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RESEARCH ARTICLE

Microbiological and Chemical Properties of Bonito Fish *(Sarda sarda)* Fillets Packaged with Chitosan Film, Modified Atmosphere and Vacuum [1]

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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

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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

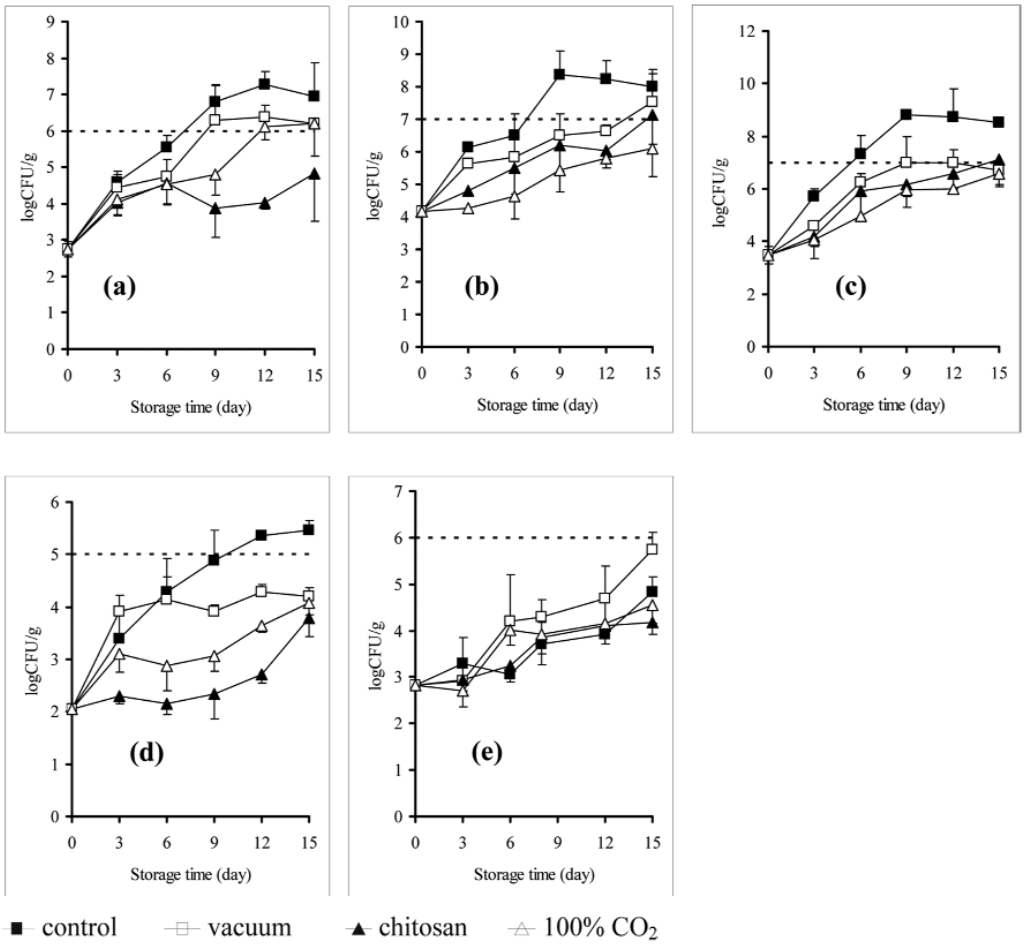

Fig 1. *Mesophilic* ***(a),*** *psychrotrophic* ***(b),*** *Pseudomonas* ***(c),*** *Enterobacteriaceae* ***(d)*** *and lactic acid bacteria* ***(e)*** *counts on bonito fillets with chitosan film or VP, MAP at 4°C. Upper areas of horizontal lines are unacceptable in each figure. Each value represents mean ±SD (n=3)*

Şekil. 1. *Palamut balığında Mezofilik* ***(a),*** *psikrotrofik* ***(b),*** *Pseudomonas* ***(c),*** *Enterobacteriaceae* ***(d)*** *and laktik asit bakteri* ***(e)*** *değerleri*

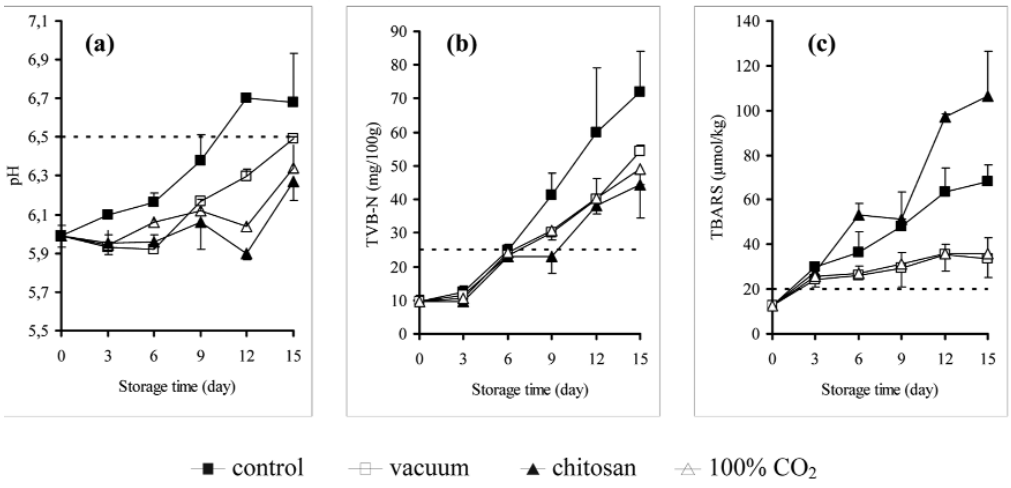

Fig 2. *pH values* ***(a),*** *TVB-N* ***(b)*** *and TBARS level* ***(c)*** *of bonito fillets with chitosan film or VP, MAP at 4°C. Upper areas of horizontal lines are unacceptable in each figure. Each value represents mean ±SD (n=3)*

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.

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