and faecal samples from schools. The prevalence of intestinal parasites in the children included in the study was determined as 8.1%. The most frequently observed parasite species were *E. vermicularis* and *G. intestinalis*. The total rate of infection was 12.1% in boys and 3.7% in girls. The highest prevalence of intestinal parasites was observed in the children aged 11 years. Gender, average age and number of rooms were determined to be markedly correlated with parasitosis [31].

In a study carried out in the Mother and Child Health and Family Planning Centre of Isparta province, 566 faecal samples (277 of which were taken from girls and 289 from boys) were examined for the presence of

parasites. Parasites were detected in 383 of the samples (121 of which belonged to girls and 262 to boys). Two hundred and sixty two of the cases of parasitic infection were in preschool children aged 0-6 years, and the most common parasite species was *E. vermicularis* [32].

Of the 3628 patients, who were referred to the clinics of the Faculty of Medicine of Dokuz Eylul University in the year 2003, 237 (6.53%) and of the 4084 patients, who were referred to the clinics in the year 2004, 258 (6.31%) were diagnosed with parasitic infection [27].

In a study conducted in primary school children in the city centre of Malatya province, of the 1838 students examined, 415 (22.5%) were diagnosed with parasitic infection. The most common parasite species was *E. vermicularis* (10.6%) [33].

In a study carried out in 110 higher education students in Van province, 34 (30.9%) were determined to be infected with at least one parasite species [34].

Doğan et al, in a study carried out at two different orphanages in children aged 0-6 years and 7-12 years, reported the prevalence of parasitic infection to be 51% and 54% in the 0-6 and 7-12 age groups, respectively [35].

Durmaz et al.[7] in a study in which they investigated the prevalence of intestinal parasites in primary school and orphan children in Malatya province, examined faecal samples belonging to 94 orphan children and 560 primary school children aged 7-11 years. Of the orphans, 89.4% were diagnosed with parasitic infection and the most frequently observed parasite was *E. vermicularis*. The rate of intestinal parasitosis was 77.1% in the primary school children with the most common parasite species being *G. lamblia*.

Yılmaz et al.[11] reported that of the 3505 children aged 0-13 years, who were referred to the Parasitology Laboratory of the Faculty of Medicine of 100. Yıl University in the year 1996, 779 were diagnosed with parasitic infection. The researchers determined that the most common parasites species were *E. histolytica* and *A. lumbricoides*.

In Niğde province, Topçu and Uğurlu [10] have reported on the species associated with parasitic infection in primary schools with different conditions, and have also demonstrated the distribution of thse parasites for age, gender and socioeconomic level.

In the present study the incidence of parasitosis in children cared for at the Niğde Orphanage was determined as 37.1%. In particular, due to the cellophane tape method not being able to be used for the diagnosis of *E. vermicularis* infections, it is considered that values lower than the actual parasitosis incidence may have been obtained. In the present study, 61% of the parasitosis cases were determined to be caused by protozoa, while 39% were ascertained to be caused by helminths.

As observed in the present study, differences in the aforementioned factors influence the incidence and prevalence of parasitosis. The species *A. lumbricoides*, which is very common in Eastern, South-eastern and Central Anatolia, was also determined at a high rate in this study. Factors, which determine the occurrence of parasites, are frequently observed in locations where people reside collectively, such as orphanages. In such places, age is observed to be a more influential factor. In view of the wealth of information gained to date, for the community to overcome this problem, it is essential that public awareness is raised and each individual takes responsibility. Appropriate sanitation and food habits should be established and maintained. Medical staff appointed for such services should be well-educated. The significance of preventive medicine should be communicated to the public. Accordingly, to achieve this target, the relationship between medical staff and families should be strengthened. Furthermore, all departments, units, institutions and organizations involved in public service should fulfil their responsibilities with a high level of awareness. In view of the fact that the source of many parasites is humans and that cysts excreted in the faeces are transmitted by the faecal-oral route, adequate importance should be attached to infrastructure and environmental health services. Responsible institutions and organizations should cooperate in harmony. Prevailing infections should be eliminated and prevented through the treatment of infected persons and the improvement of environmental conditions. In other words, treatment and prevention should be implemented together. In locations, where prevalence and incidence rates are already high or are expected to be high, people should be subjected to regular health checks, surveys should be conducted and people should be educated with a view to raise public awareness. In terms of preventive medicine and treatment, the education of the public and the raising of public awareness bear great significance.

REFERENCES

1. Yorulmaz M, Durmaz R, Saygı G: Malatya ili Tecde yöresinde 5-15 yaş grubu çocuklarda parazit sıklığı ve buna çevresel faktörlerin etkisi. *T Parasitol Derg,* 21 (2): 153-158, 1997.

2. Erdemir F, Özçelik S, Saygı G: *Enterobius vermicularis*'li çocukların annelerinin sorunu tanımlama, sorunu çözme ve sağlık personelinden aldıkları yardım özelliklerinin saptanması. *T Parazitol Derg,* 16 (2): 46-52, 1992.

3. Kasım H, Ay YD, Oğuz MC, Öztürk MO, Coşkun ŞZ: Bursa yöresi ilkokul çocuklarında gastro-intestinal parazitlerin yayılışı. *T Parazitol Derg,* 20 (2): 191-197, 1996.

4. Öztürkcan S, İçağasıoğlu D, Yalçın AN, Saygı G: Sivas Çocuk Yuvasında intestal parazitler ve deri lezyonları arasındaki ilişki. *T Parazitol Derg,* 18 (3): 308-312, 1994.

5. Saygı G, Kirişçi MF: İlkokul kız öğrencilerinde selofan bant preparatı ile *Enterobius vermicularis* ve *Taenia saginata* infeksiyonlarının araştırılması. *T Parazitol Derg,* 14 (3-4): 77-84, 1990.

6. Söyletir G, Doğanay M: İnfeksiyon Hastalıkları. Nobel Tıp Kitabevleri, s. 630-641, 1996.

7. Durmaz B, Yakıncı C, Rafia M, Durmaz R: Malatya'daki ilkokul ve yetiştirme yurdu çocuklarında barsak parazitlerinin prevalansı. *T Parazitol Derg,* 21 (4): 391-394, 1997.

8. Saygı G: Son yirmi yılda barsak parazitleri ile ilgili olarak yapılan yayınların incelenmesi. *T Parazitol Derg,* 16 (3-4): 161-189, 1992.

9. Taşçı S, Balcıoğlu İC: Manisa Celâl Bayar Üniversitesi Tıp Fakültesi Sağlık Uygulama ve Araştırma Merkezinde 1995 yılında saptanan barsak parazitlerinin değerlendirilmesi. *T Parazitol Derg,* 20 (4): 383-387, 1996.

10. Topçu A, Uğurlu K: Niğde ve yöresinde ilkokul çocuklarında görülen barsak parazitlerinin yaşa, cinsiyete ve sosyo-ekonomik duruma göre dağılımı. *T Parazitol Derg,* 23 (3): 281-290, 1999.

11. Yılmaz H, Cesur Y, Özkaya E, Gödekmerdan A, Gül A: Yüzüncü Yıl Üniversitesi Tıp Fakültesi Parazitoloji Laboratuarına başvuran 0-13 yaş grubu çocuklarda barsak parazitlerinin dağılımı. *T Parazitol Derg,* 21 (4): 347-350, 1997

12. Kuman HA, Altıntaş N: Protozoon Hastalıkları. 1. Baskı, s. 27-28, Ege Üniversitesi Matbaası, Bornova-İzmir, 1996.

13. Unat EK, Yücel A, Atlaş K, Samaası M: Unat'ın Tıp Parazitolojisi. 5. Baskı, İstanbul Üniv. Cerrahpaşa Tıp Fakültesi Yayınları, 1995.

14. Dickson R, Awasthi S, Williamson P, Demellweek C, Garner P: Effects of treatment for intestinal helminth infection ongrowth and cognitive performance in children: Systemic review of randomised trials. *Br Med J,* 320, 1697-1701, 2000.

15. Ak M, Ok Ü, Gürüz Y, Turgay N, Kırağı D, Özcel MA: İzmir Şirinyer Çocuk Islahevi hükümlü ve personelinde barsak parazitlerinin araştırılması. *T Parazitol Derg,* 19 (2): 243-248, 1995.

16. Mandell GL, Douglas G, Bennett JE: Principles and Practice Infection Disease. 3rd ed., pp. 380-393, New York Churchill Liwingstone Inc, 1994.

17. Markel EK, Voge M, John DT: Medical Parasitology: Sings and Symptoms of Pparasitic Disease. 7th ed., 380-393, W. B. Sounders Co, Philedelphia, 1992.

18. Unat EK, Yücel A, Altaş K, Samastı M: Unat'ın Tıp Parazitolojisi. s. 19-49, İstanbul Üniv. Yayınları, 1995.

19. Aşçı Z, Yılmaz M, Ay S, Barlas HH: Harput Çocuk Yuvası 6-12 yaş grubu çocuklarında parazitolojik incelemeler. *T Parazitol Derg,* 15 (3-4): 83-87, 1991.

20. Aşçı Z, Seyrek A, Kizirgil A, Yılmaz A: Harput Çocuk Yuvasındaki 13-18 yaş grubu çocuklarda parazitolojik araştırma. *T Parazitol Derg,* 21 (2): 169-171, 1997.

21. Ay S, Yılmaz M, Aşçı Z, Barlar HH, Yücal A: Fırat Üniversitesi Tıp Fakültesi Mikrobiyoloji Laboratuarına başvuran hastalarda barsak parazitlerinin dağılımı. *T Parazitol Derg,* 15 (3-4): 88-91, 1990.

22. Dardal N: Parazitoloji laboratuarına başvuran kişilerde barsak parazitlerinin dağılımı. *T Parazitol Derg,* 9 (1-2): 5-11, 1986.

23. Köseoğlu V, Yakıncı C, Durmaz B, Akın R: İnönü Üniversitesi Tıp Fakültesi ve Malatya Askeri Hastanesi Çocuk Polikliniklerine başvuran 0-7 yaş grubu çocuklarda barsak parazitlerinin dağılımı. *T Parazitol Derg,* 16 (3-4): 115-121, 1992.

24. Özçelik S, Sümer Z, Çeliksöz A, Poynaz Ö, Koçoğlu F, Demirtaş S, Saygı G: Sivas Çocuk Yuvasındaki çocuklarda barsak parazitlerinin incelenmesi. *T Parazitol Derg,* 19 (2): 254-257, 1995.

25. Şimşekcan D, Ersöz V, Toker R, Coşkun S, Keskin M: İzmir Bölge Hıfzısıhha Enstitüsü Parazitoloji Laboratuarına başvuran 651 hastada barsak parazitlerinin dağılımı. *T Parazitol Derg,* 15 (3-4): 58-66, 1991.

26. Unat GK: Tıp Helmintolojisi Ders Kitabı. 2. Baskı, İstanbul Üniv Tıp Fak Yay, No: 474, İstanbul.

27. Usluca S, Yalçın G, Över L, Tuncay S, Şahin S, İnceboz T, Aksoy Ü: Dokuz Eylül Üniversitesi Tıp Fakültesi Araştırma ve Uygulama Hastanesi'nde 2003-2004 yılları arasında saptanan bağırsak parazitlerinin dağılımı. *T Parazitol Derg,* 30 (4): 308-312, 2006.

28. Özcan S, Özcan H, Sönmez E, Yazar S: Kayseri'de dört ilköğretim okulundaki öğrencilerde *Enterobius vermicularis* yaygınlığının araştırılması. *T Parazitol Derg,* 28 (1): 24-26, 2004.

29. Eren ŞH, Oğuztürk H: İshalli kişilerde bağırsak protozoonlarının prevalansı. *Cukurova Univ Tıp Fak Derg,* 27 (1): 11-14, 2005.

30. Gülden ST, Şeyda Ç, Ayşe W: Kocaeli Üniversitesi Tıp Fakültesi Hastanesi Parazitoloji Laboratuvarına başvuran hastalarda bağırsak parazitlerinin dağılımı. *T Parazitol Derg,* 32 (2): 126-129, 2008.

31. Kılınç AS: Burdur il merkez ilköğretim okullarındaki çocuklarda bağırsak parazitlerinin görülme sıklığı. *Yüksek Lisans Tezi*. Suleyman Demirel Üniv Sağ Bil Enst, Isparta, 2006.

32. Aydın S, Yaylı G: Ana Çocuk Sağlığı ve Aile Planlaması Merkezine başvuran çocuklarda barsak parazitleri sıklığı. *Klimik Dergisi,* 15 (2): 54-55, 2002.

33. Çelik T, Daldal N, Karaman Ü, Aycan ÖM, Tambay M: Malatya ili merkezinde üç ilköğretim okulu çocuklarında bağırsak parazitlerinin dağılımı. *T Parazitol Derg,* 30 (1): 35-38, 2006.

34. Yetkin A, Değer S, Özdal N: Intestinal parasites in the students of Van Health High School and Faculty of Veterinary Medicine. *Kafkas Univ Vet Fak Derg,* 16 (1): 81-84, 2010.

35. Doğan N, Akgün Y, Akşit F, Şengül M: Toplu halde yaşayan çocuk ve yaşlı bakımevlerinde farklı yöntemlerle yapılan kopro-parazitolojik inceleme. *T Parazitol Derg,* 17 (3-4): 48-56, 1993.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S141-S146, 2010
DOI:10.9775/kvfd.2009.2555

RESEARCH ARTICLE

Evaluation of Ticks Biting Humans in Thrace Province, Turkey

Ayşen GARGILI * | Sırrı KAR ** | Nadim YILMAZER **
Çiğdem CERİT *** | Gülden SÖNMEZ **** | Fikret ŞAHİN *****
Hatice GÜNSELİ ALP ****** | Zati VATANSEVER *******

* Marmara Üniversitesi, Enfeksiyon Hastalıkları ve Epidemiyoloji Araştırma Merkezi, TR-34660 İstanbul - TÜRKİYE
** Namık Kemal Üniversitesi, Biyoloji Bölümü, TR-59100 Tekirdağ - TÜRKİYE
*** Kırklareli Sağlık Müdürlüğü, Bulaşıcı Hastalıklar Bölümü, TR-39020 Kırklareli - TÜRKİYE
**** Edirne Sağlık Müdürlüğü, Bulaşıcı Hastalıklar Bölümü, TR-22030 Edirne - TÜRKİYE
***** Tekirdağ Sağlık Müdürlüğü, Bulaşıcı Hastalıklar Bölümü, TR-59100 Tekirdağ - TÜRKİYE
****** Pendik Veteriner Kontrol ve Araştırma Enstitüsü, Parazitoloji Teşhis Laboratuarı, TR-34893 İstanbul - TÜRKİYE
******* Kafkas Üniversitesi, Veteriner Fakültesi, TR-36100 Kars - TÜRKİYE

Makale Kodu (Article Code): KVFD-2009-2555

Summary

This study examined 1478 tick samples taken from humans who applied to the hospitals with tick bites in the provinces of Tekirdag, Kirklareli and Edirne in Thrace region of Turkey between May and November, 2007. The samples were evaluated in terms of species, developmental stage, gender, season and locality. The ticks were identified as Hyalomma spp. (76.05%), *Rhipicephalus* spp. (11.71%), *Ixodes* spp. (10.55%), *Haemaphysalis* spp. (1.48%), *Dermacentor* spp. (0.07%), *Ornithodoros* spp. (0.07%), and *Argas* spp. (0.07%). Majority of the tick samples were *Hyalomma aegyptium* nymphs (68.54%). According to the hospital application dates, tick bite cases occurred in August at most (30.11%). Tick bites by *Ixodes* spp. were abundant in the Black Sea coasts of the region. Based on localities, majority of the cases were reported from the urbanized areas (61.30%).

Keywords: *Tick, Thrace, Turkey, Human*

Trakya İllerinde İnsanları Tutan Kenelerin Değerlendirilmesi

Özet

Bu çalışmada, 2007 yılı Mayıs ve Kasım ayları arasında, Trakya Yöresindeki Tekirdağ, Kırklareli ve Edirne illerinde kene tutunma şikayeti ile hastanelere başvuran insanlardan alınan 1478 kene örneği incelenmiştir. Örnekler tür, gelişim dönemi, cinsiyet, tutunma zamanı ve bölgesi yönünden değerlendirilmiştir. Teşhis edilen keneler *Hyalomma* spp. (%76.05), *Rhipicephalus* spp. (%11.71), *Ixodes* spp. (%10.55), *Haemaphysalis* spp. (%1.48), *Dermacentor* spp. (%0.07), *Ornithodoros* spp. (%0.07) ve *Argas* spp. (%0.07)'dir. En fazla *Hyalomma aegyptium* nimflerine (%68.54) rastlanmıştır. Hastaneye başvuru zamanı bakımından, kene tutunma olguları en çok Ağustos ayında (%30.11) gerçekleşmiştir. *Ixodes* spp. kaynaklı olgular bölgenin Karadeniz kıyısında yoğunlaşmıştır. Olguların büyük çoğunluğu (%61.30) şehirsel alanlardan gelmiştir.

Anahtar sözcükler: *Kene, Trakya, Türkiye, İnsan*

INTRODUCTION

Distributed worldwide, there are some 899 described tick species (Argasidae, Ixodidae and Nuttalliellidae consisting of 185, 713 and 1 species, respectively), 10% of which significantly impact human and animal health, by transmitting pathogens of around 200 diseases [1,2].

İletişim (Correspondence)
☎ +90 216 3999371
✉ aysen.gargili@marmara.edu.tr

Ticks exhibit varying degrees of host selection, depending on species and developmental stages. However, they use different animals as host when they could not quest for their own appropriate host. Although there is not a known tick species which tends to complete its life cycle on humans, it is reported that around 200 tick species, can attach to and feed on humans. Among these, 28 species can play a direct role in transmission of several diseases [3,4].

Risk of tick attachment and related tick-borne diseases in a given region are certainly related to vector tick abundance and human activities. Also, geographic and climatic conditions, and presence and habitat preferences of host animals are closely related with the risk factors [4-6].

In the present study, tick bite cases were evaluated in terms of tick species, seasonal activity and characteristics of locality in Thrace region between May and November, when the most tick species were active, in 2007.

MATERIAL and METHODS

Description of the Study Area

Thrace region constitutes the European part of Turkey. It is bounded on the west by Greece and Bulgaria, on the east by Bosphorus strait, on the north by the Black Sea, on the south by the Sea of Marmara, and on the southwest by the Aegean Sea. Of the study provinces, Tekirdag is situated on the northern coast of the Sea of Marmara, Kirklareli is located along the Bulgarian border and on the west coast of the Black Sea, and Edirne which is in Eastern Thrace bordering the Aegean Sea to the south, Bulgaria to the north and Greece to the west. Large plains and hilly surfaces occupy the interior and south parts of the region. Thrace has high mountains to the north, which run parallel to the Black Sea, and are densely forested. The second largest longos forest in Europe is in this region. Vegetation is composed of steppe predominantly, and maquis in places, and forests in highlands, while the interior and south parts of the region contain cultivable areas. The diverse and transitional weather conditions are the results of the interplay of the different climatic zones in the region; coasts are affected by the climate of the sea nearby, whereas inland exhibits continental climate to some degree. The summers are generally hot (average temperature 27°C) and moderately rainy, except the Black Sea coast receiving high precipitation. The winters are cold (average temperature 4°C) and rainy, especially inland experiencing heavy snow and freezing temperatures below 0°C in places. Total population of the region is 1.502.358 (Tekirdag: 521.554; Edirne: 262.039; Kirklareli: 218.071), and population density is 79.23 per km^2 (Tekirdag: 122.09 per km^2; Edirne: 64.72 per km^2; Kirklareli: 51.44 per km^2) in 2008. The percentage of the total population living in urban areas is 66.67%, while rural population is 33.33%.

Tick Collection and Identification

Ticks were obtained from humans who applied to the regional hospitals with complaints of tick bites between the dates of 01.05.2007 and 30.11.2007. Ticks were removed from the patients by the physicians and sent to our laboratory for identification. Adult ticks were identified to the species level, while larvae and nymphs to the genus level with the exception of *Hyalomma aegyptium* nymphs which could be identified to the species level. Identification was done using taxonomic keys [7-10].

Determination of Seasonal and Regional Distribution of Tick Bites

Tick bites were recorded for each case, based on species and developmental stage of the tick, and date and place of the attachment. From the anamnesis records, possible attachment localities were further determined, and those localities were classified as rural and urban areas.

RESULTS

Data on Tick Species and Developmental Stages

A total of 1478 tick samples were obtained from the overall tick bite cases. Based on developmental stages and numbers, distribution of the species is given in Table 1. In most cases, ticks were in the nymphal stage (74.77%), while adults (23.54%) and larvae (1.69%) succeeded respectively *(Table 1)*.

Data on Localities Where Tick Bites Occurred

Of 1478 cases, 572 were reported from rural areas including villages, farms, grasslands and picnic sites (38.70%), whereas 906 from urban areas (61.30%). While *H. aegyptium* nymphs led to cases of bites in the latter mainly (72.26%), adults of *H. marginatum, H. aegyptium, R. bursa* and *I. ricinus,* and nymphs of *Ixodes spp.* bite humans in rural areas (76.67%, 53.57%, 68.18%, 71.43% and 71.43%, respectively). The number of biting cases by *Rhipicephalus sanguineus* group ticks *(R. sanguineus* and *R. turanicus*) was almost the same for urban and rural areas (51.46% and 58.54% respectively). Tick species, of which number of samples was less than 25, were not taken into account for this evaluation *(Table 2)*.

Table 1. *Species, developmental stages and numbers of the ticks detached from humans*
Tablo 1. *İnsanlardan toplanan kenelerin tür, gelişme dönemi ve sayıları*

Total	Number of Larvae	Number of Nymphs	Number of Adults	Total
Hyalomma spp.	16	-	-	16
H. marginatum	-	-	60	60
H. aegyptium	-	1013	28	1041
H. detritum	-	-	7	7
Ixodes spp.	9	84	-	93
I. ricinus	-	-	63	63
Rhipicephalus spp.	-	4	-	4
R. sanguineus gr.	-	-	103	103
R. bursa	-	-	66	66
Haemaphysalis spp.	-	4	-	4
H. parva	-	-	15	15
H. punctata	-	-	2	2
H. sulcata	-	-	1	1
Dermacentor marginatus	-	-	1	1
Ornithodorus sp.	-	-	1	1
Argas sp.	-	-	1	1
Total	25	1105	348	**1478**

Of the 156 *Ixodes spp.* collected, 120 (76.92%) occurred in Kirklareli located on the west coast of the Black Sea, whereas 29 (18.59%) and 7 (4.49%) in Tekirdag and Edirne, respectively, the former being situated on the coast of the Sea of Marmara, and the latter being in the inner of the region. *Table 2* summarizes the related data.

Seasonal Distribution of Cases

During the period from May to November, cases of tick bites were reported each month. Most cases were occurred between June and September, reaching to a peak with 445 cases (30.11%) in August. Larval infestations of *Hyalomma spp.* were occurred between May and September, mostly in June and July with the

Table 2. *Cases of tick bites by species, type of locality and provinces*
Tablo 2. *Tür, tutunma yeri tipi ve illere göre kene tutma olguları*

Tick Species	Tick Numbers				
	Type of Locality		The Provinces		
	Rural Area	Urban Area	Tekirdag	Kirklareli	Edirne
Hyalomma spp. (Larvae)	6	10	9	5	2
H. marginatum	46	14	13	24	23
H. aegyptium (Nymph)	281	732	558	236	219
H. aegyptium	15	13	9	12	7
H. detritum	3	4	1	4	2
Ixodes spp. (Larva)	6	3	3	6	0
Ixodes spp. (Nymph)	60	24	11	68	5
I. ricinus	45	18	15	46	2
Rhipicephalus spp. (Nymph)	2	2	1	3	0
R. sanguineus gr.	50	53	48	38	17
R. bursa	45	21	29	27	10
Haemaphysalis spp. (Nymph)	1	3	2	2	0
H. parva	8	7	11	3	1
H. punctata	1	1	2	0	0
H. sulcata	1	0	1	0	0
Dermacentor marginatus	0	1	0	1	0
Ornithodorus sp. (Adult)	1	0	0	1	0
Argas sp. (Adult)	1	0	0	1	0
Total (1478)	572 (38.70%)	906 (61.30%)	713 (48.24%)	477 (32.27%)	288 (19.49%)

case numbers 6 and 7, respectively. Infestations with *H. aegyptium* nymphs were reported between May and October, and there was a peak in August (394 cases) and September (391 cases), while adults of this species were recorded between June and September with a peak of 13 cases in June. *H. marginatum* adults were seen between May and August, and July showed a peak with 29 cases. Little cases by nymphs of *Haemaphysalis spp.* were occurred between July and September. Fifteen (15) cases of *H. parva* infestation were seen predominantly in autumn months, October being the month in which 7 cases occurred. *Rhipicephalus spp.* were recorded between May and September, especially in June and July. Adults of *I. ricinus* infestations were recorded each month during the study period, and both adults and nymphs were predominant in June and July. *Table 3* summarizes the related data.

Table 3. *Cases of tick bites by months*
Tablo 3. *Aylara göre kene tutma olguları*

Tick Species	Tick Numbers by Months						
	May	June	July	August	September	October	November
Hyalomma spp. (Larva)	1	6	7	1	1	0	0
H. marginatum (Adult)	3	22	29	6	0	0	0
H. aegyptium (Nymph)	1	25	148	394	391	54	0
H. aegyptium (Adult)	0	13	9	4	2	0	0
H. detritum (Adult)	1	3	1	2	0	0	0
Ixodes spp. (Larva)	1	0	5	3	0	0	0
Ixodes spp. (Nymph)	9	41	32	2	0	0	0
I. ricinus (Adult)	6	37	8	4	3	2	3
Rhipicephalus spp. (Nymph)	0	1	1	1	1	0	0
R. sanguineus gr. (Adult)	11	60	23	8	1	0	0
R. bursa (Adult)	1	23	24	16	2	0	0
Haemaphysalis spp. (Nymph)	0	0	1	2	1	0	0
H. parva (Adult)	1	1	0	1	1	7	4
H. punctata (Adult)	0	0	0	0	2	0	0
H. sulcata (Adult)	0	0	0	0	1	0	0
D. marginatus (Adult)	0	0	0	1	0	0	0
Ornithodorus sp. (Adult)	0	1	0	0	0	0	0
Argas sp. (Adult)	0	0	1	0	0	0	0
Total (1478)	35	233	289	445	406	63	7
(%)	(2.37)	(15.76)	(19.55)	(30.11)	(27.47)	(4.26)	(0.47)

DISCUSSION

Throughout Turkey, 32 tick species (28 ixodids and four argasids) have been found so far; of these, 20 species are found in Marmara Region involving Thrace [11]. However, the present study reported 13 tick species from three provinces in Thrace. *Boophilus annulatus (R. annulatus)* and *Hyalomma anatolicum* which are known to frequently attach to livestock in the study area [11] were not encountered in biting cases evaluated with this study. This may suggest that these species indicate high host selectivity, or they do not tend to bite humans. The degree of host selection of *B. annulatus,* a one-host tick found most often on cattle, is known to be high [12]. Thus, it is more likely that this species prefers their primary host, cattle which are abundant in the study area, rather than humans and other animal hosts.

Each tick species possesses a special activity season, and activity period may differ partly, depending on weather conditions and geographical characteristics. Species of Dermacentor and Haemaphysalis are active in early Spring and late Autumn, while *Rhipicephalus spp.* appear in humid and warm months, and Ixodes species become active in cool and humid months [13]. This study revealed that most of tick biting cases occurred in hot summer months, especially in August. Overlap of periods in which a given tick species is active and humans enter the habitat of that tick is one of the most important factors which determines the possibility of human-tick contact. When considering that humans occupy grasslands, pastures and picnic sites in warm seasons, it is clear why Hyalomma species which are active in hot months cause most of biting cases.

According to the species, and even developmental stage, most of the ticks prefer a certain group of hosts. In contrast to adults larvae and nymphs exhibiting less host selectivity and readily attach to rodents and birds [12,14]. With regard to developmental stages, nymphs were

found to be dominant in this study (74.77%), and succeeded by adults and larvae (23.54% and 1.69%, respectively). Additionally, tendency of each species, even each developmental stage to bite human is quite different from each other. While 28 *H. aegyptium* adults were found infesting humans, the number of its' nymphs found on humans was 1013. Similarly, *Ixodes spp.* nymphs found outnumbered the adults (84 and 63, respectively). On the other hand, adults of Rhipicephalus and Haemaphysalis were numerous, and there were no nymphs of *H. marginatum*. Considering the biology of the latter which is a two-host tick, it is not surprising that there were no nymphs at all. But, one cannot say the same thing for Rhipicephalus and Haemaphysalis species which are three-host ticks, since there may be several different factors affecting the attachment of immature forms of these ticks to humans. 1013 *H. aegyptium* nymphs constituted the biggest portion (68.54%) of total ticks collected during the study. However, adults of this species were less in number (28 adults composing 1.89% of total ticks). Another interesting finding is that nymphs were mostly from urban areas (72.26%), whereas adults from rural areas (53.57%). It is reported that *H. aegyptium,* a three-host tick, uses turtles, other reptiles and mammals as host [8], turtles are the primary host of each of three developmental stages [15], being the first preference of the adults [16]. Larvae and nymphs may prefer also other reptiles, mammals and birds rather than turtles [7,8]. Conserving natural structure to some extent (eg. small city parks, cemetaries), urban areas in the study area possess turtles overly. This situation and the current literature may explain the abundance of *H. aegyptium* nymphs leading to human infestations. Yet, outnumbered nymphs compared to adults may be correlated to the density in environment and flexibility in host selection. However, these cannot be explanation for the adults causing biting cases in rural areas. Our observations showed that turtles in urban areas tend not to leave their limited habitat. Thereby, instead of attempting to find different hosts, huge and crawling Hyalomma adults [9,10] readily find turtles, which travel wide distances, to feed on. But, larvae and nymphs are not easy to find a host, and use the first animal which they find as host without showing host selection. Nevertheless, these assumptions cannot explain why biting cases caused by larvae of both *H. aegyptium* and other species were less in number, compared to the nymphs. On the other hand, larvae possibly prefer small animals rather than humans as host since it will be difficult for them to crawl up bigger hosts. In addition, due to their small size, larvae attached to human body might be overlooked.

Although *R. bursa* caused more biting cases in rural areas (68.18%), the number of the cases by *R. sanguineus* group was almost the same in rural and urban areas (48.54% and 51.46% respectively). The reason for this may be related to the fact that ticks of *R. sanguineus* group are three-hosted and prefer dogs and some other animals [9], and population of stray dogs is enough in both areas to support population of these ticks. On the other hand, *R. bursa* is a two-host species preferring ruminants, and its biology is mostly restricted to rural areas.

Hyalomma marginatum is the known vector of Crimean-Congo hemorrhagic fever in Turkey [17]. This species was reported not to prefer humid areas, and to feed on animals, exhibiting a tendency to use humans as host [4]. Our results also showed that *H. marginatum* is abundant in relatively dry and rural parts of the study area.

Ixodes spp. were seen to be abundant mainly in Kirklareli where rainy Black Sea climate prevails, and partially in neighboring Tekirdag. In fact, this situation is quite accordant with the known bioecological characteristics of the ticks in the genus Ixodes which is well adapted to rainy and cooler geographical areas [13]. Unlike to the fact that nymphs of *Ixodes spp.* are widely seen in Autumn in the regions where these ticks are commonly found [13], both adults and nymphs of *Ixodes spp.* were observed mainly in June and July in our study. Considering that the present study was a passive survey, rather than the actual annual distribution of this tick, the situation can be related to the dense human activity in the natural habitat of this species in summer months.

66.67% and 33.33% of the people live in urban and rural areas respectively in the study area, and the percentages of biting cases were 61.30% for urban and 38.70% for rural areas. As seen from the data, the population density and tick bite cases in the study area are quite parallel to each other.

Factors such as temperature, humidity, precipitation, vegetation and altitude affect activities of ticks substantially [18]. Furthermore, tick infestation, vector potential of the relevant ticks and the accompanying problems are closely connected to some factors including host species, habitat and geographic conditions [4-6,19]. In conclusion, our findings confirmed by current literature reveal that there are many factors which determine level of risk to humans resulting from tick attachment and tick-borne diseases.

REFERENCES

1. Jongejan F, Uilenberg G: The global importance of ticks. *Parasitol,* 129, 3-14, 2004.

2. Labuda M, Nuttall PA: Tick-borne viruses. *Parasitol,* 129, 221-45, 2004.

3. Anderson JF, Magnarelli LA: Biology of ticks. *Infect Dis Clin North America,* 22, 195-215, 2008.

4. Estrada-Peña A, Jongejan F: Ticks feeding on humans: A review of records on human-biting Ixodoidea with special reference to pathogen transmission. *Exp Appl Acarol,* 23, 685-715, 1999.

5. Vatansever Z, Gargili A, Aysul NS, Sengoz G, Estrada-Pena A: Ticks biting humans in the urban area of Istanbul. *Parasitol Res,* 102 (3): 551-553, 2008.

6. Qiu W, Dykhuizen DE, Acosta MS, Luft BJ: Geographic uniformity of the Lyme disease spirochete *(Borrelia burgdorferi)* and its shared history with tick vector *(Ixodes scapularis)* in the Northeastern United States. *Genetics,* 160, 833-849, 2002.

7. Apanaskevich DA: The diagnostics of *Hyalomma (Hyalomma) aegyptium* (Acari: Ixodidae) (in Russian). *Parazitologiia,* 37 (1): 47-59, 2003.

8. Apanaskevich DA: Host-parasite relationships of the genus *Hyalomma* Koch (Acari, Ixodidae) and their connection with microevolutionary processes (in Russian). *Parazitologiia,* 38, 515-523, 2004.

9. Estrada-Peña A, Bouattour A, Camicas JL, Walker AR: Ticks of domestic animals in the Mediterranean region. A guide of identification of species. University of Zaragoza Press, Zaragoza, p. 131, 2004.

10. Walker AR, Bouattour A, Camicas JL, Estrada-Peña A, Horak IG, Latif AA, Pegram RG, Preston PM: Ticks of domestic animals in Africa. A guide to identification of species. Bioscience Reports, Scotland, UK, 2003.

11. Aydin L, Bakirci S: Geographical distribution of ticks in Turkey. *Parasitol Res,* 101 (2): 163-166, 2007.

12. Sonenshine DE: Biology of Ticks. Volume 2. Oxford University Press, p. 488, 1993.

13. Gray JS: Biology of Ixodes species in relation to tick-borne zoonoses. *Wien Klin Wochenschr,* 114, 473-478, 2002.

14. Valenzuela JG: Exploring tick saliva: from biochemistry to 'sialomes' and functional genomics. *Parasitol,* 129, 83-94, 2004.

15. Siroky P, Petrzelkova KJ, Kamler M, Mihalca AD, Modry D: *Hyalomma aegyptium* as dominant tick in tortoises of the genus Testudo in Balkan countries, with notes on its host preferences. *Exp Appl Acarol,* 40, 279-290, 2006.

16. Petney TN, Al-Yaman F: Attachment sites of the tortoise tick *Hyalomma aegyptium* in relation to tick density and physical conditions of the host. *J Parasitol,* 71, 287-289, 1985.

17. Ergonul O: Crimean-Congo haemorrhagic fever. *Lancet Infect Dis,* 6 (4): 203-214, 2006.

18. Urquhart GM, Armour J, Duncan JL, Dunn AM, Jennings FW: Veterinary Parasitology. 179-181, Churchill Livingstone Inc, New York, 1987.

19. Randolph SE: How does tick ecology determine risk? http://www.lymediseaseaction.org.uk/conf2008/randolph.pdf, 2008. *Accessed:* 15 January 2010.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S147-S152, 2010
DOI:10.9775/kvfd.2010.2646

RESEARCH ARTICLE

Ege ve Marmara Denizi'nde Avlanılan Midye ve İstiridyelerin Felç Yapıcı ve İshal Yapıcı Kabuklu Su Ürünü Toksinleri Yönünden Araştırılması [1]

Sadık KÜÇÜKGÜNAY * Ali BİLGİLİ **

[1] *Bu çalışma doktora tezinden güncellenerek özetlenmiştir*
* Et ve Balık Kurumu Genel Müdürlüğü Sincan Et Kombinası, TR-06930 Ankara - TÜRKİYE
** Ankara Üniversitesi Veteriner Fakültesi, Farmakoloji ve Toksikoloji Anabilim Dalı, TR-06110 Ankara - TÜRKİYE

Makale Kodu (Article Code): KVFD-2010-2646

Özet

Araştırma, Ege ve Marmara Denizi'nden avlanılan midye ve istiridyelerde felç yapıcı ve ishal yapıcı kabuklu su ürünü toksinlerinin bulunup bulunmadığını, varsa insan ve hayvan sağlığı açısından risk oluşturacak düzeylerde olup olmadığını, hangi bölgelerde ve hangi mevsimlerde rastlanıldığını ortaya koymak amacıyla yapıldı. Analizler Mayıs, Temmuz ve Kasım aylarında toplanan 72 grup midye ve istiridye örneği üzerinde yapıldı. Yöntem olarak bu tip zehirlenmelerin tespitinde en güvenilir test olarak kabul edilen "fare biyolojik metot" kullanıldı. Analiz sonuçlarına göre ishal yapıcı kabuklu su ürünü toksinlerine hiç rastlanmadığı, felç yapıcı kabuklu su ürünü toksinlerinin ise İzmir ve Balıkesir'de ilkbahar sonu ve yaz aylarında bulunabileceği, ancak insan ve hayvan sağlığı açısından risk oluşturacak miktarlarda olmadığı görüldü.

Anahtar sözcükler: *Dinofisistoksin, Fare biyolojik metot, İstiridye, Midye, Okadaik asit, Saksitoksin*

The Investigation of The Presence of Paralytic and Diarhetic Shellfish Toxins in Commercially-Cought Mussels and Oysters from Aegian and Marmara Seas

Summary

The aim of this study was to research whether there were paralytic and diarhetic shellfish toxins in mussels and oysters that were commercially-cought from Aegian and Marmara Seas, and in case of presence, to evaluate whether these toxins were hazardous for human health, and to assert the seasons and regions in which these toxins were abundant. Samples from 72 group of mussels and oysters which were collected in May, July and October were analyzed in the study. 'Mouse Biological Method', which was accepted as the most certain test for such toxication tests, was used as the main method. The data showed that diarhetic shellfish toxins were not found in any region, but paralytic shellfish toxins may be found in İzmir and Balıkesir during summer and at the end of spring. In addition, the amount of toxins did not carry risk for human health.

Keywords: *Dinophysistoxin, Mouse biological method, Mussel, Oyster, Ocadaic acide, Saxitoxin*

GİRİŞ

Dinoflagellate türleri, çok değişik türde doğal toksin üretebilir. Algler tarafından da üretilebilen bu toksinlerle kontamine kabuklu su ürünlerinin tüketilmesi sonucu, felç yapıcı kabuklu su ürünü zehirlenmesi (FKZ), nörotoksik kabuklu su ürünü zehirlenmesi (NKZ), amnezik kabuklu su ürünü zehirlenmesi (AKZ), ishal yapıcı kabuklu su ürünü zehirlenmesi (İKZ), ciguatera balık zehirlenmesi ve azaspirasid kabuklu su ürünü zehirlenmesi meydana gelir [1].

İletişim (Correspondence)
☎ +90 312 2701932
✉ skgunay@hotmail.com

Kontamine kabuklu su ürünlerini tüketen insanlarda görülen zehirlenmeler sonucunda FKZ toksinleri tespit edilmiştir [2]. Aynı toksinler tatlı su siyanobakterilerince de üretilmekte olup [2,3], bu toksinlerle kirlenmiş suları içen insan ve hayvanlarda da sağlık açısından istenmeyen etkiler meydana getirmektedir [3]. Tüm dünyaya dağılım göstermiş olan bu organizmalar içme su kaynaklarında da yaygın olarak bulunmaktadır. Zehirli toksinlerin içme sularındaki düzeyleri kabuklu su ürünlerindekinden daha düşüktür [2].

FKZ toksinleri; ısıya ve soğuğa [4], asit ortama, pişirme ve haşlama işlemlerine dayanıklı, bazik ortamda ise dayanıksızdır. Tetrodotoksin gibi guanidium bileşiğidirler [5,6] ve tetrohidropurin yapısı içerirler [7,8]. *Protogonyaulax* türü dinofilagellatalardan orijin alan saksitoksin, gonyatoksin 1, gonyatoksin 2, gonyatoksin 3, gonyatoksin 4 [9], gonyatoksin 5, gonyatoksin 6, gonyatoksin 7 ve neosaksitoksin olarak anılan dokuz farklı tip izole edilmiştir. En zehirlileri saksitoksin ve gonyatoksin 3'dür [8,10-13]. Öldürücü dozları türe ve canlının ağırlığına göre değişir [12]. Toksinler, sinir ve kas hücre zarlarında sodyum kanallarını bloke ederek sodyum geçişini [5,6,14-16] ve çevresel sinir iletimini engellerler [7,17]. Solunum felcine [18,19], solunum güçlüğüne, yüz felcine [20] ve kan basıncının düşmesine sebep olurlar [6,7].

Kabuklu su ürünleri, toksin üreten mikroorganizmaları taşımak suretiyle zehirlenmeye sebep olurlar [21,22]. İnsanlardaki öldürücü dozu 0.3-1 mg kadardır [9,10,22-24]. Su ürünlerinin yenilebilir et kısmı için belirlenen en düşük saksitoksin miktarı 80 µg/100 g'dır [17,25-32].

İKZ'leri gıda zehirlenmelerinin özel bir tipidir. Bu zehirlenmeyle ilgili Dinophysis türü dinoflagellatalardan orijin alan [17] okadaik asit, dinofisistoksinler ve pektenotoksinler ve yessotoksinler olarak anılan 4 farklı toksin grubu saptanmıştır [28].

Bu grup toksinler, mide bağırsak ve iskelet sistemi üzerine etkilidirler [30]. Farelere periton içi yolla uygulanan pektenotoksinlerin karaciğer nekrozuna, yessotoksinlerin ise kalp kasında hasar yaptığı tespit edilmiştir. Azaspirasidler dahil [28], toksinlerin tümü mide-bağırsak kanalı belirtileriyle karakterize ishal yapıcı kabuklu su ürünü zehirlenmesine sebep olurlar [31]. Tolerans düzeyi 0-60 µg/100 g'dır [29].

Fare biyolojik metot, toksisitenin belirlenmesi için kullanılan en güvenilir testtir ve bu test bir kontaminanttan daha fazlasının değerlendirilmesini de sağlar. Antikor ölçümlerine dayanan testleri de içine alan diğer birçok metot, dönüşüm faktörlerini kullanarak hesaplanan toksisitenin hangi toksin yoğunluklarında olduğunun tahminini sağlamaktadır [32]. Etik ve teknik nedenler fare deneylerine alternatif olarak yeni tekniklerin bulunmasını teşvik etmiştir. Fakat antikor ve saksitoksin çipleri kullanılarak yapılan yeni testlerin çok fazla iş yükü getirdiği belirtilmiştir [27]. Kabuklu deniz ürünlerinde FKZ toksinlerini izleme şu anda uluslararası akredite "Association of Official Analytical Chemists (AOAC)" fare biyolojik metodu kullanılarak yapılmaktadır. Fare biyolojik metodunun FKZ toksinlerinin analizleri için kullanılmasının gerekliliği Avrupa Komisyonu direktiflerinde de belirtilmiştir [33].

FKZ toksinlerinin fare biyolojik metotla analizlerinde sonuçlar; 100 g kabuklu su ürünü etinde µg saksitoksin olarak hesap edilir ve saksitoksin tespiti için elde edilen süzüntünün fareye enjeksiyonundan sonra bir saat süreyle fare ölümlerinin gözlenmesi ile değerlendirilir [26,34]. Süzüntü işlemi genellikle 0.1 N HCl ile gerçekleştirilir ve pH değeri tercihen 2.5-3'e ayarlanır. PH ayarlaması için HCl asit (5N HCl) ve NaOH (0.1 N NaOH) kullanılır. Örnek 5 dak kaynatılır, sonra soğutulur ve tekrar pH ayarlaması yapılır [34,35]. İshal yapıcı kabuklu su ürünü toksinlerinin fare biyolojik metotla analizlerinde sonuçlar; kabuklu su ürünü etinden elde edilen süzüntülerin farelere enjeksiyonundan sonra 24 saat süreyle fare ölümlerinin gözlenmesi ile değerlendirilir [34,36].

Bu çalışma ile, Ege ve Marmara Denizi'nden avlanılan midye ve istiridyelerde felç yapıcı ve ishal yapıcı kabuklu su ürünü toksinlerinin bulunup bulunmadığının, varsa insan ve hayvan sağlığı açısından risk oluşturacak düzeylerde olup olmadığının, hangi bölgelerde ve hangi mevsimlerde rastlanıldığının ortaya konulması amaçlandı.

MATERYAL ve METOT

Çalışmada, 1999 yılında Ege ve Marmara Denizi'nden avlanılan midye ve istiridyeler kullanıldı. Midye ve istiridye örnekleri, Ege Denizi'nin İzmir, Balıkesir (Ayvalık) ve Çanakkale sahilleri ile Marmara Denizi'nin Çanakkale, Balıkesir (Bandırma) ve İstanbul sahillerinden, Mayıs, Temmuz ve Kasım aylarında ikişer defa olmak üzere her noktadan toplam altı kez toplandı. Kış mevsiminde midye ve istiridye avlanılamadığı için örnek toplanmadı. Toplanan her grup örnek en az ellişer adet olmak üzere toplam 36 grup midye ve 36 grup istiridye örneği toplandı.

Toplanan bu örnekler, 18°C'de muhafaza edilerek analizleri 1999 yılı içerisinde tamamlandı ve yerel etik kurul ilkelerine uyuldu.

FKZ Toksin Tespiti

Midye ve istiridye örneklerinin ayıklanan 150 g kadar et kısmı parçalandıktan sonra, 100 g örnek bir behere

tartıldı. Üzerine 100 ml 0.1 N HCI asit ilave edilerek iyice karıştırıldı. Karışım, pH metre ile pH'sı 2.5'a ayarlandı. Hafif ateşte beş dakika kaynatıldı. Oda sıcaklığına kadar soğutuldu. pH'sı tekrar 2.5'a ayarlandı. Karışım, dereceli kaba aktarılarak distile su ile 200 ml'ye tamamlanarak karıştırıldı. Santrifüj tüplerine bir miktar alındı ve 3000 devirde beş dakika santrifüj edildi. Her süzüntüden, 18-21 ağırlığındaki üç adet test faresine periton içi yolla birer ml enjekte edildi. Fareler birer saat gözlem altında tutuldu. Ölüm görülen örneklerde 0-80 μg/100 g miktarlarında FKZ toksinlerinin bulunduğu tespit edildi.

İKZ Toksin Tespiti

Midye ve istiridye örneklerinin iç kısımlarından 20-25 g bir cam balona tartıldı. Örnek, bir parçalayıcıda parçalandı ve sonra 20 g tartıldı. Tartılan 20 g örnek üzerine 100 ml aseton ilave edilerek, karıştırıcı vasıtasıyla beş dakika karıştırıldı. 500 ml'lik bir balon içerisinde üstteki sıvı kısmı süzgeç kağıdı yardımıyla süzülerek alındı. Bu işlem üç defa yapıldı. Elde edilen süzüntü (yaklaşık 300 ml) 40-50°C'de bir evaporatorda buharlaştırıldı. Asetonik faz tamamen buharlaştırıldıktan sonra 15 ml distile su ile tekrar süspanse edildi. 100 ml'lik ayırma hunisinde 50 ml etil eter ile süzüntü işlemi gerçekleştirildi. Eter ile su fazının süzüntü işlemi 2 kez tekrar edildi. Eter süzüntüleri 1 balon içinde toplandı. Evaporatorda buharlaştırıldı. Son kalıntıya 4 ml %1'lik Tween 60 ilave edilerek, %1'lik Tween 60 içinde çözdürüldü. Her süzüntüden, 18-21 g ağırlığındaki üç adet test faresine periton içi yolla birer ml enjekte edildi. Fareler, 24 saat gözlem altında tutuldu. Ölüm görülen örneklerde 0-80 μg/100 g miktarlarında İKZ toksinlerinin bulunduğu tespit edildi.

BULGULAR

FKZ toksini bulunup bulunmadığının ortaya konulması için; Ege Denizi, İzmir kıyılarından Temmuz ayında toplanan 2 grup midye ve Mayıs ayında toplanan ikinci grup istridye örneğinden elde edilen süzüntülerin farelere enjeksiyonu sonucunda her 1 gruptan birer fare ölümü görüldü. Balıkesir kıyılarından, Mayıs ayında ikinci defa toplanan midye ve Temmuz ayında toplanan birinci grup istiridye örneğinden elde edilen süzüntünün farelere enjeksiyonu sonucunda birer fare ölümü görüldü. Çanakkale kıyılarından toplanan midye ve istiridye örneklerinin analizinde hiçbir fare ölümü görülmedi. Marmara Denizi, Balıkesir kıyılarından Temmuz ayında toplanan birinci grup midye ve istiridye örneklerinden elde edilen süzüntülerin farelere enjeksiyonu sonucunda birer fare ölümü görüldü. Toplanan diğer midye ve istiridye örneklerinden elde edilen süzüntülerin farelere enjeksiyonu sonucunda hiçbir fare ölümü görülmedi *(Tablo 1)*.

İKZ toksini bulunup bulunmadığının ortaya konulması için; midye ve istiridye örnekleri üzerinde yapılan analizlerde farelere yapılan enjeksiyonlar sonucunda hiçbir fare ölümü görülmedi *(Tablo 2)*.

TARTIŞMA ve SONUÇ

Kabuklu su ürünü zehirlenmesi tüm dünyanın bir problemidir. Dünya genelinde proteinli besin maddesi ihtiyacının artması ile su ürünlerine yönelimin hız kazanması nedeniyle bu zehirlenmelere maruz kalma riski de artmaktadır. Ciddi planlama, düzenli olarak kabuklu su

Tablo 1. *Ege ve Marmara Denizi'nden toplanan midye ve istiridye örneklerindeki saksitoksin bulguları (ölen fare sayısı/süzüntü uygulanan fare sayısı)*

Table 1. *Paralytic shellfish toxin findings in mussel and oyster samples obtained from Aegian and Marmara Seas (died mouse number/mouse number processed extraction)*

Örnek Çeşidi	Örnek No	Ege Denizi			Marmara Denizi			Toplam
		İzmir	Balıkesir	Çanakkale	Çanakkale	Balıkesir	İstanbul	
Ölen fare/süzüntü uygulanan fare Midye	Mayıs-1	0/3	0/3	0/3	0/3	0/3	0/3	0/18
	Mayıs-2	0/3	1/3	0/3	0/3	0/3	0/3	1/18
	Temmuz-1	1/3	0/3	0/3	0/3	1/3	0/3	2/18
	Temmuz-2	1/3	0/3	0/3	0/3	0/3	0/3	1/18
	Kasım-1	0/3	0/3	0/3	0/3	0/3	0/3	0/18
	Kasım-2	0/3	0/3	0/3	0/3	0/3	0/3	0/18
Ölen fare/süzüntü uygulanan fare İstiridye	Mayıs-1	0/3	0/3	0/3	0/3	0/3	0/3	0/18
	Mayıs-2	1/3	0/3	0/3	0/3	0/3	0/3	1/18
	Temmuz-1	0/3	1/3	0/3	0/3	1/3	0/3	2/18
	Temmuz-2	0/3	0/3	0/3	0/3	0/3	0/3	0/18
	Kasım-1	0/3	0/3	0/3	0/3	0/3	0/3	0/18
	Kasım-2	0/3	0/3	0/3	0/3	0/3	0/3	0/18
Toplam	**12**	**3/36**	**2/36**	**0/36**	**0/36**	**2/36**	**0/36**	**7/216**

Tablo 2. *Ege ve Marmara Denizi'nden toplanan midye ve istiridye örneklerindeki İKZ toksin bulguları (Ölen fare/Süzüntü uygulanan fare)*

Table 2. *Diarhetic shellfish toxin findings in mussel and oyster samples obtained from Aegian and Marmara Seas (Died mouse number/Mouse number processed extraction)*

Örnek Çeşidi	Örnek Sayısı	Ege Denizi			Marmara Denizi			Toplam
		İzmir	Balıkesir	Çanakkale	Çanakkale	Balıkesir	İstanbul	
Midye	6	0/18	0/18	0/18	0/18	0/18	0/18	0/108
İstiridye	6	0/18	0/18	0/18	0/18	0/18	0/18	0/108
Toplam	**12**	**0/36**	**0/36**	**0/36**	**0/36**	**0/36**	**0/36**	**0/216**

ürünlerinde toksin düzeylerinin izlenmesi ve yaygın halk sağlığı programları ile bu tür zehirlenmelerin önüne geçilebilir [14].

FKZ toksinlerinin tespitinde kullanılan ELISA, yüzey plazmon rezonans biosensör deneyleri ve fare deneyleri gibi testlerin kullanımı, son kullanıcının amaçlarına yönelik olarak seçilip kullanılmalıdır [37]. Kromatografik metotlar her zaman toksisitenin güvenilir indikatörü değildir. Fare deneyi AB direktiflerinde kabuklu su ürünlerinin analizlerinde kullanılan geçerli metot olarak yer almıştır [33].

İKZ toksinlerin identifikasyonu amacıyla 2002, 2006 ve 2007 yıllarında zehirlenme olgularında toplanan numunelerde fare deneyi, yüksek performanslı likid kromatografisi ve protein fosfataz 2A inhibisyon testleri kıyaslanmış ve uygulamalar sonrası bu testler arasındaki korelasyonların çok yüksek olduğu saptanmıştır [38-40].

Ham İKZ toksinleri üzerinde 1978-1982 yıllarında Osaka'da meydana gelen zehirlenmelerde rol oynayan deniztarağından asetonla ekstraksiyon yapılarak izole edilen dinofisistoksin 1, dinofisistoksin 3, pektenetoksin 1 ve okadaik asit gibi toksinlerle süt emen fare ve tavşanlar üzerinde çeşitli çalışmalar yapılmış, ince bağırsak incelemesinde epitelyum hasarı ve lamina propriada ödem görülmüştür [31].

Bu tip zehirlenmelerin daha çok Mayıs ve Eylül ayları arasında ve kırmızı renk değişikliği (red-tide) olayının meydana geldiği denizlerde görüldüğü bildirilmektedir [6,7,12,16]. Shanghai'da yapılan bir çalışmada kontamine örneklerin hep Mayıs, Haziran ve Temmuz aylarında olduğu tespit edilmiştir [41].

Örnekleme planında kabukluların zehirliliği ihmal edilebilir bir düzeyden öldürücü bir düzeye bir haftadan daha kısa sürede çıkabileceği göz önünde bulundurulmalıdır. Midyeler için bu süre 24 saatten bile kısa olabilir. Zehirlilik seviyesi hayvanın yaşadığı coğrafik bölgeye, su akıntısına ve denizdeki renk değişikliği olaylarına göre değişiklik gösterir [29].

Ege ve Marmara Denizi kıyılarında, literatür kayıtlarında belirtildiği gibi dünyanın çeşitli bölgelerinde meydana gelen kırmızı renk değişikliği olayları pek fazla görülmemektedir. Sadece İzmir Körfezi'nde *Protogonyaulax tamarensis*'in sebep olduğu varsayılan renk değişikliği az da olsa meydana gelmektedir [12].

Tablo 1 ve *2*'de görüldüğü şekilde FKZ toksini bulunup bulunmadığının ortaya konulması için yapılan çalışmalarda, Ege Denizi kıyılarından toplanan midye ve istiridye örneklerinin analizi sonucunda sadece Mayıs ve Temmuz aylarına ait 4 grupta (2 grup midye ve 2 grup istiridye) birer fare ölümü ve Marmara Denizi kıyılarından toplanan midye ve istiridye örneklerinin analizi sonucunda sadece Temmuz ayına ait 2 grupta birer fare ölümü görülmüş ve FKZ toksin varlığı yönünden tüm sonuçlar negatif olarak değerlendirilmiştir. İKZ toksini bulunup bulunmadığının ortaya konulması için ilkbahar, yaz ve sonbahar mevsimlerinde toplanan 36 grup midye ve 36 grup istiridye örneğinden elde edilen süzüntülerin farelere enjeksiyonu sonucunda fare ölümü görülmemiş ve tüm sonuçlar İKZ toksin varlığı yönünden negatif olarak değerlendirilmiştir.

Sonuç olarak Marmara ve Ege Denizi'nden temin edilen midye ve istiridye örneklerinin hiç birinde İKZ toksin bulunmadığı, aynı bölgelerden alınan örneklerden yapılan analizler sonucunda örneklerin bir kısmında FKZ toksin bulunabileceği, ancak bu düzeylerin insan sağlığı açısından risk oluşturacak düzeyde olmadığı sonucuna varıldı. FKZ toksin varlığı yönünden, bölgesel olarak İzmir ve Balıkesir kıyıları ile mevsimsel olarak da ilkbahar ve yaz mevsimi dikkat çekmektedir.

Midye ve istiridyelerin değişik zaman dilimlerinde farklı miktarlarda toksin içermeleri, bazılarının sadece gelişme dönemlerinde toksin taşımaları ve bunu kısa süre içerisinde vücutlarından atmaları, bazılarının ise toksini uzun yıllar bünyelerinde taşıyabilmeleri nedeniyle özellikle sahillerimizden temin edilen balık ve kabuklu su ürünleri tüketimi aşamasında meydana gelebilecek zehirlenmelerin iyi takip ve kontrolü için, renk

değişikliği olayı görülen sahillerin tespiti ve toplum sağlığı için etkin izleme programları kapsamında bu toksinlerin analizleri yapılmalıdır.

KAYNAKLAR

1. Wang DZ: Neurotoxins from marine dinoflagellates: A brief review. *Mar Drug,* 6 (2): 349-371, 2008.

2. Humpage AR, Magalhaes VF, Froscio SM: Comparison of analytical tools and biological assays for detection of paralytic shellfish poisoning toxins. *Anal Bioanal Chem,* [Epub ahead of print], 2010.

3. Humpage AR, Ledreux A, Fanok S, Bernard C, Briand JF, Eaglesham G, Papageorgiou J, Nicholson B, Steffensen D: Application of the neuroblastoma assay for paralytic shellfish poisons to neurotoxic freshwater cyanobacteria: Interlaboratory calibration and comparison with other methods of analysis. *Environ Toxicol Chem,* 26 (7): 1512-1519, 2007.

4. Ng PKL, Chia DGB, Koh EGL, Tan LWH: Poisonous Malaysian crabs. *Nature- Malaysiana,* 17, 4-9, 1992.

5. Eastaugh J, Shepherd S: Infectious and toxic syndromes from fish and shellfish consumption. *Arch Intern Med,* 149, 1735-1740, 1989.

6. Sakamoto Y, Lokey R, Krzanovski J: Shellfish and fish poisoning related to the toxic dinofilagellates. *South Med J,* 80, 860-870, 1987.

7. Noble RC: Death on the half - shell: The health hazards of eating shellfish. *Perspect Biol Med,* 33 (3): 313-322, 1990.

8. Sousa JVB, Bragana MB: Neurotoxins in bivalve molluscs and their transformation products. *Alimentaria,* 222, 29-41, 1991.

9. Asakawa M, Miyazamawa K, Noguchi T: Studies on paralytic shellfish poison (PSP) oxification of bivalwes, in association with appearance of *Alexandrium tamarensis* in Hirosima bay, Hirosima perfecture. *J Food Hyg Soc Japan,* 34, 50-54, 1993.

10. Hungerfod JM, Wekell MM: Analytical methods for marine toxins. *Food Poisoning,* 7 (16): 415-473, 1992.

11. Kelly GJ, Hallegraff GM: Dinofilagellate toxins in Australian shellfish. *Toxins and Targets,* 1-9, 1992.

12. Koray T: Az bilinen doğal bir afet: Kırmızı renkli deniz ve neden olduğu zehirlenmeler. *Bilim ve Teknik Derg,* Aralık, 9-14, 1988.

13. Lassus P, Bardouil M, Ledoux M, Murail I, Bohec M: Paralytic phycotoxin uptake by scallops *(pecten maximus). Aquatic Living Res,* 5 (4): 319-324, 1992.

14. Smart D: Clinical Toxcology of Shellfish Poisoning. **In,** Meier J, White J (Eds): Clinical Toxicology of Animals Venoms and Poisons. pp. 33-53. CRC Press, Inc. New York, London, Tokyo, 1995.

15. Özay G: Bazı deniz kabuklularında saksitoksin (paralytic shellfish poison) kontaminasyonu ve insan sağlığı açısından taşıdığı riskler. *Gıda Sanayi Derg,* 6 (1): 16-24, 1992.

16. Smith DS, Kitts DD: Development of a monoclonal-based ensyma-linked immunoassay for saxitoxin-induced protein. *Toxicon,* 32 (3): 317-322, 1993.

17. Schulze K: Shellfish poisoning caused by algal toxins. *Berl Münch Tierarztl Wshr,* 98, 383-387, 1985.

18. Saldate-Castaneda O, Vasquez-Castellaos JL, Galvan J, Sanchez-Anguiano A, Nazar A: Poisoning from paralytic shellfish toxins in Oaxaca, Mexico. *Salud Publica-de-Mexico,* 33, 240-247, 1991.

19. Tanado K, Yasuda K, Ushiyama H, Nishima T: Effect of calcium on the mouse bioassay method for paralytic shellfish poison (hygienic studies on health food (III). *J Food Hyg Soc Japan,* 32, 402-407, 1991.

20. Wu CH, Huang JMC, Vogel SM, Atchison VD, Narahashi, T: Actions of *Ptychodiscus brevis* toxins on nerve and muscle membranes. *Toxicon,* 23, 481-487, 1985.

21. Citterio B, Manzano M, Maifreni M, Comi G: Natural fish and shellfish poisons. *Ann Microbiol Enzimol,* 42, 203-216, 1992.

22. Luckas B, Thielert G, Peters K: The problem of the selective estimation of PSP toxins in mussels. *Zeitschift Lebensmitt Untersuch Forch,* 190, 491-495, 1990.

23. Ellenhorn MJ, Barceloux DG: Medical Toxicology. Elsevier Science Publishers Company Inc, USA, 1988.

24. Pirinçci İ: Deniz ürünlerinde bulunan toksinler. **In,** Kaya S (Ed): Veteriner Klinik Toksikoloji. s. 352-353, Medisan, Ankara, 1995.

25. Aran N: Gıda Kaynaklı Mikrobiyel Toksinler. *Gıda Sanayi Derg,* 7 (1): 31-46, 1993.

26. Pac: Paralytic Shellfish Poison. http:/www.pac.dfo.mpo.gc.ca/ops/fm/shellfish/ Biotoxins/PSP. *Accessed:* 06.03.2000.

27. Fonfría ES, Vilariño N, Campbell K, Elliott C, Haughey SA, Ben-Gigirey B, Vieites JM, Kawatsu K, Botana LM: Paralytic shellfish poisoning detection by surface plasmon resonance-based biosensors in shellfish matrixes. *Anal Chem,* 79 (16): 6303-6311, 2007.

28. Dominguez H J, Paz B, Daranas AH, Norte M, Franco JM, Fernández JJ: Dinoflagellate polyether within the yessotoxin, pectenotoxin and okadaic acid toxin groups: Characterization, analysis and human health implications. Toxicon, 2009 ([Epub ahead of print]).

29. Huss HH: Biotoxin. Assurance of sea food quality. *FAO Fisheries Tecnical Paper.* p. 334, 1994.

30. Vale C, Botana LM: Marine toxins and the cytoskeleton: okadaic acid and dinophysistoxins. *FEBS J,* 275 (24): 6060-6066, 2008.

31. Hamano Y, Kinishita Y, Yasumoto T: Studies on diarrhetic shellfish toxins. Enteropathogenicity of diarrhetic shellfish toxins in intestinal models. *J Food Hyg Soc Japan,* 27, 375-379, 1996.

32. Laycock MV, Donovan MA, Easy DJ: Sensitivity of lateral flow tests to mixtures of saxitoxins and applications to shellfish and phytoplankton monitoring. *Toxicon,* 55 (2-3): 597-605, 2010.

33. Campbell K, Stewart LD, Doucette GJ, Fodey TL, Haughey SA, Vilariño N, Kawatsu K, Elliott CT: Assessment of specific binding proteins suitable for the detection of paralytic shellfish poisons using optical biosensor technology. *Anal Chem,* 79 (15): 5906-5914, 2007.

34. Anon: Su Ürünleri Kalite Kontrol El Kitabı. Tarım ve Köy İşleri Bakanlığı. Ankara, 1996.

35. Quillam MA: AOAC mouse bioassay for PSP toxins. http://www.maritimes.dfo.ca/science/mesd/he/lists/phycotoxins-o/ms00541. *Accessed:* 06.03.2000.

36. WHO: Aquatic (Marine and Fresh Water) Biotoxins. *Enviromental Health Criteria, 37th World Health Organisation.* Geneva. 1984.

37. Campbell K, Huet AC, Charlier C, Higgins C, Delahaut P, Elliott CT: Comparison of ELISA and SPR biosensor technology for the detection of paralytic shellfish poisoning toxins. *J Chromatogr B Analyt Technol Biomed Life Sci,* 877 (32): 4079-4089, 2009.

38. Prassopoulou E, Katikou P, Georgantelis D, Kyritsakis A: Detection of okadaic acid and related esters in mussels during diarrhetic shellfish poisoning (DSP) episodes in Greece using the mouse bioassay, the PP2A inhibition assay and HPLC with fluorimetric detection. *Toxicon,* 53 (2): 214-227, 2009.

39. Mouratidou T, Kaniou-Grigoriadou I, Samara C, Kouimtzis T: Detection of the marine toxin okadaic acid in mussels during a diarrhetic shellfish poisoning (DSP) episode in Thermaikos Gulf, Greece, using biological, chemical and immunological methods. *Sci Total Environ,* 366 (2-3): 894-904, 2006.

40. Louppis AP, Badeka AV, Katikou P, Paleologos EK, Kontominas MG: Determination of okadaic acid, dinophysistoxin -1 and related esters in Greek mussels using HPLC with fluorometric detection, LC-MS/MS and mouse bioassay. *Toxicon,* 55 (4): 724-733, 2010.

41. Wu JY, Zheng L, Wang JH: Contamination of shellfish from Shanghai seafood markets with paralytic shellfish poisoning and diarrhetic shellfish poisoning toxins determined by mouse bioassay and HPLC. *Food Addit Contam,* 22 (7): 647-651, 2005.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S153-S158, 2010
DOI:10.9775/kvfd.2010.2654

RESEARCH ARTICLE

Chemical and Microbiological Quality of the *Chamelea gallina* from the Southern Coast of the Marmara Sea in Turkey [1]

Fatma ARIK ÇOLAKOĞLU * Hasan Basri ORMANCI * İbrahim Ender KÜNİLİ *
Serhat ÇOLAKOĞLU **

[1] *The present study was carried out with financial support of COMU BAP project 2007/52*
* Canakkale Onsekiz Mart University Faculty of Fisheries, Terzioglu Kampüsü, TR-17100 Canakkale - TURKEY
** Ministry of Agriculture and Rural Affairs, Provincial Directorates for Protection and Control, TR-17100 Canakkale - TURKEY

Makale Kodu (Article Code): KVFD-2010-2654

Summary

The aim of this work was to analyze *Chamelea gallina* harvested in the Sea of Southern Marmara in terms of heavy metal, biotoxin, and microbiological contents. Samples were collected seasonally from five stations which were determined to be their natural habitats in the between February 2008 and January 2009. Heavy metal contents of the samples revealed statistically significant ($P<0.05$) differences between seasons and stations. In two stations, Pb and Zn content of the clams were determined to exceed legal limits imposed by Turkish Fisheries Regulation and EC Shellfish Hygiene Directive (91/492/EEC). Biotoxin was not detected in any sample analyzed and the counts of *Escherichia coli* and fecal coliform bacteria of the samples were lower than the legal limits. *Salmonella* spp. was not detected in the sampling stations, whereas *Vibrio parahaemolyticus* was isolated in two stations. In conclusion, clams harvested in the southern Marmara Sea, excluding Gelibolu and Karabiga stations, were found suitable for human consumption.

Keywords: *Chamelea gallina, Heavy metals, Biotoxin, Microbiological quality, Marmara Sea*

Marmara Denizi'nin Güney Kıyılarındaki *Chamelea gallina*'ların Kimyasal ve Mikrobiyolojik Kalitesi

Özet

Bu çalışmada, güney Marmara Denizi'nden toplanan *Chamelea gallina*'ların ağır metal, biyotoksin ve mikrobiyolojik içerikleri tespit edilmiştir. Örnekler Şubat 2008-Ocak 2009 tarihleri arasında doğal yatakların bulunduğu toplam 5 istasyondan mevsimsel olarak temin edilmiştir. Örneklerin ağır metal içerikleri, mevsimler ve istasyonlar arasında istatistiksel olarak önemli derecede farklılık göstermiş olup ($P<0.05$), iki istasyonda, Pb ve Zn içeriklerinin Türkiye Su Ürünleri Yönetmeliği ve AB Kabuklu Hijyen Direktifi (91/492/EEC)'ne göre, limit değerlerin üzerinde olduğu belirlenmiştir. Hiçbir örnekte biyotoksin tespit edilmemiştir. Mikrobiyolojik analiz sonuçlarına göre, örneklerin *Escherichia coli* ve fekal koliform bakteri içeriklerinin yasal sınırların altında olduğu saptanmıştır. *Salmonella* spp. örnekleme istasyonlarında saptanamamış, *Vibrio parahaemolyticus* ise iki istasyondan izole edilmiştir. Sonuç olarak, Marmara Denizi'nin güneyinden toplanan beyaz kum midyeleri, Gelibolu ve Karabiga istasyonları hariç, tüm istasyonlarda insan tüketimine uygun bulunmuştur.

Anahtar sözcükler: *Chamelea gallina, Ağır metaller, Biyotoksin, Mikrobiyolojik kalite, Marmara Denizi*

INTRODUCTION

Chamelea gallina, also known as 'striped venus', is one of the most harvested and demanded species of bivalves and spreads across the coasts of the Black and Mediterranean Seas [1]. In Turkey, striped venus exists mostly in the coasts of the western Black Sea and the Marmara Sea [2,3] and commercial use began in 1986 [2]. As the demand increased, Turkey became the leading producer country in 2007, producing 58.3% of the world's total production [4]. Turkey was followed by Italy, Spain, and France [4].

İletişim (Correspondence)
☎ +90 286 2180018/1562
✉ arikfatmaa@yahoo.de

There are some risks affecting production quality of bivalves. These risks are mostly caused by the natural accumulation of heavy metal, biotoxin, and micro-organisms in bivalves due to their filter-feeding system [5-8].

Heavy metals, biotoxins and microorganisms were naturally present in waters [5,9] and their amounts could increase as a result of direct or indirect effects of human activities [9]. Heavy metal concentration of water is generally affected by the waste products of industry, mining and shipping operations; however, micro-organism concentration of water is affected by household and industrial wastes. Biotoxins are generated by the metabolic activities of single-celled algae [10,11]. Although these toxins are not harmful to bivalves, they could be led to serious diseases such as Amnesic Shellfish Poisoning (ASP), Azaspiracid Shellfish Poisoning (AZP), Diarrhetic Shellfish Poisoning (DSP), Paralytic Shellfish Poisoning (PSP), Neurotoxic Shellfish Poisoning (NSP) and even death in human [5,9,10,12]. Presence of algae species that produce biotoxins in Turkish seas was reported [13]; however, no biotoxin event except for DSP was reported from the Marmara Sea [14].

Mentioned risk factors must be lower than the national and international legal limits for the production and consumption of clams [15].

In this study, seasonal samplings were made in Marmara Sea, one of the most important habitats for striped venus, to analyze heavy metal, biotoxin and microbiological contents of striped venus, and their suitability for human consumption was evaluated.

MATERIAL and METHODS

Sample Collection

Striped venus samples were collected from pre-determined stations on the Northern coast of the Sea of Marmara and were located, from east to west direction, in Karabiga, Kemer, Sevketiye (Anatolian side), Bolayıraltı and Gelibolu (European side), respectively *(Fig. 1)*. Samples were collected using dredge from 5 to 10 m of the littoral zones in the above mentioned stations between February 2008 and January 2009. For each sampling, 5 kg of samples were collected from the stations and carried at +4°C in a cool box to the laboratory.

Heavy Metal Analysis

For heavy metal analysis, seasonally collected samples were stored in polyethylene bags at -18°C in laboratory. To represent each station, 10 units were randomly taken from samples. Selected samples were separated from their shells and dried at 105°C. Dried samples were ground and 0.5 g portions were put into Teflon tubes; then, they were turned into soluble forms in microwave (CEM Mars X-press) with 10 ml concentrated nitric acid (65% w/v) (Merck, Germany) addition. After the application of organic digestion process, samples were filtered and diluted with deionized water (Millipore, USA) [16]. Before heavy metal analysis, nonstandard dilutions were prepared, and ICP-AES device was calibrated. Blind sample was prepared and measured for every 5 sample to determine the effects of

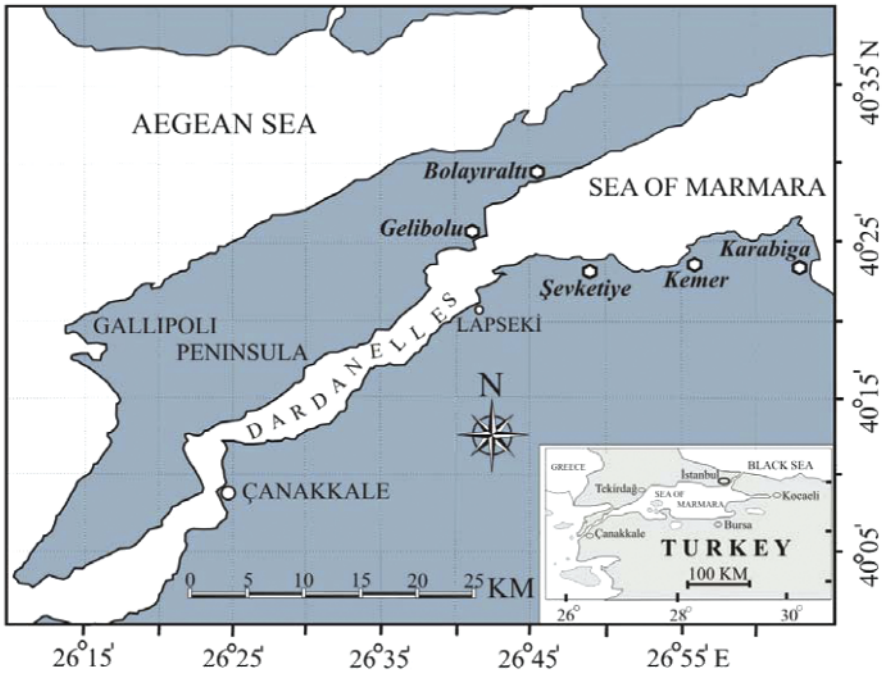

Fig 1. Sampling stations in the Marmara Sea

Şekil 1. Marmara Denizi'nde örneklerin alındığı istasyonlar

used reagents. Heavy metal (Cd, Cu, Pb, Zn, Fe, and Cr) analysis was applied using Varian Liberty AX Sequential ICP-AES [17]. Results of the all analysis were first compared to the standard results obtained with metal salts and then measured as mg kg^{-1} of wet matter.

Biotoxin Analysis

Shells of the samples used for biotoxin analysis were properly cleaned. Hepatopancreases of the clams were removed aseptically after shucking the clams. Diarrhetic shellfish poison (DSP) analysis was performed using mouse assay (*Mus musculus* var. albinus) as described by Yasumoto [18]. For this purpose, hepatopancreases were first homogenized (Ultra-Turrax; IKA Yellow line, Germany) in acetone (Merck, Germany) followed by intraperitoneal (IP) injection of the homogenized 1 ml into three mice weighing 16-20 g each. Mice were observed for 24 h, and symptoms were recorded. The amount of the toxin which killed a mouse in 24 h after injection was defined as one mouse unit (MU g^{-1} digestive gland) [19]. In the paralytic shellfish poison (PSP) analysis, clam tissue homogenized in Ultra-Turrax (IKA Yellow line, Germany) was made acidic with 0.1 N HCl (Merck, Germany) addition, centrifuged by boiling and cooling, and then 1 ml injected to three lab mice, supernatant weighs of which were recorded. After the injection, mice were observed for 1 h to record their death time, and toxin amounts were calculated with Sommer's table [20]. In the amnesic shellfish poison (ASP) analysis, prepared extract was filtered in a membrane filter (Millex-HV, Millipore, U.S.A) of 0.45 µm [21], and the amount, calibration curve of which was drawn with standard domoic acid solution (DACS-1B Reference Standard, NRC, Halifax, Canada), was determined using HPLC (Shimadzu Corporation, Kyoto, Japan) with UV detector [22].

Microbiological Analysis

Shells of the samples taken for microbiological analysis were washed, scraped free of dirt, shucked with a sterile knife and then tissue and shell fluids of the samples were weighed in the predetermined amounts [23] and homogenized by mixing them in bag mixer (Stomacher 400, A.J. Seward-London) for 2 min. Homogenized samples were diluted to 10^{-6} for the analysis of total mesophilic bacteria.

The counts of the total aerobic mesophilic bacteria was performed using Plate Count Agar and incubating plates at 35°C for 48 h [23]. Total coliform, fecal coliform, *E. coli* counts were made according to the three tube most probable number method (MPN) using Lauryl Sulphate Tryptose broth (35°C, 24 h), Brilliant Green Bile broth (35°C, 48 h), EC broth (44.5°C, 24 h), respectively. The counts of *Salmonella* spp. analysis was carried out on 25 g of blended clams which were added to 225 ml and incubated for 24 h at 35°C. After incubation second step of enrichment were performed using RVS and TT broth and incubated for 24 h at 42°C and 24°C, respectively. A loopful of both medium was streaked onto Xylose Lysine Deoxycholate Agar and incubated for 24 h at 35°C [23]. The count of *Vibrio parahaemolyticus* was made according to Colakoglu et al.[24] using Thiosulfate Citrate Bile Salts Sucrose Agar and incubating plates at 37°C for 24 h.

Statistical Analysis

The descriptive statistics (mean, standard error (SE) and range) of the findings of the chemical composition and heavy metal content and microbiological analysis and also the one-way variance analysis (ANOVA) of the interactions between seasons and stations were calculated using Microsoft Office Excel 2007 software (Seattle, USA). Significance was established at $P<0.05$ [25].

RESULTS

Heavy Metals

The clams meat was analyzed for the determination of 6 metals, ie. Cd, Cu, Pb, Zn, Fe, Cr as summarized in *Table 1*. The mean concentrations (mg kg^{-1} wet weight) of trace minerals in tissues of *C. gallina* were the following: Cd, 0.04-0.69; Cu, 0.71-5.30; Pb, 0.18-3.24; Zn, 13.08-77.76; Fe, 2.46-89.73; Cr, 0.08-1.25. According to the results of the statistical analysis, heavy metal contents showed significant variation between seasons and stations ($P<0.05$).

Biotoxins

In present study PSP, DSP, and ASP toxins were not detected in the samples seasonally collected from the research stations.

Microbiological Analysis

The striped venus meat was also analyzed for the determination of total coliform, fecal coliform, *E. coli, Salmonella* spp, and *V. parahaemolyticus.* The highest value ($P<0.05$) of total aerobic organisms was found as $2.0x10^4$ CFU/g in summer, and the lowest ($P<0.05$) was found as $2.1x10^2$ CFU/g in winter. Total amount of coliforms and fecal coliforms was found to reach its maximum level ($P<0.05$) in summer and autumn *(Fig. 2)*. Fecal coliform content was lower than coliform content, and the total coliform amount in these seasons was found to be dense (1100 MPN/100 g) in Sevketiye and Kemer stations. Maximum *E. coli* level (107 MPN/100 g) was determined in summer, and minimum *E. coli* level (2 MPN/100 g) was determined in winter *(Fig. 2)*.

Table 1. *Seasonal variation of heavy metals concentration (mg kg^{-1}) in different stations in striped venus*
Tablo 1. *Beyaz kum midyesinin farklı istasyonlarda ağır metal konsantrasyonlarının (mg kg^{-1}) mevsimsel dağılımı*

Metals	Stations	Seasons			
		April'08	July'08	October'08	January'09
Cd	Gelibolu	0.07 ^{Ec}	0.15 ^{Cb}	0.08 ^{ABc}	0.24 ^{Ba}
	Bolayıraltı	0.31 ^{Bb}	0.04 ^{Dc}	0.05 ^{Bc}	0.52 ^{Aa}
	Sevketiye	0.61 ^{Aa}	0.24 ^{Bb}	0.10 ^{Ac}	0.18 ^{BCbc}
	Kemer	0.16 ^{Da}	0.05 ^{Dc}	0.07 ^{ABbc}	0.08 ^{Db}
	Karabiga	0.22 ^{Cb}	0.69 ^{Aa}	0.08 ^{ABd}	0.14 ^{CDc}
Cu	Gelibolu	2.24 ^{Ab}	2.58 ^{Ba}	1.80 ^{Cd}	2.07 ^{Cc}
	Bolayıraltı	2.21 ^{Ab}	4.27 ^{Aa}	0.71 ^{Dd}	2.11 ^{Cc}
	Sevketiye	1.23 ^{Dc}	2.39 ^{Ca}	2.03 ^{Bb}	2.00 ^{Db}
	Kemer	1.82 ^{Cc}	2.18 ^{Da}	2.00 ^{Bb}	2.21 ^{Ba}
	Karabiga	2.02 ^{Bc}	1.98 ^{Ec}	4.87 ^{Ab}	5.30 ^{Aa}
Pb	Gelibolu	3.01 ^{Ab}	2.51 ^{Ac}	2.29 ^{Ad}	3.24 ^{Aa}
	Bolayıraltı	0.78 ^{Bc}	0.90 ^{Cb}	0.18 ^{Ed}	1.40 ^{Ca}
	Sevketiye	0.42 ^{Dc}	0.27 ^{Dd}	1.09 ^{Db}	1.40 ^{Ca}
	Kemer	0.84 ^{Bc}	0.91 ^{Cc}	1.21 ^{Cb}	1.36 ^{Ca}
	Karabiga	0.69 ^{Cd}	1.10 ^{Bc}	1.71 ^{Bb}	2.35 ^{Ba}
Zn	Gelibolu	18.88 ^{Dc}	18.65 ^{Dd}	19.73 ^{Cb}	23.54 ^{Ba}
	Bolayıraltı	17.68 ^{Eb}	25.76 ^{Ba}	13.08 ^{Ed}	16.33 ^{Ec}
	Sevketiye	27.39 ^{Ba}	26.10 ^{Cb}	21.45 ^{Bc}	17.37 ^{Dd}
	Kemer	22.35 ^{Cb}	14.61 ^{Ed}	19.12 ^{Dc}	36.91 ^{Aa}
	Karabiga	77.76 ^{Aa}	44.02 ^{Ab}	22.48 ^{Ac}	17.94 ^{Cd}
Fe	Gelibolu	2.46 ^{Ed}	4.70 ^{Ec}	89.73 ^{Aa}	27.85 ^{Bb}
	Bolayıraltı	18.81 ^{Bc}	114.22 ^{Aa}	17.89 ^{Ed}	20.73 ^{Cb}
	Sevketiye	17.00 ^{Cd}	32.82 ^{Cb}	51.56 ^{Ba}	17.46 ^{Ec}
	Kemer	16.52 ^{Dd}	46.06 ^{Ba}	26.65 ^{Db}	17.64 ^{Dc}
	Karabiga	21.39 ^{Ad}	23.47 ^{Dc}	39.49 ^{Ca}	28.36 ^{Ab}
Cr	Gelibolu	0.32 ^{Cb}	0.19 ^{Dc}	0.76 ^{Aa}	- *
	Bolayıraltı	- *	0.40 ^{Ba}	0.21 ^{Db}	0.20 ^{Cc}
	Sevketiye	0.27 ^{Dc}	0.53 ^{Ab}	0.47 ^{Bb}	1.25 ^{Aa}
	Kemer	0.46 ^{Ab}	0.23 ^{Dd}	0.34 ^{Cc}	1.13 ^{Ba}
	Karabiga	0.37 ^{Ba}	0.35 ^{Ca}	0.08 ^{Ec}	0.21 ^{Cb}

**** :*** *not detected*
Means with different superscript capital letters in the same row and lowercase letters in the same column for each metals indicate significant differences (P<0.05)

It was observed that there was no statistically significant difference (P>0.05) of *E. coli* between seasons in Sevketiye station. Seasonal changes of the amount of total aerobic, total coliform and fecal coliform organisms were significant difference (P<0.05).

Salmonella spp. was not detected and *V. parahaemolyticus* was isolated two times in summer (Sevketiye) and autumn (Karabiga).

DISCUSSION

Metal density, natural components of sea, might be increased as a consequence of human activities, and they could be remained in dangerous concentrations in the ecosystem for a long time [27-29]. Heavy metals, though in low concentrations, are accumulated in the meat of bivalve, therefore, consumption of bivalve meat containing excessive concentrations of these metals could be endangered human health [30]. Lead amounts in the meat of bivalve collected from Gelibolu station were found as 1.5 mg kg^{-1}, which was higher than the legal limits imposed by both Turkish Fisheries Regulation and European Commission for all seasons (P<0.05). In addition, lead content [15,31] was observed to be higher than the legal limits in Karabiga station in autumn (1.71 mg kg^{-1}) and winter (2.35 mg kg^{-1}) (P<0.05). Also, high level (77.76 mg kg^{-1}) of zinc content *(Table 1)* was detected in spring samples of Karabiga Station (P<0.05), which was higher than the alert levels of Turkey and NNSP (National Shellfish Sanitation Program) [15,32].

Heavy metal contents (Cd, Cu, Pb, Zn, Fe, and Cr) of the samples collected from other stations was determined to be lower than the national and international limit values [15,31]. In the studies made on heavy metal contents of striped venus in Marmara Sea, findings have been reported similarly to be lower than the limit values so far [33,34]. It is thought that the high levels of lead and zinc determined in this study could be related to the contaminated streams that converge to Black Sea, which is known to be polluted by many rivers; River of Danube being the most polluted among all [43].

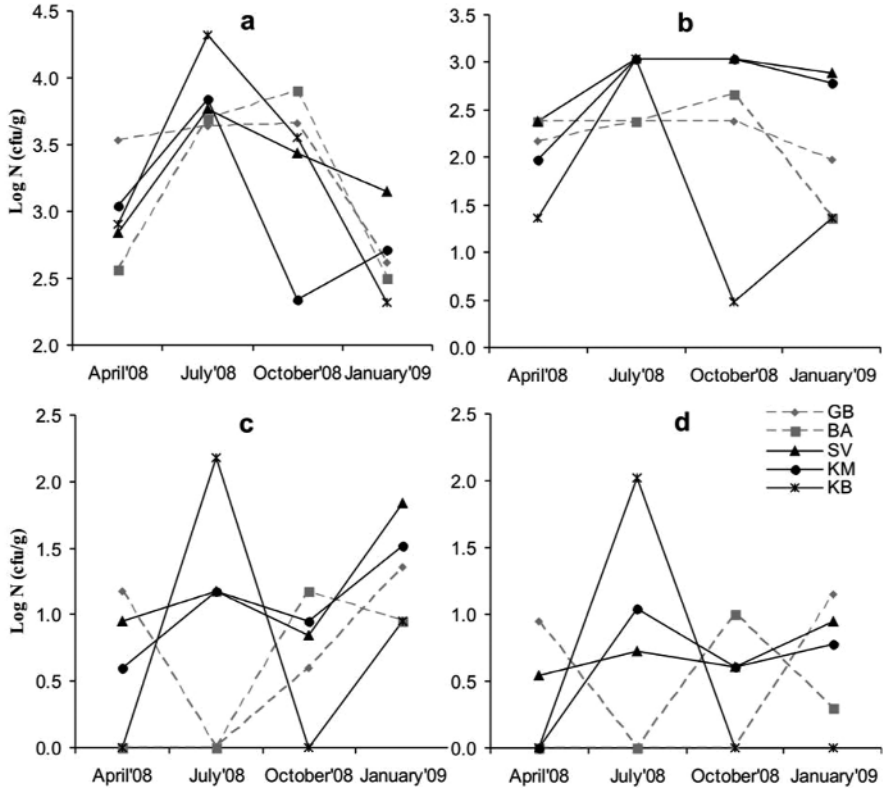

Fig 2. Levels of total aerobic counts **(a),** total coliform **(b),** fecal coliform **(c)** and *E. coli* **(d)** bacteria in striped venus of different seasons and stations

Şekil 2. Beyaz kum midyesinin farklı mevsim ve istasyonlardaki toplam aerobic **(a),** toplam koliform **(b),** fekal koliform **(c)** ve *E .coli* **(d)** bakteri miktarları

In bacteriological examinations of bivalves, total amount of aerobic bacteria was detected around 10^2-10^4 CFU/g in all stations (P<0.05) *(Fig. 2)*. Intensive amounts of coliform bacteria (1100 MPN/100 g) were found in Sevketiye and Kemer stations (P<0.05). Fecal and *E. coli* levels were found to be lower than the limit values (300 MPN/100 g for fecal coliform and 230 MPN/100 g for *E. coli*) imposed by the Turkish Fisheries Regulations and EC Shellfish Hygiene Directive (91/492/EEC) [15,31,35] *(Fig. 2)*. These bacteria reached their maximum levels in summer months (P<0.05). *Salmonella* spp. was not detected in any sample; however, *V. parahaemolyticus* was detected two times in Sevketiye and Karabiga stations. The members of *Vibrio* were frequently defined as opportunistic and potential pathogenic bacteria of the water bodies especially in warm climate zones [36,37]. Nevertheless from the public health perspective, the occurrences of these bacteria have caused concerns for authorities. However, the presences of some *Vibrio* species were also reported off Dardanelles coast in Turkey [24] and some European countries coasts' [38,39]. Bacteria levels of clams in northern Marmara Sea were found to increase in summer, and *Salmonella* spp. was detected four times between May and August [40]. Bacteria levels of the clams increase in summer because of the increase in human recreational activities, as well as the increase in industrial and household wastes in these months. In addition, it was reported that increasing temperature of sea water, wind effects, nutrient increase and currents could also be effective in the increase of bacteria amounts [41,42].

In conclusion, many of the parameters studied in this work seasonally and locally fluctuated. In particular, the microbiological; *Vibrio parahaemolyticus* and metal content; Pb and Zn did not meet the legal requirements imposed Turkish Fisheries Regulation and European Commission standards for its quality trademark. This could be related to the contaminated streams that converge to Black Sea, which is known to be polluted by many rivers; River of Danube being the most polluted among all. Due to these risks of the striped venus in Marmara Sea, continuously monitoring should be conducted.

Acknowledgements

The authors would like to thank COMU Scientific Technology Research Center for heavy metal analysis and Fikret Cakir who helped in the laboratory. Mouse assays were carried out in accordance with the ethical rules.

REFERENCES

1. Moschino V, Marin MG: Seasonal changes in physiological responses and evaluation of "well-being" in the venus clam *Chamelea gallina* from the Northern Adriatic Sea. *Comp Biochem Physiol A Mol Integr Physiol,* 145, 433-440, 2006.

2. Deval MC: The shell growth and the biometry of the stripped

venus *Chamelea gallina* (L.) in the Sea of Marmara. Turkey. *J. Shellfish Res.,* 20 (1): 155-159, 2001.

3. Deval MC, Oray IK: The shell growth and the biometry of the striped venus *Chamelea gallina,* (L.) in the Sea of Marmara and the western Black Sea, Turkey. *J Fish Aquat Sci,* 6, 127-142, 1992.

4. FAO-FIGIS: Global capture statistics of stripped venus. Food and Agriculture Organization of the United Nations-Fisheries Global Information System <http://www.fao.org/figis/servlet/SQServlet?file=/usr/local/tomcat/FI/5.5.23/figis/webapps/figis/temp/hqp_35166.xml&outtype=html> *Accessed:* 29.07.2009.

5. Ahmed FE: Seafood safety. National Academy Press, Washington, D.C., 1991.

6. Cook DW: Indicators and alternate indicators of growing waters. **In,** Ward DR, Ackney CH (Eds): Microbiology of Marine Food Products. pp. 19-39, AVI Book, Van Nostrand Reinhold, New York, NY, 1991.

7. Feldhusen F: The role of seafood in bacterial foodborne diseases. *Microbes Infect,* 2, 1651-1660, 2000.

8. Sumner J, Ross T: A semi-quantitative seafood safety risk assesment. *Int J Food Microbiol,* 77, 55-59, 2002.

9. Huss HH: Assurance of seafood quality. FAO Fisheries Technical Paper 334, FAO, Rome, p. 169, 1994.

10. Anonymous: Marine Biotoxins. FAO Food Nutr Pap 80. FAO, Rome,1994.

11. Aydın H, Uzar S: Denizel mikroalg biyotoksinleri ve etkileri. *Celal Bayar Univ Fen Bil Derg,* 5 (1): 87-100, 2009.

12. Dolah FMV: Marine algal toxins: Origins, health effects, and their increased occurrence. *Environ Health Perspect,* 108, 133-141, 2000.

13. Koray T: A Check-list for phytoplankton of Turkish Seas. *Ege Univ J Fish Aquat Sci,* 18 (1-2): 1-23, 2001.

14. Anonymous: Çanakkale Tarım İl Müdürlüğü Su Ürünleri Brifing Raporu, 2009.

15. Anonymous: Fishery products law and regulation. Turkish Republic Ministry of Agriculture and Rural Affairs Press. p. 85. Ankara, 1995.

16. EPA: Microwave assisted acid digestion of sediments, sludges, soils, and oils. Environmental Protection Agency Method 3051A. http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/3051a.pdf, *Accessed*: March 2009, 1998.

17. EPA: Inductively coupled plasma-atomic emission spectrometry. Environmental Protection Agency Method 6010C. http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/6010c.pdf, *Accessed:* March 2009, 2000.

18. Yasumoto T: Method for the bioassay of diarrhetic shellfish toxin. *Shokuhin Eiseigaku Zasshi,* 31, 515-522, 1981.

19. CRL: Report on the EU-NRLs intercalibration exercise on DSP determination (April, 2001). *European Community Reference Laboratory on Marine Biotoxins.* Vigo, Spain, 2001.

20. AOAC: Official Method 959.08. Paralytic shellfish poison, biological method. *Official Methods of Analysis of AOAC International.* Gaithersburg, Maryland, USA, 2000.

21. Quilliam MA, Sim PG, Mcculloch AW, Mcinnes AG: High-performance liquid-chromatography of domoic acid, a marine neurotoxin, with application to shellfish and plankton. *Int J Environ Anal Chem,* 36 (3): 139-154, 1989.

22. AOAC: Official Method 991.26. Domoic acid in mussels, liquid chromatographic method. *Official Methods of Analysis of AOAC International.* Gaithersburg, Maryland, USA, 2000.

23. FDA: Bacterial Analytical Manual. 8th ed. Revision A. Food and Drug Administration. *AOAC International,* Washington, DC, 1998.

24. Colakoglu FA, Sarmasik A, Koseoglu B: Occurrence of *Vibrio* spp. and *Aeromonas* spp. in shellfish harvested off Dardenelles cost of Turkey. *Food Control,* 17, 648-652, 2006.

25. Zar JH: Biostatistical Analysis. Fourth ed. Department of Biological Sciences Northern Illinois University, 1999.

26. Hallegraeff GM: Harmful algal blooms: A global overview. **In,** Hallgraeff GM, Anderson DM, Cembella AD (Eds): Manual on Harmful Marine Microalgae. pp. 25-49, UNESCO, Paris, 2004.

27. Chen MH, Shih CC, Chou CL, Chou LS: Mercury, organic-mercury and selenium in small cetaceans in Taiwanese waters. *Mar Pollut Bull,* 45, 237-245, 2002.

28. Francesconi KA, Edmonds JS: Arsenic species in marine samples. *Croat Chem Acta,* 71 (2): 343-359, 1998.

29. Renzoni A, Zino F, Franchi E: Mercury levels along the food chain and risk for exposed populations. *Environ Res,* 77, 68-72, 1998.

30. Belitz HD, Grosch W, Schieberle P: Lehrbuch der Lebensmittelchemie. Springer-Verlag, Berlin, Heidelberg, 2001.

31. Anonymous: Commission regulation as regards heavy metals, directive 2001/22/EC, No: 466/2001. Setting maximum levels for certain contaminants in food stuffs, 2001.

32. Ratcliffe S, Wilt DS: Proceedings Seventh National Shellfish Sanitation Workshop, Washington, DC, October 20-22. p. 412, 1971.

33. Ozden O, Erkan N, Deval MC: Trace mineral profiles of the bivalve species *Chamelea gallina* and *Donax trunculus. Food Chem,* 222-226, 2009.

34. Şentürk A: Çeşitli yörelerden avlanmış mollusklarda cıva kadmiyum ve kurşun düzeylerinin araştırılması. *Yüksek Lisans Tezi.* İstanbul Üniv Fen Bil Enst, 1993.

35. Anonymous: Council directive 91/492/EEC of 15 July 1991 laying down the health conditions for the production and the placing on the market of live bivalve mollusks, 1991.

36. Farmer JJ III, Hickman Brenner FW: The genera Vibrio and Photobacterium. **In,** Balows A, Tru¨per HG, Dworkin M, Harder W, Schleifer KH (Eds): The Prokaryotes. 2nd ed., pp. 2952-3011, Springer Verlag, New York, NY, 2, 1992.

37. Huss H: Control of indigenous pathogenic bacteria in seafood. *Food Control,* 8, 91-98, 1997.

38. Baffone W, Pianetti A, Bruscolini F, Barbieri E, Citterio B: Occurrence and expression of virulence-related properties of *Vibrio* species isolated from widely consumed seafood products. *Int J Food Microbiol,* 54 (1-2): 9-18, 2000.

39. Lhafi SK, Kuhne M: Occurrence of *Vibrio* spp. in blue mussels *(Mytilus edulis)* from the German Wadden Sea. *Int J Food Microbiol,* 297-300, 2007.

40. Altug G, Cardak M, Ciftci PS: Indicator and other bacteria in striped venus (*Chamelea gallina,* L.) and wedge clam *(Donax trunculus)* from the northern coast of the sea Marmara, Turkey. *J Shellfish Res,* 27 (4): 783-788, 2008.

41. Grimes DJ, Atwell RW, Brayton PR, Palmer LM, Rollis DM, Roszak DB: The fate of enteric pathogenic bacteria in estuarine and marine environments. *Microbiol Sci,* 3, 324-329, 1986.

42. Okumus I, Stirling HP: Seasonal variations in the meat weight, condition index and biochemical composition of mussels (*Mytilus edulis* L.) in suspended culture in two Scottish sea lochs. *Aquaculture,* 159 (3-4): 249-261, 1998.

43. Nisbet C, Terzi G, Pilgir O, Sarac N: Determination of heavy metal levels in fish samples collected from the Middle Black Sea. *Kafkas Univ Vet Fak Derg,* 16 (1): 119-125, 2010.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S159-S162, 2010
DOI:10.9775/kvfd.2010.2671

RESEARCH ARTICLE

Determination of Aflatoxin M_1 Level in Butter Samples Consumed in Erzurum, Turkey

Meryem AYDEMİR ATASEVER * Mustafa ATASEVER *
Korhan ÖZTURAN ** Sevda URÇAR *

* Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Ataturk University, TR-25240 Erzurum - TURKEY
** Nineth Army Cops "A" Type Food Control Detachment Commandery, Erzurum - TURKEY

Makale Kodu (Article Code): KVFD-2010-2671

Summary

In this study; the levels of aflatoxin M_1 (AFM_1) in 80 butter samples were determined. They were obtained from supermarkets in city center of Erzurum. The presence and concentration range of AFM_1 in the samples were investigated by competitive enzyme-linked immunoabsorbent assay (ELISA) method. AFM_1 was found in 66 (82.5%) samples at levels ranging from 10 to 121 ng/kg with mean concentration of 30.4±23.9 ng/kg. The levels of AFM_1 in 13 (16.3%) samples were higher than the maximum legal limit accepted by Codex Alimentarious Commission (CAC). None of the contaminated butter sample exceeded the legal limit regulated by Turkish Food Codex (TFC) for AFM_1. The results indicated that contamination of the butter samples with AFM_1 in high level could be a potential hazard for public health.

Keywords: *Aflatoxin M_1, Butter, ELISA*

Erzurum'da Tüketime Sunulan Tereyağlarında Aflatoxin M_1 Düzeyinin Belirlenmesi

Özet

Bu çalışmada 80 tereyağ örneğinde Aflatoksin M_1 (AFM_1) seviyesi belirlendi. Örnekler Erzurum şehir merkezindeki marketlerden temin edildi. Örneklerin AFM_1 içeriği ve konsantrasyonu kompetitiv ELISA metoduyla araştırıldı. AFM_1 seviyesi 66 örnekte 10 ile 121 ng/kg arasında değişmekte olup ortalama 30.4±23.9 ng/kg olarak bulundu. 13 numunede (16.3%) belirlenen AFM_1 düzeyinin Kodeks Alimentarius Komisyonu (CAC) tarafından düzenlenen yasal limitleri aştığı belirlendi. Kontamine tereyağı örneklerinin hiç birinde Türk Gıda kodeksi limitlerini aşan numuneye rastlanmadı. Bu sonuçlar, tereyağındaki yüksek AFM_1 düzeyinin halk sağlığı açısından risk oluşturabileceğini göstermektedir.

Anahtar sözcükler: *Aflatoksin M_1,Tereyağ, ELISA*

INTRODUCTION

Aflatoxin is a family of highly toxic and carcinogenic fungal metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus* and the rare *A. nomius fungi* [1,2]. *A. flavus* produces only B aflatoxins, while the other two species produce both B and G aflatoxins. Aflatoxin M_1 (AFM_1) is the principal hydroxylated aflatoxin metabolite in the milk of dairy cows fed contaminated feed with aflatoxin B_1 (AFB_1). These toxins show a serious risk for animal and human health, particularly for children, who are the major milk consumers. It has been reported several effects of aflatoxins on health such as hepato-toxic, genotoxic, carcinogenic, teratogenic, immuno-

İletişim (Correspondence)
☎ +90 442 2315544
✉ meryemataseve r@atauni.edu.tr

suppressive and antinutritional [1].

AFM_1 is mainly soluble in the aqueous phase of milk or adsorbed to casein particles; information of several studies show that a small ratio of AFM_1 in milk is carried-over to cream, and yet a smaller proportion to butter. The remainder of AFM_1 in milk, however, remains in skim milk and buttermilk [3].

To protect consumers, several countries have established legislation to regulate the levels of mycotoxins [4,5]. Several factors may influence the establishment of mycotoxin limits and regulations. These include scientific factors such as the availability of toxicological data and survey data, knowledge about the distribution of mycotoxin in commodities,and analytical methodology. Economical and political factors such as commercial interests and sufficiency of food supply have their impact as well [6]. The Codex Alimentarious Commission (CAC) [7] has set a limit of 50 ng/kg for AFM_1 in milk products while the Turkish Food Codex [8] prescribed the maximum level for AFM_1 in milk products 10-fold higher (500 ng/l) than the current level in the CAC.

Dairy products have been produced and consumed widely in Turkey. Owing to the common presence and harmful effects of aflatoxin contamination, there is a need for detection and measurement of AFM_1 in dairy products. Ascribed to scientific literature, very few studies [5,9-12] have been published on the presence and level of AFM_1 in milk and dairy products in Turkey. The present study has been designed to investigate the presence of AFM_1 in butter and to compare the results with the maximum AFM_1 tolerance limits which are accepted by the CAC and TFC.

MATERIAL and METHODS

Samples

A total of 80 butter samples were obtained randomly from supermarkets between September 2007 and September 2009 in Erzurum city. The samples were transported to the laboratory in an insulated container at about 4°C and analyzed upon arrival.

Analysis of AFM_1 by ELISA

The quantitative analysis of AFM_1 in the butter samples was performed by competitive enzyme immunoassay using RIDASCREEN Aflatoxin M_1 30/15 (Art. No: R1111, R-Biopharm, Darmstadt, Germany) [13] test kit. Immunoaffinity column (Rida Aflatoxin Column Art. No: R5001/5002) [14] were used to run ELISA analyses.

Aflatoxin Column

Sample preparation procedures were performed according to the instructions of the test kit (Rida Aflatoxin Column Art. No: R5001/5002) [14] manual. 25 ml of methanol (70%) was added to 5 g of butter. Afterwards, the solution was extracted by mixing gently for 10 min at room temperature. The extract was filtered through a paper filter and 15 ml of distilled water were added to 5 ml of filtered solution. 0.25 ml Tween 20 were added and stirred for 2 min, followed by entire amount of the sample solution (20 ml) passing over the column. Clean up procedure was performed according to the kit's manual. Eluate containing toxin was diluted 1:9 with the sample dilution buffer (supplied with the test kit) and used 100 µl per well in the assay.

Test Procedure of AFM_1

One-hundred microliter of standard solutions and prepared samples were added into separate microtitre wells and incubated for 60 min at room temperature (22-25°C) in the dark. The liquid was then poured out and the wells were washed with washing buffer (250 µl) twice. In the next stage, 100 µl of the diluted enzyme conjugate was added to the wells, mixed gently by shaking the plate manually and incubated for 15 min at room temperature in the dark. Again, the wells were washed twice with washing buffer. Afterwards, 100 µl of substrate/chromogen was added, mixed gently and incubated in the dark at room temperature for 15 min. Finally, 100 µl of the stop reagent (1 N H_2SO_4) was added into the wells and the absorbance was measured at k=450 nm in ELISA plate reader (ELx800, Bio-Tek Instruments, USA) within 10 min.

Evaluation

The samples were evaluated according to the RIDAVIN computer program prepared by R-Biopharm. According to the instructions for use of the RIDASCREEN kit; the lower detection limit was 5 ng/kg.

RESULTS

In this study, a total of 80 butter samples were analysed for AFM_1 with the competitive ELISA. The presence and the distribution of AFM_1 concentration in various ranges in butter samples are presented in *Table 1.* As shown in *Table 1,* AFM_1 was detected in 66 of 80 of the butter samples above the detectable level of 5 ng/kg. In total 16.3% of AFM_1-contaminated samples exceeded the CAC regulation (50 ng/kg). However, none of the contaminated butter sample exceeded the limit (500 ng/kg) reported by TFC for AFM_1. Quantity

(concentration) of AFM1 in butter samples ranged from 10 to 121 ng/kg while the mean value was 30.4 ng/kg (*Table 1*).

Table 1. *Presence and distribution of AFM1 in butter samples*
Tablo 1. *Tereyağ örneklerinin AFM1 içeriği ve dağılımı*

Kind of Samples	Samples Tested (n)	Proportion of Positive Samples n (%)	Distribution of Samples [a] n (%)					Proportion of Samples Exceeding the CAC Legal Limit >50 ng/kg	Proportion of Samples Exceeding the TFC >500 ng/kg	Quantity of AFM1 (ng/kg)		
			<5*	5-25	26-50	51-100	>100			x±Sx	Min.	Max.
Butter	80	(82.5)	(17.5)	(28.8)	(37.5)	(15)	(1.3)	(16.3)	ND	30.4±23.9	10	121

** distribution of negative samples, **a:** ng/kg, **CAC:** Codex Alimentarious Commission, **TFC:** Turkish Food Codex, **( ):** indicates percent, **ND:** Not Detected, **x±Sx:** mean±standart deviation*
** negatif örneklerin dağılımı, **a:** ng/kg, **CAC:** Kodeks Alimentarius Komisyonu, **TFC:** Türk Gıda Kodeksi, **( ):** yüzde ifadesi, **ND:** Saptanmadı, **x±Sx:** ortalama±standat sapma*

Table 2. *Aflatoxin M1 contents of butter reported in previous studies*
Tablo 2. *Önceki çalışmalarda tereyağında bildirilen AFM1 içerikleri*

Sample	Country	No. of Samples Positive	Range of Samples Positive (ng/kg)	Exceed Legal Limit *	References
Butter	Turkey	92/92 (100)	10-7000	26/92 (28.3)	Tekinsen and Ucar [18]
	Turkey	5/5 (100)	13.5-16.6	0/5 (0)	Bakirci [19]
	Turkey	52/61(81)	ND	10/61 (16.4)	Aycicek et al. [15]
	Turkey	25/27 (92.6)	1-100	10/27 (3.7)	Aycicek et al. [16]

** CAC limits in butter is 50 ng/kg, **ND:** Not Detected, **( ):** indicates percent*
** Kodeks Alimentarius Komisyonu tereyağı limiti 50 ng/kg, **ND:** Saptanmadı, **( ):** yüzde ifadesi*

DISCUSSION

Dairy products play a significant role in human diet since they are rich sources of bioavailable calcium and proteins. However, many of the previous studies indicate the presence of AFM1 at high concentrations in dairy products [5,9-12].

A few studies in Turkey have addressed the issue of AFM1 contamination of butter. AFM1 levels determined in the butter consumed in Turkey by previous reports are indicated in *Table 2*.

As shown in *Table 1*, AFM1 was detected in 82.5% of the butter samples. These results are in parallel with the findings of Aycicek et al.[15,16]. In present study the contamination level of AFM1(as incidence) in butter samples was found to be low as compared to the results of earlier studies in the same area [17,18]. In a study by Tekinsen and Ucar [18], the number of AFM1 positive butter samples as well as the maximum AFM1 level is higher than the values reported in our study. These differences might be due to the differences in the AFM1 levels in the milk from which the butter is produced [19] and in the processing method of milk or due to the differences in the methods of analysis [20]. Moreover, differences in the hygiene and storage conditions at the dairies and sales department are other factors on the variations of the results [18,20,21]. In addition, the AFM1 level in the milk was signigicantly affected by the geographical region and the country [20]. The lower incidence of AFM1 found in butter samples may due to the number of samples analysed than other researches done in Turkey.

In this study, AFM1 concentration in contaminated butter samples exceeding CAC legal limit was lower than the results reported by Tekinsen and Ucar [18] and were higher than the results reported by Bakirci [17] and Aycicek et al.[16]. Similarly, results reported by Aycicek et al.[15] indicated that 10/61 (16.4%) samples had levels higher than CAC regulation.

The TFC has updated the maximum allowable AFM1 limit as 500 ng/kg for the foodstuff that has a potential to contain aflatoxin. This limit have been 50 ng/kg in the previous years. In the previous studies, the number of samples exceeding the Turkish legal limits has been high owing to that reason. The fact that no samples exceeding the legal limits regulated by the TFC encountered in our study is partially due to that reason. The recent limits put forward by the latest regulations are thought to be considerably high (10 times higher than that of the CAC limits) and a re-consideration of this arrangement is

thought to be required.

Shortly, the results of this study show that there is a risk from butter produced from milk obtained from animals fed with contaminated animals feed with aflatoxins, since all the age groups including infants and children consume milk and dairy products daily. The AFM_1 level of butter samples is closely related to the aflatoxins concentration in milk used for butter production and other related factors. Therefore the prevention of aflatoxin formation in feeds is very important. Avoiding contamination appears to be the only practical way to ensure the safety of milk and milk products for human consumption. For this reason, it is considered that food substances should be produced from healthy raw material and kept in convenient conditions to prevent aflatoxin formation.

REFERENCES

1. Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D: Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences, and interventions 1-3. *Am Soc Clinical Nutr,* 80, 1106-1122, 2004.

2. Creppy EE: Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicol Lett,* 127, 19-28, 2002.

3. Prandini A, Tansini G, Sigolo S, Filippi L, Laporta M, Piva G: On the occurrence of aflatoxin M1 in milk and dairy products. *Food Chem Toxicol* doi:10.1016/j.fct.2007.10.005, 2008.

4. Rastogi S, Dwivedi DP, Khanna KS, Das M: Detection of aflatoxin M_1 contamination in milk and infant milk products from Indian Markets by ELISA. *Food Control,* 15, 287-290, 2004.

5. Sarimehmetoglu B, Kuplulu O, Celik TH: Detection of aflatoxin M1 in cheese samples by ELISA. *Food Control,* 15, 45-49, 2004.

6. Food and Agriculture Organization of the United Nations (FAO): Worldwide regulations for mycotoxins in food and feed in 2003. http://www.fao.org/docrep/007/y5499e/y5499e00.HTM. *Accessed:* 30.01.2010.

7. Codex Alimentarious Commission (CAC): Comments submitted on the draft maximum level for aflatoxin M_1 in milk. Codex Committee on food additives and contaminants 33rd session, Hague, The Netherlands, 2001.

8. Turkish Food Codex (TFC): Gıda maddelerinde belirli bulaşanların maksimum seviyelerinin belirlenmesi hakkında tebliğ. *Resmi Gazete,* 17 Mayıs 2008. Sayı: 26879 Başbakanlık Basımevi. Ankara, Turkey, 2008.

9. Tekinşen KK, Tekinşen OC: Aflatoxin M_1 in white pickle and Van otlu (herb) cheeses consumed in southeastern Turkey. *Food Control,* 16, 565-568, 2005.

10. Yaroglu T, Oruc HH, Tayar M: Aflatoxin M_1 levels in cheese samples from some provinces of Turkey. *Food Control,* 16, 883-885, 2005.

11. Aydemir Atasever M, Adiguzel G, Ozturan K, Atasever M: Determination of Aflatoxin M_1 levels in some cheese types consumed in Erzurum-Turkey. *Kafkas Univ Vet Fak Derg,* 2010 (In press).

12. Aydemir Atasever M, Adiguzel G, Atasever M, Özlü H, Ozturan K: Occurrence of Aflatoxin M_1 in UHT Milk in Erzurum-Turkey. *Kafkas Univ Vet Fak Derg,* 2010 (In press).

13. R-Biopharm GmbH: Enzyme immunoassay for the quantitative analysis of aflatoxins. Ridascreen aflatoxin M_1 Art. No: R-1101. R-Biopharm GmbH, Darmstadt, Germany, 1999.

14. R-Biopharm GmbH: Immunoaffinity column for sample clean up prior to analysis of aflatoxin. Art. No: R5001/R5002. R-Biopharm GmbH, Darmstadt, Germany, 2005.

15. Aycicek H, Yarsan E, Sarımehmetoglu B, Cakmak O: Aflatoxin M_1 in white cheese and butter consumed in Istanbul, Turkey. *Vet Hum Toxicol,* 44, 295-296, 2002.

16. Aycicek H, Aksoy A, Saygi S: Determination of aflatoxin levels in some dairy and food products which consumed in Ankara, Turkey. *Food Control,* 16, 263-266, 2005.

17. Bakirci I: Sütlerde Aflatoxin M_1 Oluşumu ve Ürünlere Geçişi Üzerinde Bir Araştırma. *Doktora Tezi.* Yüzüncüyıl Üniv Fen Bil Enst, Van, 1995.

18. Tekinsen KK, Ucar G: Aflatoxin M_1 levels in butter and cream cheese consumed in Turkey. *Food Control,* 19, 27-30, 2008.

19. Bakirci I: A study on the occurrence of aflatoxin M_1 in milk and milk products produced in Van province of Turkey. *Food Control,* 12, 47-51, 2001.

20. Galvano F, Galofaro V, Galvano G: Occurrence and stability of aflatoxin M_1 in milk and milk products. *J Food Protect,* 59, 1079-1090, 1996.

21. Wiseman DW, Marth EH: Behavior of aflatoxin M_1 during manufacturing and storage of queso blanco and bakers cheese. *J Food Protect,* 46, 910-913, 1983.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S163-S166, 2010
DOI:10.9775/kvfd.2010.2801

RESEARCH ARTICLE

Presence of *Enterobacter sakazakii* in Milk Powder, Whey Powder and White Cheese Produced in Konya

Mukadderat GÖKMEN * Kemal Kaan TEKİNŞEN ** Ümit GÜRBÜZ **

* Province Control Laboratory, 42090 Konya - TURKEY
** Department of Food Hygiene and Technology, Faculty of Veterinary, University of Selcuk, 42075 Konya - TURKEY

Makale Kodu (Article Code): KVFD-2010-2801

Summary

Enterobacter sakazakii has been caused foodborne illnesses through consumption of a variety of foods such as milk powder, cheese, sausage, particularly infant foods. In this study, the presence of *E. sakazakii* were investigated in 60 milk powder, 50 whey powder and 50 white cheese. ISO/TS 22964 (IDF/RM 210) method was used for detection *E. sakazakii*. *E. sakazakii* was identified from milk powder 5% (3/60), white cheese 4% (2/50). *E. sakazakii* was not detected in whey powder. According to Commission Regulation (EC) and Turkish Food Codex Microbiological Criteria Communique *E. sakazakii* must not present in infant formulae, formulae for special medical purposes and follow-on formulae. Also in many countries, *E. sakazakii* infections associated with most of the milk-based products that have been reported. In conclusion, the results indicate that milk powder and white cheese produced some dairy plants in Konya presents a risk in terms of human health and the necessary precaution will have to be taken to carry out effective sanitary practices in the plants.

Keywords: *E. sakazakii, Milk powder, Whey powder, White cheese*

Konya'da Üretilen Süt Tozu, Peynir Altı Suyu Tozu ve Beyaz Peynirde *Enterobacter sakazakii* Varlığının Araştırılması

Özet

Enterobacter sakazakii süt tozu, peynir, sucuk ve özellikle bebek mamaları gibi çeşitli gıdaların tüketilmesiyle gıda kaynaklı hastalıklara neden olmaktadır. Bu çalışmada 60 süt tozu, 50 peynir altı suyu tozu ve 50 beyaz peynir *E. sakazakii* varlığı yönünden araştırıldı. *E. sakazakii*'nin tespiti için ISO/TS 22964 (IDF/RM 210) metodu kullanıldı. Süt tozu örneklerinin %5 (3/60)'inde ve beyaz peynir örneklerinin %4 (2/50)'ünde *E. sakazakii* identifiye edildi. Peynir altı suyu tozu örneklerinde ise *E. sakazakii* tespit edilemedi. Commission Regulation (EC) ve Türk Gıda Kodeksi Mikrobiyolojik Kriterler Tebliği'ne göre bebek mamaları, özel tıbbi amaçlı formüller ve devam mamalarında *E. sakazakii* bulunmaması gerektiği belirtilmekte, pek çok ülkede *E. sakazakii* enfeksiyonlarının çoğunlukla süt bazlı ürünler ile ilişkili olduğu rapor edilmektedir. Sonuç olarak, bulgular Konya'da bazı işletmelerde üretilen süt tozu ve beyaz peynirin insan sağlığı açısından risk oluşturduğunu ve işletmelerde etkili sanitasyon uygulamalarının gerçekleştirilebilmesi için ilgili tedbirlerin alınması gerekliliğini ortaya koymaktadır.

Anahtar sözcükler: *E. sakazakii, Süt tozu, Peynir altı suyu tozu, Beyaz peynir*

INTRODUCTION

The ubiquitous microorganism *Enterobacter sakazakii,* a gram-negative, non-spore-forming, rod-shaped, oxidase, lactose and sorbitol negative, facultative anaerobe, motile by peritrichous flagella bacterium, is a member family [1-3]. This organism was known as 'yellow pigmented *Enterobacter cloacae*' until 1980, afterward it was designated as *E. sakazakii* due to differention of biochemical characteristic from *E. cloacae* [4]. *E. sakazakii* is an opportunistic pathogen in new-born. *E. sakazakii* infections are important cause of life-threatening cases of

İletişim (Correspondence)
☎ +90 332 2233568
✉ kktekinsen@selcuk.edu.tr

meningitis (58%), sepsis (17%), and necrotizing enterocolitis (29%) in premature and full-term infants. Mortality rates of 40-80% have been described and survivors often suffer from neurological sequelae [5,6].

Food and food ingredients may be contaminated with *E. sakazakii* under unsuitable conditions of hygiene and contaminated equipment [7]. Powdered milk formula is important source of *E. sakazakii* infections [2,8]. This bacterium is resistance to drying and acid pH, heat, biofilm formation and persistence on food preparation surfaces. Due to new-born infections *E. sakazakii* is noticed as associated with infant formula and milk powder [4,9]. Infant formula is produced from ingredients that may include milk, milk derivatives, soy protein isolates, carbohydrates, fats, minerals, vitamins and some food additives [10]. Formula preparation equipment contaminated by *E. sakazakii* has been demonstrated to have caused two outbreaks [11,12], but the original source of *E. sakazakii* was not determined in either case. Environmental swabbing of formula preparation areas in the course of outbreak investigations has not demonstrated *E. sakazakii* in the general environment. *E. sakazakii* has been identified in the environments of milk powder production facilities and other food production facilities, as well as in households [13]. Not all infants with *E. sakazakii* infection have been exposed to powdered infant formula, and *E. sakazakii* infections can also occur in adults [6]. Thus, although an environmental source of *E. sakazakii* infection other than infant formula has not been strictly identified, other sources undoubtedly exist [10].

E. sakazakii has been isolated from a range of foods including cheese, fermented bread, tofu, sour tea, cured meats, minced beef and sausage meat [4]. According to Commission Regulation (EC) [14] and Turkish Food Codex [15], Microbiological Criteria Communique *E. sakazakii* must not present (0/10 g and 0/25 g, respectively) in infant formulae, formulae for special medical purposes and follow-on formulae. The objective of this study was to investigate the presence of *E. sakazakii* in milk powder, whey powder and white cheese produced in Konya.

MATERIAL and METHODS

Samples

In this study, total of 160 samples from three different milk products (60 milk powder, 50 whey powder and 50 white cheese) were used. The samples were obtained different retail market and dairy products factory in Konya and transported to the laboratory in insulated cooler boxes immediately. Samples were stored at 4°C until analyses. The presence of *E. sakazakii* were examined in milk powder, whey powder and white cheese.

Detection of E. sakazakii

ISO/TS 22964 (IDF/RM 210) [16] method was used for detection *E. sakazakii*. To prepare the pre-enrichment dilution, 25 g sample was added to 225 ml Buffered Peptone Water (BPW). Pre-enrichment dilution was incubated at 37±1°C for 18±2 h. After incubation of the inoculated pre-enrichment medium, 0.1 ml of the obtained culture was transferred into 10 ml Modified Lauryl Sulphate Broth (mLST) (Oxoid CM 1133)/ Vancomycin (Oxoid DR 595) medium. This medium was incubated at 44±0.5°C for 24±2 h. After incubation of the inoculated mLST-Vancomycin medium, a loopful (ca. 10 µ) was streaked onto the surface of the ChromoCult® *Enterobacter sakazakii* Agar (Merck 1.0087). The plate was incubated 44±1°C for 24±2 h. Five typical turquoise colonies was streaked on Tryptone Soya Agar (TSA) (Oxoid CM 131) and TSA plates was incubated at 25±1°C for 48±4 h [16]. After incubation, one yellow colony which selected from each TSA plate was confirmed with API 20 E kit [17] and VITEC 2 compact GN card according to the manufacturer's instructions [18] *(Fig. 1)*.

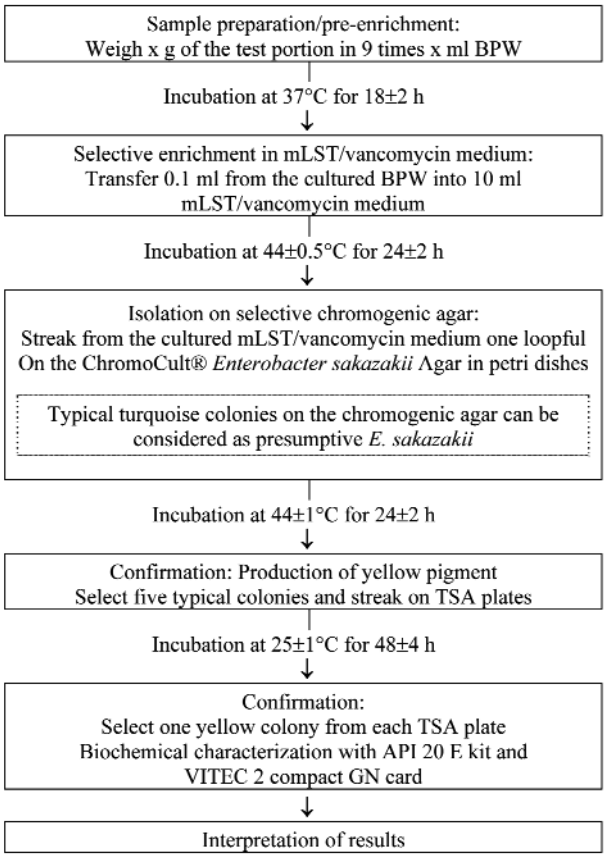

Fig 1. Identification method flow scheme of *E. sakazakii*
Şekil 1. *E. sakazakii*'nin identifikasyon metodu akış şeması

RESULTS

In this study, three different milk products total 160 samples (60 milk powder, 50 whey powder and 50 white cheese) were tested. *E. sakazakii* was isolated from 3/60 (5%) milk powder, 2/50 (4%) white cheese. But none of the samples contained *E. sakazakii* in whey powder. The summarized results of presence of *E. sakazakii* in milk powder, whey powder and white cheese are shown in *Table 1*.

Table 1. *The results of presence of E. sakazakii in milk powder, whey powder and white cheese*

Tablo 1. *Süt tozu, peynir altı suyu tozu ve beyaz peynirde E. sakazakii analiz bulguları*

Sample	No. of Sample	No. of Positive Sample
Milk Powder	60	3 (5%)
Whey Powder	50	ND
White Cheese	50	2 (4%)

Figures in parentheses are the percent of samples
ND: Not detected

Table 2. *Presence of E. sakazakii in some food products and plants*

Tablo 2. *Bazı gıda ürünlerinde ve işletmelerinde E. sakazakii varlığı*

Sample	No. of Sample	No. of Positive Sample	Country	References
Infant formula powder	40	1 (3)	Netherland	Heuvelink et al.[19]
Infant milk formula	82	2 (2)	United Kingdom	Iversen and Forsythe [20]
Infant milk formula	8	2 (25)	Jordan	Shaker et al.[3]
Infant milk formula	50	7 (14)	South Africa	Cawthorn et al.[21]
Infant milk formula	35	2 (6)	Egypt	El-Sharoud et al.[22]
Dried infant foods	49	5 (10)	United Kingdom	Iversen and Forsythe [20]
Infant food formula	15	2 (13)	Jordan	Shaker et al.[3]
Milk powder	170	7 (4)	Netherland	Heuvelink et al.[19]
Milk powder	72	3 (4)	United Kingdom	Iversen and Forsythe [20]
Full-fat milk powder	10	-	Jordan	Shaker et al.[3]
Full-fat milk powder	15	-	Egypt	El-Sharoud et al.[22]
Skimmed milk powder	37	5 (14)	Egypt	El-Sharoud et al.[22]
Stored Domiatti cheese	10	-	Egypt	El-Sharoud et al.[22]
Fresh Domiatti cheese	10	4 (40)	Egypt	El-Sharoud et al.[22]
Ras cheese	10	-	Egypt	El-Sharoud et al.[22]
Kariesh cheese	10	-	Egypt	El-Sharoud et al.[22]
Cheese products	62	2 (3)	United Kingdom	Iversen and Forsythe [20]
Milk powder factory	23	2 (9)	Switzerland	Kandhai et al.[13]*
Milk powder factory	26	9 (35)	Switzerland	Kandhai et al.[13]*
Milk powder factory	11	1 (9)	Switzerland	Kandhai et al.[13]*
Milk powder factory	8	2 (25)	Switzerland	Kandhai et al.[13]*
Environmental, Milk factory	14	6 (43)	South Africa	Cawthorn et al.[21]
Environmental, Milk factory	1	1 (100)	Egypt	El-Sharoud et al.[22]

Figures in parentheses are the percent of samples
** From different factory*

DISCUSSION

E. sakazakii has been isolated from a wide range of environmental sources and from several foods of animal and plant origin. Infant formula and milk powder have been the most common vehicles implicated in neonatal *E. sakazakii* infections and also in many countries, *E. sakazakii* infections associated with most of the milk-based products that have been reported [10]. Presence of *E. sakazakii* in some food products and plants are shown *Table 2*.

At the present study, presence of *E. sakazakii* was determined in milk powder, whey powder and white cheese which are produced in Konya city of Turkey. As depicted in *Table 1*. *E. sakazakii* was detected in 5% of the milk powder samples and in 4% of the white cheese samples, respectively. But *E. sakazakii* was not detected in whey powder samples.

These results are in parallel with the findings of some previous reports [19,20,22] which pointed out the presence of *E. sakazakii* in 4% of the milk powder, 3% cheese products and 2-6% milk-based products (infant formula powder, infant milk formula) samples *(Table 2)*. The present findings suggest that the milk which is processed into dairy products may be contaminated with *E. sakazakii*. The presence of *E. sakazakii* in these products is unlikely since the liquid milk is normally pasteurized. It has been reported that pasteurization treatment is effective in the elimination of this pathogen [3]. Nazarowec-White and Farber [1] found that *E. sakazakii* was more heat-sensitive than other pathogenic organisms like *Listeria monocytogenes*. The results confirm the findings

of some researchers [13,21,22] who reported a high occurrence of *E. sakazakii* in enviroment of milk factory and milk powder factory. Thus, as seen on *Table 2* a number of researchers [3,20-22] reported a higher occurrence in some milk-based products (infant milk formula, dried infant foods, infant food formula), skimmed milk powder and fresh Domiatti cheese samples. On the other hand Shaker et al.[3] and El-Sharoud et al.[22] could not be detected *E. sakazakii* in full-fat milk powder and some cheese kinds (stored Domiatti, Ras and Kariesh cheeses). The variations on the findings may be attributable to the fact that milk to be processed may contain different levels of *E. sakazakii* according to different processing techniques and analysis methods. Moreover, differences in the hygiene and storage conditions at the dairies and retail points are other key factors on the variations of the results [3,22,23].

E. sakazakii was not isolated from any samples of whey powder *(Table 1)*. Although it is not detected in whey powder samples, each sample must be evaluated on itself. Cross contamination must be recognized; either directly or via surface and equipment from raw milk [3].

In conclusion, this study showed that *E. sakazakii* was found in milk powder and white cheese samples produced some dairy plants in Konya. The results indicate that the milk powder and white cheese represents a potential hazard for consumer. Thus the necessary precaution will have to be taken to carry out effective cleaning of tools and equipment and personal hygiene practices in the plants.

REFERENCES

1. Nazarowec-White M, Farber JM: *Enterobacter sakazakii:* A review. *Int J Food Microbiol,* 34 (2): 103-113, 1997.

2. Drudy D, Mullane NR, Quinn T, Wall PG, Fanning S: *Enterobacter sakazakii:* An emerging pathogen in powdered infant formula. *Clin Infect Dis,* 42, 996-1002, 2006.

3. Shaker R, Osaili T, Al-Omary W, Jaradat Z, Al-Zuby M: Isolation of *Enterobacter sakazakii* and other *Enterobacter* sp. from food and food production environments. *Food Control,* 18, 1241-1245, 2007.

4. Iversen C, Forsythe S: Risk profile of *Enterobacter sakazakii,* an emergent pathogen associated with infant milk formula. *Trends Food Sci Technol,* 14, 443-454, 2003.

5. Muytjens HL, Zanen HC, Sonderkamp HJ, Kollée LA, Wachsmuth IK, Farmer JJ: Analysis of eight cases of neonatal meningitis and sepsis due to *Enterobacter sakazakii. J Clin Microbiol,* 18 (1): 115-20, 1983.

6. Lai KK: *Enterobacter sakazakii* infections among neonates, infants, children, and adults: Case reports and a review of the literature. *Medicine (Baltimore),* 80, 113-22, 2001.

7. Kuzina LV, Peloquin JJ, Vacek DC, Miller TA: Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies, *Anastrepha ludens* (Diptera: Tephritidae). *Curr Microbiol,* 42, 290-294, 2001.

8. Gurtler JB, Kornacki JL, Beuchat LR: *Enterobacter sakazakii:* A coliform of increased concern to infant health. *Int J Food Microbiol,* 104, 1-34, 2005.

9. Leclercq A, Wanegue C, Baylac P: Comparison of fecal coliform agar and violet red bile lactose agar for fecal coliform enumeration in foods. *Appl Environ Microbiol,* 68, 1631-1638, 2002.

10. FAO/WHO: *Enterobacter sakazakii* and Other Micro-organisms in Powdered Infant Formula: Meeting Report. Microbiological Risk Assessment Series 6. Food Safety Department World Health Organisation 20 Avenue Appia CH-1211 Geneva 27 Switzerland, 2004.

11. Noriega FR, Martin MA, Schwalbe RS: Nosocomial bacteremia caused by *Enterobacter sakazakii* and *Leuconostoc mesenteroides* resulting from extrinsic contamination of infant formula. *Pediatr Infect Dis J,* 9, 447-449, 1990.

12. Block C, Peleg O, Minster N, Bar-Oz B, Simhom A, Arad I, Shapiro M: Cluster of neonatal infections in Jerusalem due to unusual biochemical variant of *Enterobacter sakazakii. Eur J Clin Microbiol Infect Dis,* 21, 613-616, 2002.

13. Kandhai MC, Reij MW, Gorris LGM, Guillaume-Gentil O, van Schothorst M: Occurrence of *Enterobacter sakazakii* in food production environments and households. *The Lancet,* 363, 39-40, 2004.

14. Commission Regulation (EC): No 2073/2005 of 15 November 2005 on Microbiological Criteria for Foodstuffs. (Text with EEA relevance) OJ L 338, 1, 2005.

15. Turkish Food Codex: Microbiological Criteria Communique (in Turkish). Tebliğ No 2009/6. *Resmi Gazete,* Sayı: 27133, Tarih: 06.02.2009.

16. ISO/TS 22964 IDF/RM 210: Milk and Milk Products-Detection of *Enterobacter sakazakii*. 1st ed., ISO Copyright Office, Switzerland, 2006.

17. bioMérieux Inc: API® 20 E. Identification System for *Enterobacteriaceae* and Other Non-fastidious Gram-negative Rods. Ref: 20 100/20 160, Marcy l'Etoile, France, 2006.

18. bioMérieux Inc: VITEK® 2 Compact GN Card. Identification of *Enterobacteriaceae* and a Select Group of Glucose Non Fermenting Gram-negative Organisms. Ref: 21 341, Marcy l'Etoile, France, 2007.

19. Heuvelink AE, Kodde FD, Zwartkruis-Nahuis JTM, de Boer E: *Enterobacter sakazakii* in Melkpoeder. Project number OT 0110, Keuringsdienst van Waren Oost, 2001.

20. Iversen C, Forsythe S: Isolation of *Enterobacter sakazakii* and other *Enterobacteriaceae* from powdered infant formula milk and related products. *Food Microbiol,* 21 (6): 771-777, 2004.

21. Cawthorn DM, Botha S, Witthuhn RC: Evaluation of different methods for the detection and identification of *Enterobacter sakazakii* isolated from South African infant formula milks and the processing environment. *Int J Food Microbiol,* 127, (1-2): 129-138, 2008.

22. El-Sharoud WM, O'Brien S, Negredo, C, Iversen C, Fanning S, Healy B: Characterization of *Cronobacter* recovered from dried milk and related products. *BMC Microbiol,* 9, 24, 2009.

23. Beuchat LR, Kim H, Gurtler JB, Lin LC, Ryu JH, Richards GM: *Cronobacter sakazakii* in foods and factors affecting its survival, growth, and inactivation. *Int J Food Microbiol,* 136 (2): 204-213, 2009.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S167-S169, 2010
DOI:10.9775/kvfd.2009.2441

CASE REPORT

First Record of *Borrelia spielmani* in Turkey

Ayşen GARGILI * Kenan MİDİLLİ ** Sevgi ERGİN **
Gönül ŞENGÖZ *** Emine DERVİŞ **** Agustin ESTRADA PENA *****

* Marmara University, Infectious Diseases Epidemiology Research Center, 34660, İstanbul - TURKEY
** İstanbul University, Cerrahpaşa Medical Faculty, 34098, İstanbul - TURKEY
*** Haseki Education and Research Hospital, Department of Infectious Diseases, 34096, İstanbul - TURKEY
**** Haseki Education and Research Hospital, Department of Dermatology, 34096, İstanbul - TURKEY
***** Zaragoza University, Veterinary Faculty, 50013, Zaragoza - SPAIN

Makale Kodu (Article Code): KVFD-2009-2441

Summary

The almost worldwide distribution of *Borrelia burgdorferi* sl spirochaetes promotes the association with different tick species and thus different epidemiological pattern can be observed. Seven *B. burgdorferi* genospecies are currently recognized in Europe. Two of them (*B. bissettii* and *B. spielmani*) are still poorly characterized and most details about distribution are ignored. This paper reports the PCR detection of *B. spielmani* in a tick in the city of Istanbul (Turkey) collected while biting a human. This genospecies was previously known from The Netherlands, Germany, France, Hungary and Slovenia. This finding contributes to the further knowledge of the distribution of the different genospecies of *B. burgdorferi* sl.

Keywords: *B. burgdorferi sl*, *Tick*, *Turkey*

Türkiye'de İlk *Borrelia spielmani* Bulgusu

Özet

Borrelia burgdorferi sl. grubundaki spiroketlerin dünyadaki yayılışı farklı kene türleri ile ilişkilidir ve bu nedenle farklı epidemiyolojik özellikler gösterir. Avrupa ülkelerinde bugüne kadar yedi farklı *B. burgdorferi* genotipi bildirilmiştir. Bunlardan *B. bissettii* ve *B. spielmani* henüz yeterince tanımlanmamıştır ve yayılışları bilinmemektedir. Bu yazıda, İstanbul'da bir insanı tutmuş olan kene örneğinde PCR yöntemi ile saptanan *B. spielmani* bulgusu sunulmuştur. Bu genotip daha önce Hollanda, Almanya, Fransa, Macaristan ve Slovenya'dan bildirilmiştir. Bu bulgular, *B. burgdorferi* sl. genotipleri ve vektörlerinin yayılışları ile ilgili bilgilere katkıda bulunmaktadır.

Anahtar sözcükler: *B. burgdorferi sl.*, *Kene*, *Türkiye*

INTRODUCTION

Lyme borreliosis (LB), a tick-transmitted, systemic disease produced by *Borrelia burgdorferi* sensu lato (sl) exists as a zoonosis in Europe, North America, Asia and North Africa. In Europe, the presence of the agent has been reported in at least 26 countries [1]. Up to now, 12 *Borrelia* genospecies have been described under the broader name *B. burgdorferi* sl. Among them 3 are recognized as pathogenic for humans: *B. burgdorferi* sensu stricto (ss), *B. afzelii* and *B. garinii*. It appears that these three species cause different clinical manifestations in humans [2]. In North America, three *Borrelia* species have been reported, *B. burgdorferi* ss, *B. andersonii* and *B. bissettii*. In Asia, *B. burgdorferi* ss seems to be absent but *B. garinii, B. afzelii, B. valaisiana, B. japonica, B. tanukii, B. turdii* and *B. sinica* have been isolated. The distribution of the last four species is restricted to Asia. In North Africa, *B. lusitaniae, B. garinii* and *B. burgdorferi* ss have been described in *Ixodes ricinus* [2]. In Europe, 5

İletişim (Correspondence)
☎ +90 216 3271010
✉ aysen.gargili@marmara.edu.tr

species of *B. burgdorferi* sl have been reported in ticks: *B. burgdorferi* ss, *B. garinii, B. afzelii, B. valaisiana* and *B. lusitaniae*. In addition, two other genospecies have been obtained from patient tissues. *B. bissettii*, a species present in North America, has been also isolated from patients in Slovenia [3]. A novel *B. burgdorferi* sl genospecies (A14S) was cultured from patients with Erythema Migrans in The Netherlands, Hungary, Germany, and Slovenia [4-7] and from ticks in Ukraine, Germany and France [8-10]. *B. spielmani* was proposed as name for this spirochete [10]. Further species have been described from Spain [11] and Turkey [12]. The former has not yet been characterized. The later (named *B. turcica*) was isolated from *Hyalomma aegyptium*, a hard tick infesting tortoises, and formed a different cluster within the relapsing-fever spirochaetes [12,13]. For that last species, further microbiological, clinical and epidemiological studies are necessary to determine implications for human health. However, the high incidence of *H. aegyptium* biting humans in the same area where *B. turcica* was detected has been already reported [14].

CASE HISTORY

Ticks were passively surveyed in Istanbul since 2006 from human patients applying hospitals and reporting tick bites. After adequate determination of the ticks, pathogens were determined in these samples. A 30 years-old female who claimed to be bitten in Belgrad forest picnic area (30 km north of Istanbul) applied to the hospital as soon as she noticed the tick in her leg on May, 2007. For detection of *B. burgdorferi* sl. species, DNA was extracted from the tick using a commercial DNA extraction kit (Nucleospin tissue kit, Macherey Nagel, Germany). Primers (OSPA FW1: ttg gga ata ggt cta ata tta gc, OSPA FW2: atg yaa gca aaa tgt tag c, BOR R: act aat gtt ttv cca tct tc) amplifiying a 247 bp long part of outer surface protein A (ospA) of *B. burgdorferi* sl were used with a nested protocol. The reactions were performed in a final volume of 50 µl, comprising 0.5 µM each primer, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl (pH 8.3), 0.2 mM each deoxynucleoside triphosphate (Fermentas®, Lithuania), and 1.25 U of Taq DNA polymerase (Fermentas®, Lithuania) and 10 µl of DNA template. Mixture was subjected to an initial denaturation at 94°C for 2 min followed by 50 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min. Final extension was performed at 72°C for 10 min. For the second round amplification, 3 µl of the first round product was added to the 47 µl reaction mixture with the same concentrations and thermal cycling conditions of the first round mixture. The amplification products were visualized on 1.5% agarose gel electrophoresis under UV-light. After purification with a commercial PCR product purification kit (Roche®, Germany), the second round PCR products were subjected to the cycle sequencing using big-dye terminator kit (ABI®, USA). Following the cleaning-up procedure through sephadex-G50 fine columns the cycle sequencing products were run on an automated sequencer (ABI®, 310). The obtained sequence was edited and aligned using lasergene (DNA Star®) and Bioedit software packages [15] and was compared against data available in GenBank. The detected pathogen had a 99.59% of identity with the ospA sequences of *B. spielmani* (AY 995900, AF 102057) according to the molecular study results. The sequence was deposited in to the GenBank under the accession number EU545183.

DISCUSSION

In endemic areas in Europe, 6 *B. burgdorferi* sl genospecies may circulate between vertebrate hosts and ticks. Interestingly, in North Africa [2], *B. lusitaniae* is very frequent and greatly exceeds the other genospecies in ticks, whereas *B. lusitaniae* is only sporadically reported in ticks from other areas in Europe. The fact that *B. lusitaniae* is by far the dominant species in *I. ricinus* ticks in Portugal, Morocco and Tunisia indicates that the genospecies diversity of *B. burgdorferi* sl decreases towards the southern margin of its European distribution. *B. spielmani* has till now been detected in The Netherlands, Germany, France, Hungary, Ukraine and Slovenia [2,4-6,8-10]. Previous reports of *B. burgdorferi* sl in western Turkey including the city of Istanbul referred to *B. valaisiana, B. afzelii, B. garinii, B. lusitaniae* and *B. burgdorferi* sl [13]. Thus, the record of *B. spielmani* in the region considerable expands the geographical range of that spirochaete.

REFERENCES

1. Hubalek Z, Halouzka J: Distribution of *Borrelia burgdorferi* sensu lato genomic groups in Europe, a review. *Eur J Epidemiol,* 13, 951-957, 1997.

2. Gray J, Kahl O, Lane RS, Stanek G: Lyme Borreliosis: Biology, Epidemiology and Control. 201-250, CABI International, New York, 2002.

3. Picken RN, Cheng Y, Strle F, Picken MM: Patient isolates of *Borrelia burgdorferi* sensu lato with genotypic and phenotypic similarities to strain 25015. *J Inf Dis,* 174, 1112-1115, 1996.

4. Fingerle V, Michel H, Schulte-Spechtel U, Göttner G, Hizo-Teufel C, Hofmann H: A14S - A new *Borrelia burgdorferi* sl genospecies as relevant cause of human disease. *Int J Med Microbiol,* 294 (Suppl 1): 207, 2004.

5. Földvari G, Farkas R, Lakos A: *Borrelia spielmanii* Erythema migrans, Hungary. *Emerg Inf Dis,* 11, 794-795, 2005.

6. Maraspin V, Ruzic-Sablzic E, Strle F: Lyme Borreliosis and

Borrelia spielmanii. Emerg Inf Dis, 12, 1117, 2006.

7. Wang GQ, Van Dam AP, Dankert J: Phenotypic and genetic characterization of a novel *Borrelia burgdorferi* sensu lato isolate from a patient with Lyme borreliosis. *J Clin Microbiol,* 37, 3025-3028, 1999.

8. Nefedova VV, Korenberg EI, Andreichuk IV, Gorelova NB, Markov AV, Fadeeva IA, Evstafev IL: Genetic characterization of pathogenic Borrelia, group A14S, isolated in Ukraine. *Zh Mikrobiol Epidemiol Immunobiol,* 4, 23-7, 2005.

9. Rauter C, Oehme R, Diterich I, Enegele M, Hartung T: Distribution of clinically relevant Borrelia genospecies in ticks assessed by a novel single-run, real-time PCR. *J Clin Microbiol,* 40, 36-43, 2002.

10. Richter D, Schlee DB, Allgower R, Matuschka FR: Relationships of a novel Lyme disease spirochete, *Borrelia spielmani sp.* nov., with its hosts in Central Europe. *Appl Env Microbiol,* 70, 6414-6419, 2004.

11. Gil H, Barral M, Escudero R, Garcia-Perez AL, Anda P: Identification of a new *Borrelia* species among small mammals in areas of northern Spain where Lyme disease is endemic. *Appl Environ Microbiol,* 71, 1336-1345, 2005.

12. Güner ES, Watanabe M, Hashimoto N, Kadosaka T, Kawamura Y, Ezaki T, Kawabata H, Imai Y, Kaneda K, Masuzawa T: *Borrelia turcica sp.* nov., isolated from the hard tick *Hyalomma aegyptium* in Turkey. *Int J Syst Evol Microbiol,* 54, 1649-52, 2004.

13. Güner ES, Hashimoto N, Takada N, Kaneda K, Imai Y, Masuzawa T: First isolation and characterization of *Borrelia burgdorferi* sensu lato strains from *Ixodes ricinus* ticks in Turkey. *J Med Microbiol,* 52, 807-13, 2003.

14. Vatansever Z, Gargili A, Aysul NS, Sengoz G, Estrada-Peña A: Ticks biting humans in the urban area of Istanbul. *Parasitol Res,* 102, 551-553, 2008.

15. Hall TA: BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp,* 41, 95-98, 1999.

Kafkas Univ Vet Fak Derg
16 (Suppl-A): S171-S178, 2010
DOI:10.9775/kvfd.2010.1949

REVIEW

Cronobacter sakazakii (Enterobacter sakazakii): Only An Infant Problem?

Emek DUMEN *

* Istanbul University, School of Veterinary Medicine, Department of Food Hygiene and Technology, TR-34320 Avcılar, İstanbul - TURKEY

Makale Kodu (Article Code): KVFD-2010-1949

Summary

Cronobacter sakazakii has emerged as a rare cause of neonatal meningitis, septicemia and enterocolitis. Contaminated infant milk formula (IMF) has been identified as one infection route. Currently no agreed standardized typing protocol has been developed to trace *Cronobacter sakazakii.* This review article aims to inform the readers about the agent's taxonomy, isolation and typing, epidemiology, incidence in foods, and behavior in powdered infant formula.

Keywords: *Cronobacter sakazakii, Enterobacter sakazakii, Powdered infant formula, Neonatal meningitis*

Cronobacter sakazakii (Enterobacter sakazakii): Sadece Bebeklerin Problemi mi?

Özet

Cronobacter sakazakii, neonatal meningitis, septisemi ve enterokolitis hastalıklarının ana etkenlerinden biri olarak bildirilmektedir. Kontamine toz formül bebek mamaları ise önemli bir kontaminasyon kaynağıdır. *Cronobacter sakazakii*'nin izini sürmek için kullanılan standardize edilmiş bir izolasyon ve identifikasyon prosedürü bulunmamaktadır. Bu derlemede, etkenin taksonomisi, izolasyon ve tiplendirilmesi, epidemiyolojisi, gıdalardaki insidensi ve formül bebek mamalarındaki davranışları hakkında okuyuculara bilgi verilmesi amaçlanmışır.

Anahtar sözcükler: *Cronobacter sakazakii, Enterobacter sakazakii, Formül bebek mamaları, Neonatal meningitis*

INTRODUCTION

Cronobacter sakazakii (C. sakazakii) is an opportunistic pathogen causing meningitis, septicaemia and enterocolitis in neonates [1,2]. Preterm, low-birth-weight or immuno-compromised infants exposed to *C. sakazakii* are at particular risk [3]. Mortality rates of 10-80% have been described and survivors often suffer from neurological sequel [3-5]. Clinical outbreaks of infection in neonatal intensive care units associated with contaminated infant milk formula (IMF) have been reported [6,7] .

C. sakazakii is a ubiquitous organism [8]. The source of *C. sakazakii* and vehicle of transmission is not always clear however infant formula has been epidemiologically implicated as the source of *C. sakazakii* in several clinical cases [7,9]. The source of contamination of IMF is thought to include a broad range of dry blended raw material, together with possible environmental sources associated with the production environment. To minimize possible contamination of IMF both the raw materials and the production environment must be constantly monitored.

Molecular subtyping has been applied as a useful tool to facilitate surveillance, tracing routes from a source to an infected individual. Importantly, this approach makes it possible to distinguish a persistent environmental strain that could intrinsically contaminate IFM from extrinsic isolates introduced post-manufacturing. Generally methods based on phenotype analysis are acknowledged to be unreliable due to the unstable expression of the corresponding marker(s). For this

İletişim (Correspondence)
☎ +90 212 4737070/17154
✉ emekdumen@yahoo.com

reason DNA-based protocols offer an attractive alternative. Furthermore, DNA fingerprinting allows for a direct comparison of isolates in outbreaks. Previously, non-standardized DNA fingerprinting protocols have been applied to *C. sakazakii* [10]. Reported methods used include ribotyping, pulsed-field gel electrophoresis (PFGE) and random amplification of polymorphic DNA (RAPD) [10]. These molecular tools facilitate the trace back of outbreak isolates from clinical sources to the contaminated batch of powdered infant milk formula and/or the manufacturing environment. In addition, they are useful tools to target control strategies and reduce the risk of transmission. The only comparison between various molecular subtyping protocols in the literature was reported by Nazorowec - White and Farber [10].

In this paper it is aimed to inform the readers about *C. sakazakii*, which is a very important food borne pathogen that causes serious infections at almost all different age groups but especially neonates.

TAXONOMY and CHARACTERIZATION

Taxonomy, classification and nomenclature of genera in the family *Enterobacteriaceae* have evolved over the years based on various distinctions in serology, morphology, biochemical traits and genetic characteristics. There are 14 species or biogroups in the genus *Enterobacter* [11], however, for the recent years, the agent has been included in *Cronobacter* genus and this genus currently consists of 6 different species; *C. sakazakii, C. malonaticus, C. dublinensis, C. muytjensii,C. turicensis and C. genomospecies* [12]. This recently defined nomenclature is the result of a polyphasic taxonomic investigation aimed at re-defining this group of organisms. *Cronobacter spp.* are described as opportunistic pathogens, causing bacteremia, necrotizing enterocolitis (NEC), and meningitis in immunocompromised neonates [13]. More recently it has also emerged that *Cronobacter spp.* may cause infections among immunocompromised adults, in particular, elderly. Due to their ubiquitous nature, *Cronobacter spp.* have been isolated from a wide variety of foods. While primary reservoir of *Cronobacter* has yet to be defined, plant material is believed to be the likely source [2,12,13]. Major differences between *C. sakazakii* and other Enterobacter species have been traditionally thought to be its inability to ferment d-sorbitol and its ability to produce an extracellular deoxyribonuclease [14] . However, some strains of *C. sakazakii* more recently have been shown to ferment d-sorbitol [15].

Based on DNA-DNA hybridization showing yellow-pigmented strains to have less than 50% homology with non-pigmented strains [16], suggested that yellow-pigmented *E. cloacae* should comprise a new species. Phenotypic characterization and differentiation based on biochemical traits, serotyping, bacteriophage typing and antibiotic resistance are frequently among the first steps used to distinguish characteristics of isolates [10,17,18]. Some have suggested using phenotype tests (eg, biotyping, bacteriocin typing, serotyping and phage typing) to differentiate *Enterobacter* species; however, none of these tests has proven effective in distinguishing strains within the species, nor can they be used for all species of *Enterobacter* [10,19]. Iversen et al.[20], investigated the phylogenetic relationships of *C. sakazakii* using 16S ribosomal DNA and hsp60 sequencing. They found that strains were distributed among four clusters, indicating taxonomic heterogeneity. The type strain 16S rDNA sequence was 97.8% similar to that of *Citrobacter roseri* and 97.0% similar to that of *E. cloacae*. Studies have shown that both *Enterobacter* and *Cronobacter* genus are polyphyletic [21]. Strains currently classified as *C. sakazakii* fall into two distinct groups which can be further subdivided based on hsp60 sequences. Both genotypes include clinical strains and do not correspond to biochemical profiles.

Farmer et al.[14], extended the work of Brenner [16] and Brenner et al.[22] by further distinguishing 57 strains of yellow-pigmented *C. sakazakii* based on DNA hybridization, antibiotic susceptibility and biochemical reactions. Other distinguishing characteristics of the bacterium include greater pigment production at temperatures less than 36.8°C, with optimum pigment production at 25.8°C, survival of cells in stock cultures stored at 17-30.8°C without transfer for up to 8 years, utilization of citrate as a sole carbon source, 31-49% DNA-DNA homology with *E. cloacae*, and 57% guanine + cytosine ratio [14]. Production of the diffusible yellow pigment is unstable with repeated subculturing.

In addition to phenotypic characterization of *C. sakazakii*, advances have been made in fingerprinting DNA and RNA by several techniques, eg, PCR, randomly amplified polymorphic DNA (RAPD) PCR, pulsed-field gel electrophoresis (PFGE), chromosomal DNA restriction analysis, ribotyping and plasmid typing [23]. Nazarowec-White and Farber [10] ribotyped *C. sakazakii* with the *EcoR1* restriction endonuclease and found that 18 isolates were represented by 10 ribotypes. This analysis has been determined to be more discriminatory than that of restriction endonuclease analysis (REA) [24]. In another study [30], *C. sakazakii* isolates from an infant formula factory comprised only 8 ribotypes [25]. Kornacki [26] isolated 17 *EcoR1* ribotypes from a factory environment. Nazarowec-White and Farber [10] analyzed 18 isolates by

PFGE using the restriction endonuclease *Xba1* and found each to have a distinct pattern. Characterization was superior to ribogrouping in that two sets of three isolates, comprising only two ribogroups, were distinguishable as six distinct pulsovars.

C. sakazakii has been shown to exhibit substantial resistance to acid pH. Edelson-Mammel and Buchanan [27] examined survival characteristics of 12 strains of *C. sakazakii* in tryptic soy broth adjusted to pH 3.0 and 3.5 with HCl. Ten of twelve strains showed less than a l-log decline over a 5-h period at 37.8°C; reductions in TSB at pH 3.0 were 4.9 to 6.3 log CFU/ml. There was no correlation in acid resistance based on 1-h/pH 3.0 results and previously determined heat resistance of test strains [27]. Skladal et al.[28] examined the fermentation of milk inoculated with 10-15 CFU of *C. sakazakii* per 500 ml and incubated at 30.8°C. Changes in pH and the production of L-lactate and D-lactate were monitored. *C. sakazakii* fermented milk rapidly, reducing the pH from 6.6 to 5.6 in less than 20 h. Concentrations of L-lactate and D-lactate reached 0.40 mM and 10.7 mM, respectively.

ISOLATION, IDENTIFICATION and TYPING

FDA [29,30] developed a method to isolate and enumerate *C. sakazakii* in dehydrated powdered infant formula. *Table 1* indicates the enumeration procedure described above.

The method of ISO31 to isolate and enumerate *C. sakazakii* in dehydrated powdered infant formula is indicated in *Table 2* below.

Further characterization of *C. sakazakii* isolated from food and environmental samples can be accomplished by using pulsed PFGE, RFLP, multilocus enzyme electro-phoresis tests, or ribotyping. Other potential methods of analyses include testing for antibiotic resistance patterns (antibiograms), toxin assays, hemagglutination, serotyping and phage typing.

INCIDENCE in FOODS

C. sakazakii has been isolated from a wide spectrum of environmental sources [32] including water waste [33] and thermal spring water [34], soil, dust from households and food production-lines [8]. *C. sakazakii* has an unusual surviving ability under dry conditions [35], but the thermal tolerance of *C. sakazakii* strains may differ [36]. Pasteurization is effective in destroying *C. sakazakii* [37]. Acidification reduced the concentration of *C. sakazakii* in different types of infant formula and vegetable based food products [38]. In juices of vegetables, the reduction of pH after 48 h was correlated with a reduction of the numbers of *C. sakazakii*, but with increasing numbers of *C. sakazakii* in juices of different fruits [39].

Table 1. *Enumeration procedure of C. sakazakii for infant formula according to FDA*

Tablo 1. *C. sakazakii için, FDA'ya gore formül mamalardaki sayım prosedürü*

Step	Time	Temperature
Dilute 100 g, 10 g, 1 g of powdered infant formula with pre-warmed sterile distilled water at 1:10 ratio. Mix and incubate	Overnight	36ºC
Add 10 ml of each suspension to 90 ml of *Enterobacteriaceae* enrichment broth and incubate	Overnight	36ºC
Mix suspensions and surface plate 0.1 ml on VRBG agar, streak on VRBG agar with a 10 Al inoculating loop onto three quadrants for isolation and incubate	Overnight	36ºC
Pick five presumptive-positive *C. sakazakii* colonies from both sets of VRBG plates and subculture by streaking onto TSA and incubate	48-72 h	25ºC
Select yellow-pigmented colonies only and confirm per manufacturer's instructions for the API 20E biochemical confirmation system	-	-
Calculate the most probable number (MPN) after determining the number of positive tubes at each dilution	-	-

Table 2. *Enumeration procedure of C. sakazakii for infant formula according to ISO*

Tablo2. *C. sakazakii için, ISO'ya gore formül mamalardaki sayım prosedürü*

Step Number	Name of the Procedure	Description	Time	Temperature
1	Pre - enrichment in non selective liquid medium	The pre - enrichment medium is inoculated with the test portion	16 h to 20 h	37ºC±1ºC
2	Enrichment in selective liquid medium	The selective enrichment medium is inoculated with culture obtained in step 1	22 h to 26 h	44ºC±0.5ºC
3	Plating and identification	A chromogenic agar is inoculated with the enrichment culture obtained in step 2	22 h to 26 h	44ºC±1ºC
4	Confirmation	Typical colonies are selected from the chromogenic agar and isolates producing yellow pigment on tryptone soya agar are biochemically characterized	-	-

There is no essential need for special microbiological criteria of *C. sakazakii* in food other than infant formula, because *C. sakazakii* is a ubiquitous opportunistic micro-organism. *C. sakazakii* will be detected only in studies with the aim of differentiating genus *Cronobacter* or specially of tracking *C. sakazakii*. Iversen and Forsythe [40] referred in their risk-profile to *C. sakazakii* contamination in food. In 2004, they published a survey about the isolation of *C. sakazakii* from a variety of powdered infant formulas, milk powders and related food products [36]. Drudy et al.[32] compared biochemical and molecular-genetic methods in the investigation of 57 European and Australasian *C. sakazakii* isolates. The researchers indicated that 51 isolates of 57 were food originated.

C. sakazakii was isolated from wheat [41] and as endophytic bacteria from the leaves of rice plants [42]. Kanivets and Pishchur [43] detected *C. sakazakii* in the bacterial colonization flora of disinfected sugar beet seeds. As *C. sakazakii* belongs to the cultivable endophytic and epiphytic flora of rice [42] and soy bean plants [44], it could be isolated from related food products. Some traditional cereal, herb and legume-based food and beverages were found to be contaminated with *C. sakazakii* [45]. *C. sakazakii* may be part of starter cultures for fermentation of traditional vegetarian food products. Osterblad et al.[46] detected *C. sakazakii* in mixed salad vegetables and imported fresh and deep-frozen vegetables at retail level.

C. sakazakii contaminated food of animal origin comprise a variety of meat and meat products from camel, pig, beef and poultry, and, additionally, eggs, raw milk and different dairy products and, less frequently, fish. *C. sakazakii* was isolated from a variety of raw and ready-to-eat meat and its products. Watanabe and Esaki [47] isolated *C. sakazakii* during a complicated curing process of meat products. *C. sakazakii* is a histamine forming microorganism in the ripening process of cheese [48]. *C. sakazakii* has been isolated from a cheese whey substrate [49]. Lipolytic activity of a *C. sakazakii* strain was demonstrated by Chaves-Lopez et al.[50]. *C. sakazakii* has been detected in fresh and prepared fish. Miranda et al.[51] isolated a tetracycline-resistant *C. sakazakii* strain from a Chilean freshwater salmon farm with no history of recent antibiotic use. *C. sakazakii* has been isolated from smoked sardines after 12 weeks of storage after irradiation [52].

Schindler and Metz [53] found *C. sakazakii* in total frequencies of 1.8% (10/564 strains) and 0.4% (1/256 strains) investigating central and local drinking water supplies. Lee and Kim [54] identified *C. sakazakii* as bacteria indigenous to the water distribution system during their investigations for biofilm formation. Even bottled beverages should not be considered as free of micro-organisms, as shown by the results of Schindler [53] for *C. sakazakii* contaminated bottled mineral water.

EPIDEMIOLOGY

Many reservoirs exist for these bacteria, including water, soil, food, and the intestines of humans and animals. There are several modes of transmission for these organisms, including exogenous, such as fecal-oral, person-person, mother-child, food, hospital equipment, and personnel, and endogenous, from the patient's own intestinal flora. Passive carriage on the hands of medical personnel constitutes the major mode of transmission. *C. sakazakii* can also be isolated from tap and bottled water and can survive and multiply on or in hospital equipment such as hemodialysis and respiratory instruments [11].

Respiratory tract infections are often caused by gram-negative bacteria. Indeed, sputum is the first or second most common clinical specimen to yield *Enterobacter* isolates and, although these bacteria do not represent the most predominant pathogens causing respiratory infections, they are significant because of their antibiotic resistance. *E. cloacae, E. amalonaticus, E. agglomerans, E. amnigenus, E. asburiae, E. cancerogenus, E. gergoviae, E. normaechei,* and C*. sakazakii (Enterobacter sakazakii)* are species that have been isolated from respiratory tract infections [11]. These bacteria can be transmitted exogenously through hospital procedures, such as surgery or with intubation, inhalation / aspiration, or hematogenous spread to the lungs. Prior antibiotic treatment may predispose patients to *Enterobacter pneumonia,* and *Cronobacter* are a major cause of pneumonia in early post-lung transplant patients, with the bacteria originating from the donor [55].

Meningitis and brain abscesses resulting from *Citrobacter* or *Cronobacter* infections occur most often in neonates, but can also appear in immunocompromised patients and following neurosurgery [56]. The causative agents are primarily *Citrobacter koseri (diversus)* and *Cronobacter sakazakii* and, occasionally, *Citrobacter freundii* [57]. Transmission of the bacteria to the infant can occur horizontally during nosocomial outbreaks in neonatal hospital wards or from contaminated infant formula/powdered milk [58]. They can also be transmitted vertically from a colonized mother [59]. While premature or low birth-weight babies are more susceptible to such infections, any neonate can be affected. Among neonates, the meningitis often results in vasculitis, cerebritis and/or ventriculitis, the development of hydrocephalus, and a surprising rate of brain abscesses and cyst

formation [59]. The brain abscess can persist with little response to antimicrobial therapy.

CLINICAL ETIOLOGY and PATHOGENICITY

Cronobacter species can create community infections, are responsible for approximately half of all nosocomially acquired infections and are often implicated in co-infections. Infections reported in infants include meningitis leading to ventriculitis, brain abscess, and infarction and cyst formation [13]. *C. sakazakii* can cause also systemic respiration, cardiovascular and neurologic symptoms such as destruction of the frontal lobes of the brain, seizures, spastic quadriplegia, hypothermia, fever Cheyne-Stokes respirations, bradycardia, poor feeding, irritability, jaundice, grunting respirations, instability of body temperature, hemorrhagic cerebral necrosis, meningo encephalitis, necrotic softened brain, cyst formation, liquefaction of cerebral white matter and severe neurologic complications at infants, adults and also at elderly patients, too [60].

The symptoms of respiratory tract infections are similar to those seen with *Streptococcus pneumoniae*. Symptoms, which generally occur gradually, include malaise, slowly increasing fever, and/or chills and a cough. The cough will eventually produce sputum, which may be discolored and foul smelling, and the patient may experience shortness of breath. In cases of chronic pneumonia or lower respiratory tract infection, the individual may also experience appetite and weight loss [61]. A chest radiograph and culture of sputum samples are useful in identifying the etiological agent.

Gastroenteritis infections produce symptoms similar to those that occur with other enteropathogenic bacteria such as *E. coli* or *Shigella spp*. The symptoms generally appear suddenly, with loss of appetite, nausea, vomiting, intestinal/abdominal cramps, gas, and watery diarrhea. A fever and mylagia may also be present. Also drop in blood pressure may come out from loss of electrolytes due to dehydration at the infected cases. Patients with hemorrhagic colitis may experience little or no fever and bloody and/or watery diarrhea. Some may develop hemolytic-uremic syndrome that can lead to kidney failure, anemia, seizures, strokes, and nerve or brain damage [62].

Sepsis occurs when bacterial numbers in the blood are too high for efficient removal by white blood cells leading to septic shock. Bacteria normally enter the bloodstream and cause sepsis when there is an infection elsewhere in the body. Symptoms include fever, chills, shaking, nausea, vomiting, diarrhea, and general malaise. The patient will normally have a high white blood cell count. Sepsis can also lead to infections in other parts of the body, such as the brain (meningitis), heart (endocarditis), bone (osteomyelitis), or soft tissue [63].

Meningitis and brain abscesses most commonly occur in neonates and present with fever, vomiting, lack of appetite, irritability, high-pitched crying, and seizures. The forehead may bulge and the head may swell. In those older than one year of age, fever, irritability, drowsiness, confusion, and a painful stiff neck are common. The symptoms can progress to coma and death very rapidly. A lumbar puncture is required to determine the cause of infection if meningitis is suspected [63].

TREATMENT

Cronobacter and Enterobacter infections are treated with antibiotics. Care must be taken when choosing an antibiotic because of the intrinsic resistance to beta-lactams and cephalosporins, and the emergence of plasmid-mediated resistance to aminoglycosides, quinolones, and third-generation cephalosporins [64].

For *Cronobacter* and *Enterobacter* infections, carbapenems or antipseudomonal penicillins (ie, mezlocillin, piperacillin, piperacillin/tazobactam, ticarcillin, and ticarcillin/clavulanate) are recommended as drugs of choice, with ciprofloxacin as an alternative [64]. It is recommended that the drug resistance pattern of the organism be established early on so the infection can be treated properly form the beginning [65]. In the case of brain abscesses, surgery may be necessary to drain the abscess, as they do not always respond to antibiotic therapy [66]. Patients with gastrointestinal illness must have fluid and electrolytes replaced.

RISK MANAGEMENTS, CRITICAL CONTROL POINTS and HAZARD ANALYSIS

The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) jointly convened a workshop on *C. sakazakii* in early 200467 in response to a request for scientific advice from the Codex Committee on Food Hygiene to provide input for the revision of the Recommended International Code of Hygienic Practice for Goods and Infants and Children. An extensive list of recommendations to FAO, WHO, Codex Committee on Food Hygiene, their member countries, and Non-Governmental Organizations (NGOs) was issued [67] *(Table 3)*.

Table 3. *Joint FAO/WHO recommendations to the powdered infant formula industry and infant caregivers concerning processing, preparing and handling powdered and reconstituted products*

Tablo 3. *FAO/WHO kurumlarının formül mama endüstrisi ve sektörle ilgili kuruluşlar için tavsiye ettikleri hazırlama, üretim, proses prosedürleri için tavsiyesi*

Recommendations to the Powdered Infant Formula Industry By FAO/WHO
In situations where infants are not breast-fed, caregivers, particularly of infants at high risk, should be regularly alerted that powdered infant formula is not a sterile product and can be contaminated with pathogens that can cause serious illness and provided with information that can reduce the risk
In situations where infants are not breast-fed, caregivers of high-risk infants should be encouraged to use, whenever possible and feasible, commercially sterile liquid formula or formula which has undergone an effective point of use decontamination procedure (e.g., use of boiling water to reconstitute or by heating reconstituted formula)
Guidelines should be developed for the preparation, use and handling of infant formula to minimize risk
Research should be promoted to gain a better understanding of the ecology, taxonomy, virulence and other characteristics of *C. sakazakii* and on ways to reduce its levels in reconstituted powdered infant formula
Investigation and reporting of sources and vehicles, including powdered infant formulae, of infection by *C. sakazakii* and other Enterobacteriaceae and *Cronobacter* genus should be encouraged. This could include the establishment of a laboratory-based network
The use of internationally validated detected and molecular typing methods for *C. sakazakii* and other relevant microorganisms should be promoted
FAO/WHO should address the particular needs of some developing countries in establishing effective measures to minimize risk in situations where breast-milk substitutes may be used in exceptionally difficult circumstances, e.g., feeding infants of HIV-positive mothers or low-birth-weight infants
In revising its Code of Practice, Codex should better address the microbiological risks of powdered infants formula and, if deemed necessary, include the establishment of appropriate microbiological specifications for *C. sakazakii* in powdered infant formula
The infant food industry should be encouraged to reduce the concentration of prevalence of *C. sakazakii* in both the manufacturing environment and powdered infant formula. To this end, the infant food industry should consider implementing an effective environmental monitoring program
The infant food industry should be encouraged to develop a greater range of commercially sterile alternative formula products for high-risk groups

Summarized from FAO/WHO (2004) [67]

CONCLUSION

C. sakazakii can be found in a wide range of foods and beverages, many of which are not subjected to treatments or processes that inactivate the pathogen. Its ability to survive and grow in these products raises concern about safety risks not only to neonates and infants but also to older immunocompromised consumers. The suitability of infant cereals and some types of fresh fruits and vegetables to support luxuriant growth of *C. sakazakii* is of particular concern. The ability of the pathogen to produce biofilms, coupled with its resistance to sanitizers and disinfectants when present in organic matrices, emphasizes the importance of properly cleaning and sanitizing food preparation areas and utensils and containers used to prepare and serve foods to neonates and others in hospital, daycare center, and home settings.

Studies involving *C. sakazakii* have focused on methods to eliminate the coliform from powdered infant formula, to determine thermal resistance, environmental reservoirs, pathogenicity, antibiotic resistance, exopolysaccharide production and to develop rapid methods detection, enumeration and identification, subtyping and predictive modeling, but additional researches in these and other areas are needed. One study using a suckling mouse model to determine virulence mechanisms and minimum infectious dose has suggested the possibility of enterotoxin production by *C. sakazakii*.

Studies to determine conditions that influence survival and growth or cause death of *C. sakazakii* in dry and reconstituted infant formulae are needed, given the likelihood that post-process contamination is the principle route of contamination. Other areas in need of research attention include studies of conditions affecting biofilm formation by *C. sakazakii* in processing plants and hospital settings (eg, in tubes used for enteral feeding), competitive exclusion to control or prevent growth, efficacy of sanitizers, methods to recover and and evaluation of practices associated with preparing and feeding infant formulae in hospitals and in the home. Surveys of neonatal wards, Neonatal Care Units and food processing environments for the presence of *C. Sakazakii* and an evaluation of hygienic practices in hospitals and the home that may contribute to neonatal infections would also provide information of value when developing intervention strategies to eliminate *C. sakazakii* infection.

REFERENCES

1. Bar-Oz B, Preminger A, Peleg O, Block C, Arad I: *Enterobacter sakazakii* infection in the newborn. *Acta Paediatr,* 90, 356-358, 2001.

2. Muytjens HL, Kollee LA: Neonatal meningitis due to *Enterobacter sakazakii. Tijd Kindergeneeskunde,* 50, 110-112, 1982.

3. Lai KK: *Enterobacter sakazakii* infections among neonates, infants, children, and adults. Case reports and a review of the literature. *Medicine (Baltimore),* 80, 113-122, 2001

4. Gallagher PG, Ball WS: Cerebral infarctions due to CNS infection with *Enterobacter sakazakii. Pediatr Radiol,* 21, 135-136, 1991.

5. Ries M, Harms D, Scharf J: Multiple cerebral infarcts with resulting multicystic encephalomalacia in a premature infant with *Enterobacter sakazakii* meningitis. *Klin Pediatr,* 206, 184-186, 1994.

6. Block C, Peleg O, Minster N, Bar-Oz B, Simhon A, Arad I, Shapiro M: Cluster of neonatal infections in Jerusalem due to unusual biochemical variant of *Enterobacter sakazakii. Eur J Clin Microbiol Infect Dis,* 21, 613-616, 2002.

7. van Acker J, de Smet F, Muyldermans G, Bougatef A, Naessens A, Lauwers S: Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. *J Clin Microbiol,* 39, 293-297, 2001.

8. Kandhai MC, Reij MW, Gorris LG, Guillaume-Gentil O, van Schothorst M: Occurrence of *Enterobacter sakazakii* in food production environments and households. *Lancet,* 363, 39-40, 2004.

9. Weir E: Powdered infant formula and fatal infection with *Enterobacter sakazakii. Can Med Assoc J,* 166, 1570, 2002.

10. Nazarowec-White M, Farber JM: Phenotypic and genotypic typing of food and clinical isolates of *Enterobacter sakazakii. J Med Microbiol,* 48, 559-567, 1999.

11. Farmer, JJ: *Enterobacteriaceae:* Introduction and identification. **In,** Murray P, Baron EJ, Pfaller MA, Tenover FC, Yolken RH (Eds): Manual of Clinical Microbiology. pp. 442-458, ASM Press, Washington, DC, 1999.

12. Iversen C, Mullane N, McCardell B, Tall BT, Lehner A, Fanning S, Stephan R, Joosten H: *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus sp.* nov., *Cronobacter turicensis sp.* nov., *Cronobacter muytjensii sp.* nov., *Cronobacter dublinensis sp.* nov., *Cronobacter genomospecies* 1, and of three subspecies, *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov., *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov. and *Cronobacter dublinensis* subsp. *lactaridi* subsp. nov. *Int J Syst Evol Microbiol,* 58, 1442-1447, 2008.

13. Baumgartner A, Grand M, Liniger M, Iversen C: Detection and frequency of *Cronobacter (Enterobacter sakazakii)* in different categories of ready-to-eat foods other than infant formula. *Int J Food Microbiol,* 136, 189-192, 2009.

14. Farmer JJ, Asbury MA, Hickman FW, Brenner DJ: *Enterobacter sakazakii*, new species of *Enterobacteriaceae* isolated from clinical specimens. *Int J Syst Bacteriol,* 30, 569-584, 1980.

15. Heuvelink AE, Kodde FD, Zwartkruis-Nahuis JTM, de Boer E: *Enterobacter sakazakii* in melkpoeder. Keuringsdienst van Waren Oost. Project number OT 0110, 2001.

16. Brenner DJ: DNA reassociation for the clinical differentiation of enteric bacteria. *Public Health Lab,* 32, 118-130,1974.

17. Arbeit RD: Laboratory procedures for the epidemiologic analysis of microorganisms. **In,** Murray PR, Baron EJ, Pfaller MA, Tenover RC, Yolken RD (Eds): Manual of Clinical Microbiology. 6th ed., American Society for Microbiology, Washington, DC, pp. 190-208, 1995.

18. Einstein BI: New molecular techniques for microbial epidemiology and the diagnosis of infectious diseases. *J Infect Dis,* 161, 595-602, 1990.

19. Poilane I, Cruaud P, Lachissinne E, Grimont, F, Grimont P, Collin M, Gaudelus J, Torlotin JC, Collignon A: *Enterobacter cloacae* cross-colonization in neonates demonstrated by ribotyping. *Eur J Clin Microbiol Infect Dis,* 12, 820-826, 1993.

20. Iversen C, Forsythe S: Isolation of *Enterobacter sakazakii* and other *Enterobacteriaceae* from powdered infant formula milk and related products. *Food Microbiol,* 21, 771-777, 2004.

21. Loc-Corrillo C, Waddington M, Lu X, Forsythe S: Phylogenetic relationship of *Enterobacter sakazakii* and related organisms. Abstract P-005, 104th Gen Mt Am Soc Microbiol, 23-27 May, New Orleans, LA, USA, 2004.

22. Brenner DJ, Farmer JJ, Hickman FW, Asbury MA, Steigerwalt AG: Taxonomic and Nomenclature Changes in *Enterobacteriaceae*. Centers for Disease Control and Prevention, Atlanta, GA, 1977.

23. Grant KA, Kroll RG: Molecular biology techniques for the rapid detection and characterization of foodborne bacteria. *Food Sci Technol,* 7, 80-88, 1993.

24. Clark NC, Hill BC, O'Hara CM, Steingrimsson O, Cooksey RC: Epidemiologic typing of *Enterobacter sakazakii* in two neonatal nosocomial outbreaks. *Diagn Microbiol Infect Dis,* 13, 467-472, 1990.

25. Anonymous: *Enterobacter sakazakii* in Infant Formula. Riboprinterk Microbial Characterization System, Application Profile: Dupont Central Research and Development, Wilmington, DE, 1996.

26. Kornacki JL: *Enterobacter sakazakii*: Pursuit of a putative pathogen in a dairy powder Factory (a case study). *American Dairy Science Association Annual Meeting,* Denver, CO, 1998.

27. Edelson-Mammel SG, Buchanan RL: Thermal inactivation of *Enterobacter sakazakii* in rehydrated infant formula. *J Food Prot,* 67, 60-63, 2004.

28. Skladal P, Mascini M, Salvadori C, Zannoni G: Detection of bacterial contamination in sterile UHT milk using an l-lactate biosensor. *Enzyme Microbiol Technol,* 15, 508-512,1993.

29. Anonymus: U.S. Food and Drug Administration, 2002a. Isolation and enumeration of *Enterobacter sakazakii* from dehydrated powdered infant formula. http://www.cfsan.fda.gov/~comm/mmesakaz.html. *Accessed:* 19.09.2003.

30. Anonymus: U.S. Food and Drug Administration, 2002b. Questions and answers on method for *E. sakazakii* in powdered infant formula. http://www.cfsan.fda.gov/~comm/mmesakqa.html. *Accessed:* 10.10.2003.

31. Inernational Standardization for Organmization (ISO): Technical Specification, ISO / TS 22964, 2006.

32. Drudy D, O'Rourke M, Murphy M, Mullane NR, O'Mahony R, Kelly L, Fischer M, Sanjaq S, Shannon P, Wall P: Characterization of a collection of *Enterobacter sakazakii* isolates from environmental and food sources. *Int J Food Microbiol,* 110, 127-134, 2006.

33. Dudley DJ, Guentzel MN, Ibarra MJ, Moore BE, Sagik BP: Enumeration of potentially pathogenic bacteria from sewage sludges. *Appl Environ Microbiol,* 39, 118-126, 1980

34. De Los AngelesMosso MD, Delarosa MD, Vivar C, Medina MD: Heterotrophic bacterial populations in the mineral waters of thermal springs in Spain. *J Appl Bacteriol,* 77, 370-381, 1994.

35. Gurtler JB, Kornacki JL, Beuchat LR: *Enterobacter sakazakii*: A coliform of increased concern to infant health. *Int J Food Microbiol,* 104, 1-34, 2005.

36. Edelson-Mammel SG, Buchanan RL: Acid resistance of twelve strains of *Enterobacter sakazakii*. Abstract P170, Program and Abstract Book, 91st Annu Mtg Int. Assn. Food Prot, 8-11 August, Phoenix, AZ, 2004.

37. Iversen C, Waddington M, On SLW, Forsythe S: Identification and phylogeny of *Enterobacter sakazakii* relative to *Enterobacter* and *Citrobacter* species. *J Clin Microbiol,* 42, 5368-5370, 2004.

38. Joosten HM, Lardeau A: Enhanced microbiological safety of acidified infant formulas tested *in vitro.* SAJCN, 17, 87-92, 2004.

39. Kim H, Beuchat LR: Survival and growth of *Enterobacter sakazakii* on fresh-cut fruits and vegetables and in unpasteurized juices as affected bystorage temperature. *J Food Prot,* 68, 2541-2552, 2005.

40. Iversen C, Forsythe S: Risk profile of *Enterobacter sakazakii,* an emergent pathogen associated with infant milk formula. *Trends Food Sci Technol,* 14, 443-454, 2003.

41. Forlani G, Mantelli M, Branzoni M, Nielsen E, Favilli F: Differential sensitivity of plant-associated bacteria to sulfonylurea and imidazolinone herbicides. *Plant Soil,* 176, 243-253, 1995.

42. Yang HL, Sun XL, Song W, Wang YS, Cai MY: Screening, identification and distribution of endophytic associative diazotrophs isolated from rice plants. *Acta Botan Sin,* 41, 927-931, 1999.

43. Kanivets VI, Pishchur IN: Bacterial microflora on disinfected sugar beet seeds. *Microbiology,* 70, 316-318, 2001.

44. Kuklinsky-Sobral J, Araujo WL, Mendes R, Pizzirani-Kleiner AA, Azevedo JL: Isolation and characterization of endophytic bacteria from soybean *(Glycine max)* grown in soil treated with glyphosate herbicide. *Plant Soil,* 273, 91-99, 2005.

45. Nassereddin RA, Yamani MI: Microbiological quality of sous and tamarind, traditional drinks consumed in Jordan. *J Food Prot,* 68, 773-777, 2005.

46. Osterblad M, Pensala O, Peterzens M, Heleniuse H, Huovinen P: Antimicrobial susceptibility of *Enterobacteriaceae* isolated from vegetables. *J Antimicrob Chemother,* 43, 503-509, 1999.

47. Watanabe I, Esaki M: Studies on an unusual case of fermentation of meat-products during the curing process. *J Antibact Antifung Agents,* 22, 9-14, 1994.

48. Morales P, Feliu I, Fernandez-Garcia E, Nunez M: Volatile compounds produced in cheese by *Enterobacteriaceae* strains of dairy origin. *J Food Prot,* 67, 567-573, 2004.

49. De Haast J, Britz TJ: Characterization of aerobic and facultative anaerobic bacteria from the liquid phase of an anaerobic fixed-bed digester treating a cheese whey substrate. *Microb Ecol,* 12, 173-179, 1986.

50. Chaves-Lopez C, De Angelis M, Martuscelli M, Serio A, Paparella A, Suzzi, G: Characterization of the *Enterobacteriaceae* isolated from an artisanal Italian ewe's cheese (Pecorino Abruzzese). *J Appl Microbiol,* 101, 353-360, 2006.

51. Miranda CD, Kehrenberg C, Ulep C, Schwarz S, Roberts MC: Diversity of tetracycline resistance genes in bacteria from Chilean salmon farms. *Antimicrob Agents Chemother,* 47, 883-888, 2003.

52. Nketsia-Tabiri J, Adu-Gyamfi A, Montford KG, Gbedemah CM, Sefa-Dedeh S: Optimising processing conditions for irradiated cured fish. *Int Atomic Energy Agency Techn Doc,* 1337, 207-216, 2003.

53. Schindler PRG, Metz H: Coliform bacteria in drinking water from South Bavaria: Identification by the API 20E-system and resistance patterns. *Water Sci Technol,* 24, 81-84, 1991.

54. Lee DG, Kim SJ: Bacterial species in biofilm cultivated from the end of the Seoul water distribution system. *J Appl Microbiol,* 95, 317-324, 2003.

55. Sinave C: Enterobacter Infections. E-Medicine: Instant Access to the Minds of Medicine. E - Medicine, 2002.

56. Huang CR, Lu CH, Chang WN: Adult *Enterobacter* meningitis: A high incidence of coinfection with other pathogens and frequent association with neurosurgical procedures. *Infection,* 29, 75-79, 2001.

57. Tang LM, Chen ST, Lui TN: Citrobacter meningitis in adults. *Clin Neurol Neurosurg,* 96, 52-57, 1994.

58. Biering G, Karlsson S, Clark NC, Jonsdottir KE, Ludvigsson P, Steingrimsson O: Three cases of neonatal meningitis caused by *Enterobacter sakazakii* in powdered milk. *J Clin Microbiol,* 27, 2054-2056, 1989.

59. Doran TI: The role of Citrobacter in clinical disease of children: review. *Clin Infect Dis,* 28, 384-394, 1999.

60. Willis J, Robinson JE: *Enterobacter sakazakii* meningitis in neonates. *Pediatr Infect Dis J,* 7, 196-199, 1988.

61. Ray CG, Ryan KJ: Middle and Lower Respiratory Tract Infections. **In,** Sherris J (Ed): Medical Microbiology: An Introduction to Infectious Diseases, pp. 584-591, Elsevier Science Publishing Co. Inc., New York, 1984.

62. Ryan KJ: Enteric Infections and Food Poisoning. **In,** Sherris J (Ed): Medical Microbiology: An Introduction to Infectious Diseases, pp. 592-600, Elsevier Science Publishing Co. Inc., New York, 1984.

63. Arseni A, Malamou-Ladas E, Koutsia C, Xanthou M, Trikka E: Outbreak of colonization of neonates with *Enterobacter sakazakii. J Hosp Infect,* 9, 143-150, 1987.

64. Gardam MA, Burrows LL, Kus JV, Brunton J, Low DE, Conly JM, Humar A: Is surveillance for multidrug-resistant *Enterobacteriaceae* an effective infection control strategy in the absence of an outbreak. *J Infect Dis,* 186, 1754-1760, 2002.

65. Wickwire C, Gilbert D, Moellering RC, Sande MA: The Sanford Guide to Antimicrobial Therapy. Edition 33, pp. 33-36, Jeb C. Sanford, Antimicrobial Therapy Inc., Hyde Park, VT, 2003.

66. Kline MW, Mason EO Jr, Kaplan SL: Characterization of *Citrobacter diversus* strains causing neonatal meningitis. *J Infect Dis,* 157, 101-105, 1988.

67. Food and Agriculture Organization/World Health Organization: Workshop on *Enterobacter sakazaki*i and other microorganisms in powdered infant formula, Geneva, 2004.

YAZIM KURALLARI

1- Yılda 6 (Altı) sayı olarak yayımlanan Kafkas Üniversitesi Veteriner Fakültesi Dergisi'nde (Kısaltılmış adı: Kafkas Univ Vet Fak Derg) Veteriner Hekimlik ve Hayvancılıkla ilgili (klinik ve paraklinik bilimler, hayvancılıkla ilgili biyolojik ve temel bilimler, zoonozlar ve halk sağlığı, hayvan besleme ve beslenme hastalıkları, hayvan yetiştiriciliği ve genetik, hayvansal orijinli gıda hijyeni ve teknolojisi, egzotik hayvan bilimi) orijinal araştırma, kısa bildiri, ön rapor, gözlem, editöre mektup, derleme ve çeviri türünde yazılar yayımlanır. Dergide yayımlanmak üzere gönderilen makaleler Türkçe, İngilizce veya Almanca dillerinden biri ile yazılmış olmalıdır.

2- Dergide yayımlanması istenen yazılar Times New Roman yazı tipi ve 12 punto ile A4 formatında, 1.5 satır aralıklı ve sayfa kenar boşlukları 2.5 cm olacak şekilde hazırlanmalı ve resim, tablo, grafik gibi şekillerin metin içindeki yerlerine Türkçe ve yabancı dilde adları ve gerekli açıklamaları mutlaka yazılmalıdır.

Dergiye gönderilecek makale ve ekleri (şekil vs) *http://vetdergi.kafkas.edu.tr* adresindeki online makale gönderme sistemi kullanılarak yapılmalıdır.

Başvuru sırasında yazarlar yazıda yer alacak şekilleri (13 X 18 cm boyutlarından büyük olmamalı) online makale gönderme sistemine yüklemelidirler. Yazının kabul edilmesi durumunda tüm yazarlarca imzalanmış Telif Hakkı Devir Sözleşmesi editörlüğe gönderilmelidir.

3- Yazarlar yayınlamak istedikleri makale ile ilgili olarak gerekli olan etik kurulu onayı aldıkları kurumu ve onay numarasını Materyal ve Metot bölümünde belirtmelidirler. Yayın kurulu gerekli gördüğünde etik kurul onay belgesini ayrıca isteyebilir.

4- Orijinal Araştırma Makaleleri, yeterli bilimsel inceleme, gözlem ve deneylere dayanarak bir sonuca ulaşan orijinal ve özgün çalışmalardır. Türkçe yazılmış makaleler Türkçe başlık, Türkçe özet ve anahtar sözcükler, yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, giriş, materyal ve metot, bulgular, tartışma ve sonuç ile kaynaklar bölümlerinden oluşur ve toplam (metin, tablo, şekil vs dahil) 10 sayfayı geçemez. Yabancı dilde yazılmış makaleler yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Türkçe başlık, Türkçe özet ve anahtar sözcükler dışında Türkçe makale yazım kurallarında belirtilen diğer bölümlerden oluşur. Türkçe ve yabancı dilde özetlerin her biri yaklaşık 200±20 sözcükten oluşmalıdır.

Kısa Bildiri, konu ile ilgili yeni bilgi ve bulguların bildirildiği fakat orijinal araştırma olarak sunulamayacak kadar kısa olan yazılardır. Kısa bildiriler, orijinal araştırma makalesi formatında olmalı, fakat özetlerin her biri 100 sözcüğü aşmamalı, referans sayısı 15'in altında olmalı ve 4 sayfayı aşmamalıdır. Ayrıca, en fazla 4 şekil veya tablo içermelidir.

Ön Rapor, kısmen tamamlanmış, yorumlanabilecek aşamaya gelmiş orijinal bir araştırmanın kısa (en çok 2 sayfa) anlatımıdır. Bunlar orijinal araştırma makalesi formatında yazılmalıdır.

Gözlem, uygulama, klinik veya laboratuar alanlarında ender olarak rastlanılan olguların sunulduğu makalelerdir. Bu yazıların başlık ve özetleri orijinal makale formatında yazılmalı, bundan sonraki bölümleri giriş, olgunun tanımı, tartışma ve sonuç ile kaynaklardan oluşmalı ve 4 sayfayı geçmemelidir.

Editöre Mektup, bilimsel veya pratik yararı olan bir konunun veya ilginç bir olgunun resimli ve kısa sunumudur ve 1 sayfayı geçmemelidir.

Derleme, güncel ve önemli bir konuyu, yazarın kendi görüş ve araştırmalarından elde ettiği bulguların da değerlendirildiği özgün yazılardır. Bu yazıların başlık ve özet bölümleri orijinal araştırma makalesi formatında yazılmalı, bundan sonraki bölümleri giriş, metin ve kaynaklardan oluşmalı ve 10 sayfayı geçmemelidir.

Çeviri, makalenin orijinal formatı dikkate alınarak hazırlanmalıdır.

Yazarla ilgili kişisel ve kuruma ait bilgiler ana metin dosyasına değil, on-line başvuru sırasında sistemdeki ilgili yerlere unvan belirtilmeksizin eklenmelidir.

5- Makale ile ilgili gerek görülen açıklayıcı bilgiler (tez, proje, destekleyen kuruluş vs) makale başlığının sonuna üst simge olarak işaret konularak makale başlığı altında italik yazıyla belirtilmelidir.

6- Kaynaklar, metin içinde ilk verilenden başlanarak numara almalı ve metin içindeki kaynağın atıf yapıldığı yerde parantez içinde yazılmalıdır. Kaynak dergi ise, yazarların soyadları ve ilk adlarının başharfleri, makale adı, dergi adı (orijinal kısa ad), cilt ve sayı numarası, sayfa numarası ve yıl sıralamasına göre olmalı ve aşağıdaki örnekte belirtilen karakterler dikkate alınarak yazılmalıdır.

Örnek: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.

Kaynak kitap ise yazarların soyadları ile adlarının ilk harfleri, eserin adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı olarak yazılmalıdır.

Editörlü ve çok yazarlı olarak yayınlanan kitaptan bir bölüm kaynak olarak kullanılmışsa, bölüm yazarları, bölüm adı, editör(ler), kitap adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı sırası dikkate alınarak aşağıdaki örneğe göre yazılmalıdır.

Örnek: **Mcllwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed. 339-447, Lea and Febiger, Philadelphia, 1988.

Online olarak ulaşılan kaynaklarda web adresi ve erişim tarihi kaynak bilgilerinin sonuna eklenmelidir.

Diğer kaynakların yazımında bilimsel yayın ilkelerine uyulmalıdır.

Kaynak listesinde "et al." ve "ve ark." gibi kısaltmalar yapılmaz.

7- Bakteri, virus, parazit ve mantar tür isimleri ve anatomik terimler gibi latince ifadeler orijinal şekliyle ve italik karakterle yazılmalıdır.

8- Editörlük, dergiye gönderilen yazılar üzerinde gerekli görülen kısaltma ve düzeltmeleri yapabileceği gibi önerilerini yazarlara iletebilir. Yazarlar, düzeltilmek üzere yollanan yazıları online sistemde belirtilen sürede gerekli düzeltmeleri yaparak editörlüğe iade etmelidirler. Editörlükçe ön incelemesi yapılan ve değerlendirmeye alınması uygun görülen makaleler ilgili bilim dalından bir yayın danışmanı ve iki raportörün olumlu görüşü alındığı takdirde yayımlanır.

9- Yayınlanan yazılardan dolayı doğabilecek her türlü sorumluluk yazarlara aittir.

10- Yazarlara telif ücreti ödenmez.

11- Resim ve baskı masrafları için yazarlardan ücret alınır. Ücret bilgileri *http://vetdergi.kafkas.edu.tr/* adresinden öğrenilebilir.

12- Yazarlara 50 adet ayrı baskı ücretsiz olarak yollanır.

ABONE KOŞULLARI

Yıllık: 60 TL

Banka Hesap No: T.C. Ziraat Bankası Kars Şubesi, 26146825-5001 Nolu Hesap, Kars - TÜRKİYE

IBAN: TR830001000147261468255001

INSTRUCTIONS FOR AUTHORS

1- The Journal of the Faculty of Veterinary Medicine, University of Kafkas (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly, covers original papers, short communication and preliminary scientific reports, case reports, observation, letter to the editor, review and translation on all aspects of veterinary medicine and animal science (clinical and paraclinical sciences, biological and basic sciences, zoonoses and public health, animal feeding and nutritional diseases, animal breeding and genetics, hygiene and technology of food from animal sources, exotic animal science). Manuscripts submitted for publication should be written in Turkish, English or German.

2- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure, table and graphic must clearly be written in both Turkish and foreign language and their appropriate position should be indicated in the text.

The manuscript and its supplementary (figure etc.) should be submitted by using online manuscript submission system at the address of *http://vetdergi.kafkas.edu.tr/*

During the submission, the authors should upload the figures of the manuscript (the dimensions must not to exceed 13 X 18 cm) to the online manuscript submission system. If the manuscript is accepted for publication, the copyright transfer agreement form signed by all the authors should be send to the editorial office.

3- Authors should indicate the name of institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, editorial board may also request the official document of the ethical commission report.

4- Original (full-length) manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts written in Turkish consist of the title in Turkish, summary and keywords in Turkish, introduction, material and methods, results, discussion and references and it should not exceed 10 pages including text, tables and illustrations. Manuscripts written in a foreign language should follow the title in foreign language, summary and keywords in foreign language, title in Turkish, summary and keywords in Turkish and the remaining sections described above for the manuscripts written in Turkish.

Summaries written in Turkish or foreign language should contain 200±20 words.

Short communication manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but each of the summaries should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 4 pages. Additionally, they should not contain more than 4 figures or tables.

Preliminary scientific reports are short description (maximum 2 pages) of partially completed original research findings at interpretable level. These should be prepared in the format of full-length original articles.

Case reports describe rare significant findings encountered in the application, clinic and laboratory of related fields. The title and summary of these articles should be written in the format of full-length original articles and the remaining sections should follow introduction, case history, discussion and references without exceeding the total of 4 pages.

Letters to the editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases without exceeding 1 page.

Reviews are original manuscripts gather the literature on current and significant subject along with the commentary and findings of the author on the particular subject. The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should follow introduction, text and references without exceeding 10 page.

Translations should be prepared based on the format of original document being translated.

The information about author/s and institution/s should be added during the online submissoin and the main documant should be free of these information.

5- The necessary descriptive information (thesis, projects, financial supports etc) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of title.

6- References should be listed with numerical order as they appear in the text and the reference number should be indicated inside the parentheses at the cited text place. References should have the order of surnames and initial letters of the authors, title of the article, title of the journal (original abbreviated title), volume and issue numbers, page numbers and the year of publication and the text formatting should be performed as shown in the example below.

Example: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg*, 15 (2): 225-236, 2009.

If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

Example: **Mcllwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed. 339-447, Lea and Febiger, Philadelphia, 1988.

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must be complied with the other references.

Abbreviations, such as "et al" and "and friends" should not be used in the list of the references.

7- The Latin expression such as species names of bacterium, virus, parasite and fungus and anatomical terms must be written in italic character keeping their original forms.

8- The editorial board has the right to perform necessary modifications and reduction on the manuscript submitted for publication and to express recommendations to the authors. The manuscripts sent to authors for correction should be returned to the editorial office within a month. After pre-evaluation and agreement of the submitted manuscripts by editorial board, the article can only be published after the approval of the publication advisor and two referees specialized in the particular field.

9- All responsibilities from published articles merely belong to the authors.

10- There is no copyright fee for the authors.

11- A fee is charged from the authors to cover printing cost and other expenses. This payment information can be found at *http://vetdergi.kafkas.edu.tr/*

12- Reprints (in multiples of 50) of the article are sent to the authors for free.

SUBSCRIPTION

Yearly: $40

Account: T.C. Ziraat Bank, Kars Branch, **Account No:** 26146825-5004

IBAN: TR020001000147261468255004