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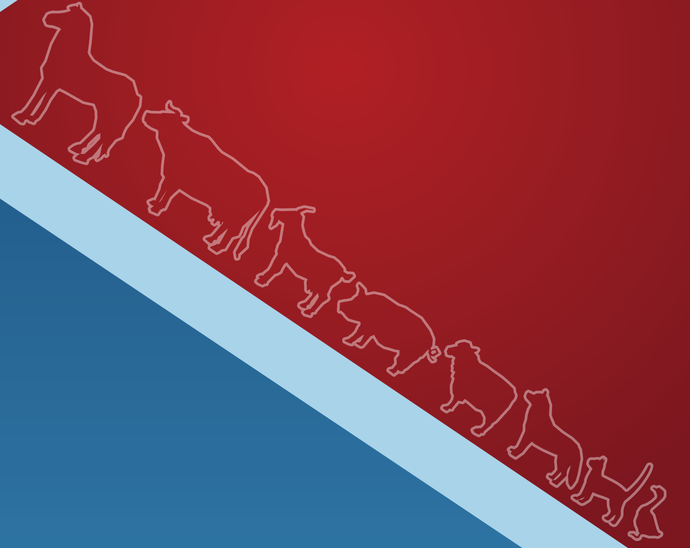

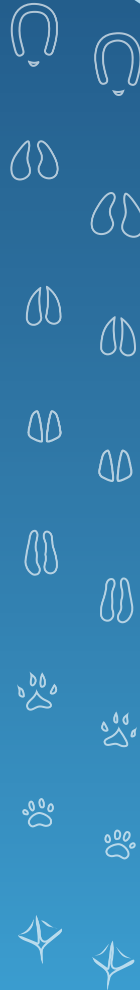

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Research Article

Effect of Antimicrobial Peptides from Fly Maggots on Immunity of Yellow-feathered Broilers

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Abstract: The aim of this experiment was to investigate the effect of dietary supplementation of fly maggot antimicrobial peptides on immune biochemical indicators, the effect of immune organ index, small intestinal bacteria and small intestinal mucosal cell count in yellow-feathered broilers. Three hundred clinically healthy 1-day-old yellow-feathered broilers were randomly divided into 3 treatment groups, 10 replicates in each group and 10 broilers in each replicate. The groups were called hereafter as basal diet group (control group), basal diet + 100 mg/kg fly maggot antimicrobial peptide (AMPs) group, basal diet + 15% bacitracin zinc group. The test period was 42 d. The results showed that the contents of albumin, IgG and IgM in the antimicrobial peptide group were significantly higher than those in the control group (P<0.05). The spleen index and thymus index in the AMPs group were higher than those in the control group and the bacitracin zinc group (P<0.05). The number of *E. coli* in each segment of the small intestine of broilers in the control group was significantly higher than that in the AMPs group and the bacitracin zinc group (P<0.05). The number of goblet cells in each segment of the small intestine of broilers in the bacitracin zinc group was higher than that in the AMPs group, but there was no significant difference (P>0.05). In conclusion, these findings that revealed maggot antimicrobial peptides as dietary supplementation can improve the immunity of the yellow-feathered broilers.

Keywords: *Broilers, Immune system organ index, Immunobiochemical parameters, Intestinal bacteria, Maggot antimicrobial peptide*

Sinek Larvalarından Elde Edilen Antimikrobiyal Peptidlerin Sarı Tüylü Piliçlerin Bağışıklığı Üzerine Etkisi

Öz: Bu çalışmanın amacı, sinek larvalarından elde edilen antimikrobiyal peptitlerinin sarı tüylü piliçlerin diyetine takviyesinin bağışıklık biyokimyasal göstergeleri, bağışıklık organ indeksi, ince bağırsak bakterileri ve ince bağırsak mukozal hücre sayısı üzerine etkisini araştırmaktı. Klinik olarak sağlıklı 300 adet 1 günlük sarı tüylü civciv, her grupta 10 hayvan ve her grubun 10 tekrarı olmak üzere rastgele 3 grubua ayrıldı. Gruplar böylelikle, bazal diyet grubu (kontrol grubu), bazal diyet + 100 mg/kg sinek larvası antimikrobiyal peptid (AMP) grubu ve bazal diyet + %15 basitrasin çinko grubu olarak adlandırıldı. Deney süresi 42 gün olarak gerçekleştirildi. Sonuçlar, AMP grubunun albümin, IgG ve IgM seviyesinin kontrol grubuna oranla önemli ölçüde yüksek olduğunu gösterdi (P<0.05). AMP grubunun dalak ve timus indeksi, kontrol ve basitrasin çinko grubundan daha yüksekti (P<0.05). Kontrol grubuna ait piliçlerin ince bağırsak kısımlarındaki *E. coli* sayısı, AMP ve basitrasin çinko grubundan önemli ölçüde daha yüksekti (P<0.05). Basitrasin çinko grubuna ait piliçlerin ince bağırsağının kısımlarındaki goblet hücrelerinin sayısı AMP grubundan daha fazlaydı, ancak anlamlı bir fark yoktu (P>0.05). Sonuç olarak, diyet takviyesi olarak kullanılan larval antimikrobiyal peptitlerin ortaya çıkaran bu etkinlikleri, sarı tüylü piliçlerde bağışıklığı güçlendirebilir.

Anahtar sözcükler: *Piliç, İmmün sistem organ indeksi, İmmünobiyokimyasal parametreler, Bağırsak bakterileri, Larval antimikrobiyal peptit*

Introduction

In the poultry breeding industry, the massive use of antibiotics will cause increased drug resistance of strains, and drug residues appear in by-products such as eggs, which seriously threaten human health. Therefore, a green, safe and pollution-free antibiotic alternative is eagerly sought. During the last years, host antimicrobial peptides have been recognized as key mediators of the innate immune response in many vertebrate species, providing the first line

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of defense against potential pathogens [1,2]. Antimicrobial peptides (AMPs) are important small peptides of the innate immune system of the body, which are widely present in organisms in nature and can effectively improve the performance of livestock and poultry and enhance immunity [3-5], and they have a very broad application prospect as a new antibiotic alternative. Currently, there are 2961 antimicrobial peptides in the Antimicrobial Peptide Database (APD), of which as many as 40 fly antimicrobial peptides have been isolated. Antimicrobial peptides from fly maggots have the advantages of high antibacterial activity, wide antibacterial spectrum, and green safety without residues [6,7]. We also show that the CATH peptides 1, 2, 3 and their amide-modified structures possess potent antimicrobial activities against both Gram-positive and Gram-negative pathogens, with these bacteria being affected to different extents [8]. The presence of the antimicrobial peptides in a broad range of tissues and their largely enhanced expression during development is suggestive of their potentially important role in early host defense and disease resistance of chickens. Nowadays, fly maggot antimicrobial peptides have been widely used in poultry disease treatment and aquatic research, but they have not been found to be used as feed additives in broiler humoral immunity and intestinal health [9].

Therefore, this experiment intends to add fly maggot antimicrobial peptide into the diet of yellow-feathered broilers, study its effects on immune biochemical parameters, immune organ index, small intestinal bacteria and small intestinal mucosal cell count in broilers, determine its appropriate amount in the diet, and finally lay a theoretical foundation for the application of fly maggot antimicrobial peptide replacement antibiotics in the poultry breeding industry.

Material and Methods

Ethical Statement

The study was approved by the Animal Experimentation Ethics Committee of the School of Animal Science and Technology, Shihezi University. All chickens were kept experimentally and euthanized in strict accordance with the guidelines of the committee. During the test, all efforts were made to minimize the suffering of the animals.

Animals, Experimental Design and Feed

In this study, 300 yellow-feathered broilers with similar healthy body weight were randomly divided into 3 treatment groups, 10 replicates in each group, 10 broilers in each group, which were called hereafter as basal diet group (control group), basal diet + 100 mg/kg fly maggot antimicrobial peptides group (AMPs), and basal diet + 15% bacitracin zinc group. Corn-soybean meal diet was used in the experiment, and the basal diet was prepared according to The National Research Council (NRC) (1994) broiler nutritional standard, and its composition and nutritional level were listed in *Table 1*. All tests were performed in the same chicken house, and the house temperature was monitored thermostatically throughout the study. The temperature, which was 32-35°C on the first day, was lowered and maintained gradually at 22°C for the last two weeks. The artificial light program was implemented in accordance with commercial conditions (23 h of lighting throughout the experiment per day). The chickens had free access to food and water. Other immunization and disinfection measures were performed in strict accordance with the farm procedures, and the test period was 42 days. The basal diet used in this experiment was purchased from Xinjiang Tiankang Feed Technology Co., Ltd. (China). Antimicrobial peptides used in the study were supplied from a commercial company (Guangzhou Yingbao Biotechnology Co., Ltd., China). 15% bacitracin zinc premix was purchased from Lukang Biotechnology Co., Ltd. (China).

Immune Biochemical Indicators

At the end of the experiment, blood samples were taken from the wing vein of the animals to the vacuum blood collection tube, allowed to stand for 24 h, and then centrifuged at 3000 rpm/min for 30 min to collect serum

***Table 1.** Dietary levels at different stages in each experimental nutrient group*

Items	Content, %
Ingredients	
Corn	62.85
Soymeal bean	31.50
Soyoil bean	1.30
Limestone	1.50
$CaHPO_4$	1.50
NaCl	0.35
Met	0.08
Premix	0.92
Total	100
Nutrient levels	
ME/(MJ/Kg)	12.08
CP	19.00
Ca	0.95
AP	0.41
Lys	0.923
Met	0.393

The premix provided the following per kg of diet (without antibiotics): Cu: 5 mg, Fe: 75 mg, Mn: 56 mg, I: 0.35 mg, Se: 0.14 mg, Zn: 38 mg, Vit. A: 1420 IU, Vit D_2: 190 IU, Vit. E: 9.5 IU, Vit. K: 0.38 mg, Riboflavin: 3.4 mg, Pantothenic acid: 9.4 mg, Nicotinic acid: 26 mg, Vit. B_{12}: 0.009 mg, Choline: 1225 mg, Biotin: 0.14 mg, Folic acid: 0.52 mg, Thioflavin: 1.0 mg, Pyridoxic acid: 2.8 mg

for determination of total protein and albumin. Total protein and albumin contents were determined using commercial kits (Nanjing Jiancheng Technology Co., Ltd., China). After the chickens were euthanized, the livers were separated, washed with normal saline, 2 g of the analytical balance was weighed, cut with scissors, placed into a beaker to add 10 mL of normal saline, placed into a homogenization mechanism to prepare the homogenate, centrifuged at 2500 r/min for 10 min, and the supernatant was taken and stored at −20°C for the determination of serum immunoglobulin, that is, IgG, IgM, and IgA contents using ELISA kits (Shanghai Yanchun Biotechnology Co., Ltd., China).

Immune Organ Index

On the 42nd day of the feeding, the yellow-feathered broilers were weighed and slaughtered. The thymus, spleen, and bursa of Fabricius of the animals were harvested, and the adipose tissue on each organ was removed. Surface water was cleaned with filter paper, and each immune organ was weighed and calculated as follows.

Immune Organ Index = Immune Organ Weight/Pre-Slaughter Live Weight

Number of Intestinal Bacteria

After the euthanasia, 1 g of duodenal, jejunal and ileal contents was quickly collected into a microcentrifuge tube and suspended with physiological saline to obtain a 1×10^{-7} sub-dilution. According to the dilution selected for the preliminary experiment, 40 μL of the sub-dilution was inoculated onto the Eosin Methylene Blue (EMB) and Salmonella Shigella (SS) medium for *Escherichia coli* and *Salmonella* spp. culture, respectively. For the cultivation of the *Lactobacillus* spp., the sub-dilution was inoculated onto the Man-Rogosa-Sharpe (MRS) agar plates and incubated at microaerophilic conditions at 37°C for 48 h.

Small Intestinal Mucosal Cell Count

The left and right intestinal tissues of the 5 cm group were respectively taken from the duodenum, jejunum and ileum, which were soaked and fixed in 4% paraformaldehyde. The fixed samples were dehydrated with 70%, 85%, 95%, 100% and 100% ethanol, respectively. The samples were cleared with xylene and embedded with an embedding machine after wax transmission. The samples were sectioned with a microtome and stained with hematoxylin and eosin. Five sections were randomly selected, and images were collected using an HMIAS-200 optical microscope color image analysis system under a (10 x 40) x microscope field of view. The changes in the number of lymphocytes and cup-shaped cells were counted.

Statistical Analysis

The one-way analysis of variance (ANOVA) method was used for the statistical analysis of the groups. Statistical differences and trend analysis were considered significant at $P \leq 0.05$. The statistical analysis was done with the SPSS software package.

Results

The effect of antimicrobial peptides from fly maggots on immune biochemical parameters in the yellow-feathered broilers was investigated. It can be seen from *Table 2* that there was no significant difference in the serum total protein and IgA contents among the study groups ($P>0.05$). The contents of albumin, IgG and IgM in the AMPs group and the bacitracin zinc group were higher than those in the control group ($P>0.05$). The contents of IgM in the AMPs group were higher than those in the bacitracin zinc group and the control group ($P>0.05$). There was no significant difference ($P>0.05$) in the contents of albumin and IgG in the AMPs group and bacitracin zinc group.

The changes in the immune organ index of the fly maggot antimicrobial peptide of yellow-feathered broilers were shown in *Table 3*. The spleen index and thymus index in the AMPs group were higher than those in the control group and the bacitracin zinc group ($P<0.05$). Compared with the control group, there was no significant difference in bursa of Fabricius index in the bacitracin zinc group and the AMPs group ($P>0.05$).

The changes in the number of *E. coli* and Lactobacilli in different parts of the chicken small intestine were shown in *Fig. 1* and *Fig. 2*. The number of *E. coli* in the duodenum,

Table 2. *Effect of maggot antimicrobial peptides on the biochemical immune indexes of the yellow-feathered broilers*

Parameters	Groups		
	Control	AMPs	Bacitracin Zinc Group
Total Protein (mg/mL)	38.24±0.66	38.89±0.10	38.31±0.49
Albumin (mg/mL)	21.99±0.87^b	23.10±0.23^a	22.35±0.15^a
IgG (mg/mL)	3.39±0.11^b	4.35±0.21^a	4.12±0.07^a
IgM (mg/mL)	0.76±0.03^c	1.13±0.06^a	0.85±0.06^b
IgA (mg/mL)	0.69±0.04	0.76±0.02	0.73±0.03

^{a,b,c} *Means within a row followed by the different superscripts differ significantly (P<0.05)*

Table 3. Effect of maggot antimicrobial peptides on the immune organ index of the yellow-feathered broilers

Parameters	Groups		
	Control	AMPs	Bacitracin Zinc Group
Thymus index (g/kg)	3.53±0.38[b]	3.91±0.35[a]	3.50±0.22[b]
Spleen index (g/kg)	1.58±0.66[b]	1.69±0.29[a]	1.55±0.58[b]
Bursa index (g/kg)	1.31±0.11	1.61±0.43	1.31±0.25

[a,b,c] *Means within a row followed by the different superscripts differ significantly (P<0.05)*

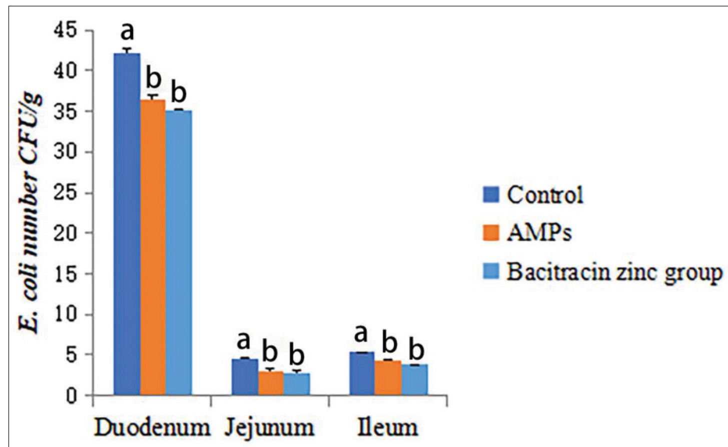


Fig 1. Changes in the number of *E. coli* in the small intestine of the yellow-feathered broilers. Differences in [a,b,c] means significant difference between the groups after different superscripts (P<0.05)

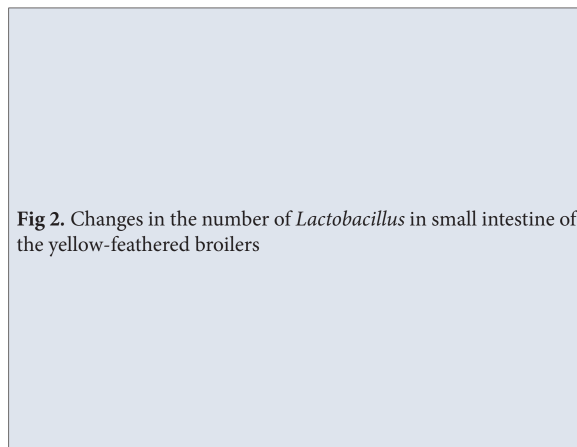
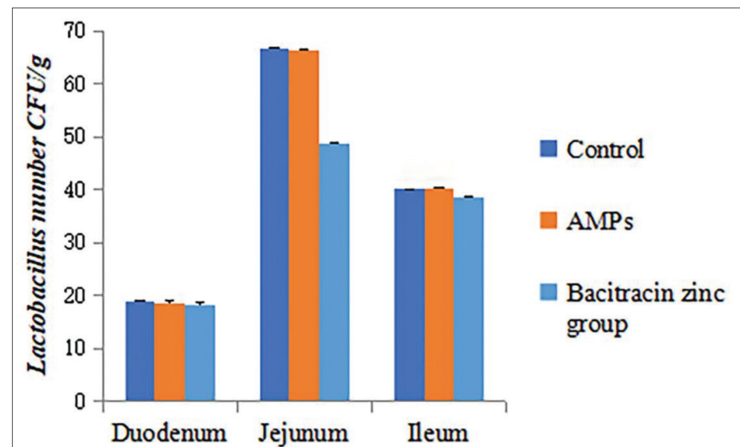


Fig 2. Changes in the number of *Lactobacillus* in small intestine of the yellow-feathered broilers



jejunum, and ileum of broilers in the AMPs and bacitracin zinc groups were lower than that in the control group (P<0.05). However, there was no significant difference in the number of *Lactobacillus* in the small intestine in each segment (P>0.05).

The number of lymphocytes in the small intestine in the AMPs group was higher than that in the bacitracin zinc group and the control group. Moreover, the number of lymphocytes in the duodenum in the bacitracin zinc peptide group was higher than that in the control group. However, there was no difference between the bacitracin zinc group and the control group in the ileum of the small intestine (P>0.05). The influence of the antimicrobial peptides on the number of lymphocytes was given in *Table 4*.

The changes in the distribution of goblet cells in different parts of the small intestine of broilers were shown in *Table 5*. The number of goblet cells in the duodenum and jejunum of the small intestine was higher in the AMPs group than in the bacitracin zinc group and the control group (P<0.05). However, there was no difference in the number of *Lactobacillus* in the small intestine in each segment (P>0.05).

Discussion

Humoral immunity plays an indispensable and important role in animal immunity, and the health status of livestock and poultry is closely related to serum protein. The increase of serum total protein and albumin contents is the embodiment of vigorous protein metabolism, indicating

Table 4. Effects of each experimental group on the number of intestinal mucosal lymphocytes

Parameters	Groups		
	Control	AMPs	Bacitracin Zinc Group
Duodenum	101.00±0.02[c]	108.00±0.14[a]	104.00±0.44[b]
Jejunum	89.00±0.02[b]	99.00±0.37[a]	89.00±0.29[b]
Ileum	85.00±0.06[b]	94.00±0.16[a]	86.00±0.43[b]

[a,b,c] *Means within a row followed by the different superscripts differ significantly (P<0.05)*

Table 5. Effects of each experimental group on the number of goblet cells in the chicken small intestinal mucosa

Parameters	Groups		
	Control	AMPs	Bacitracin Zinc Group
Duodenum	98.00±0.21[b]	100.00±0.11[a]	99.00±0.71[b]
Jejunum	105.00±0.34[b]	108.00±0.63[a]	106.00±0.71[b]
Ileum	124.00±0.83	127.00±0.11	125.00±0.53

[a,b,c] *Means within a row followed by the different superscripts differ significantly (P<0.05)*

that the absorption and utilization rate of amino acids and proteins in the body is improved. Immunoglobulins are the most important molecules in the immune response and play an important role in the process of anti-infection. Studies have shown [9,10] that the AMPs participate in the first immune defense and regulate body immunity through different mechanisms, which has attracted much attention. Wang et al.[11] showed that the addition of the antimicrobial peptides decreased the concentration of TNF-α, IL-1β, and IL-6 and effectively improved the performance, systematic inflammation, and improved fecal microbiota composition of the broilers. Jozefiak et al.[12] reported that dietary supplementation of fly maggot powder significantly enhanced the immune capacity of broilers, enhanced the body's immune response, and played a role in preventing livestock and poultry diseases. In this experiment, the contents of albumin, IgG and IgM in the AMPs group were significantly higher than those in the control group (P<0.05), indicating that the antimicrobial peptide of the fly maggots can induce the activation of immune function in the yellow-feathered broilers, promote the synthesis of immunoglobulin, and maintain the active immune ability of the body, which is basically consistent with the above reports.

The immune organ index is an important reflection of the growth and development of the immune organs. The development status and function of the immune organs directly affect the immune level of poultry. At present, relevant studies have shown that the changes in the volume of immune organs are actually affected by the rate of apoptosis and cell proliferation of the lymphocytes [13], and antimicrobial peptides can play an indispensable role in immune regulation and immune homeostasis [14]. The spleen index and the thymus index in the AMPs group were higher than those in the control group and the bacitracin zinc group (P<0.05). This indicates that the antimicrobial peptide of fly maggots could promote the development and maturation of the immune system organs and improve the immune activity of the yellow-feathered broilers. In addition, the antimicrobial peptides and bacitracin zinc showed different results possibly due to their different mechanisms of action. Yang et al.[15] showed that the antimicrobial peptides are capable of promoting systemic humoral immune responses of chickens at an early age. The increased content of the immunoglobulins in serum and the antibody-forming cells in the bursa of Fabricius strengthen the viability of chicken. In addition to the direct eradication of microorganisms, antimicrobial peptides may be used as a signal to modulate or amplify adaptive immune responses. Antimicrobial peptides serve as 'alarm' signals in mobilizing the immune system and activating innate and adaptive immune systems.

The homeostasis of the intestinal bacteria in poultry plays an important role in nutrition, immunity and metabolism [16]. The gastrointestinal tract of poultry consists of the esophagus, crop, proventriculus, gizzard, duodenum, jejunum, ileum, cecum, colon, and cloaca. The poultry gastrointestinal tract is much shorter as compared to other mammals relative to their body length. Thus, microbiota that grows in such a small gastrointestinal tract with a relatively short transit time requires unique adaptations to adhere to the mucosal wall and proliferation. The ceca has a lower passage rate and is favorable to diverse groups of bacteria, which affect nutrient utilization and the overall health of poultry [17]. According to Hirsch et al.[18], antimicrobial peptides from rat-tailed maggots of the drone fly eristalis tenax show potent activity against multidrug-resistant Gram-negative bacteria. And

novel AMPs highlight the potent and broad spectrum of antibacterial activity, a safe and stable tryptophan-rich amphiphilic peptide, called WRK-12, has a broad spectrum of antimicrobial activity against a variety of multidrug-resistant bacteria, including Methicillin-resistant *Staphylococcus aureus* (MRSA), colistin-resistant and tigecycline-resistant *E. coli* [19]. At present, although no exact mechanism has been found for the AMPs to affect intestinal bacteria, in explaining their antibacterial activity it is attributed to the different surface charges of peptides and pathogens. Specifically, the AMPs are positively charged and can attach to negatively charged cell membranes by electrostatic interaction. Thereby, they physically disrupt the phospholipid bilayer of the bacteria by blocking enzyme activity or inhibiting the synthesis of proteins and nucleic acids [20,21]. At the same time, the AMPs can selectively inhibit the growth of bacteria in the intestine, which may indicate a significant competitive advantage of the AMPs compared with the antibiotics [22,23]. Furthermore, antimicrobial peptides are critical components of host defense limiting bacterial infections at the gastrointestinal mucosal surface. Rowan et al.[24] found through computer model studies that the synthetic form of a chicken novel beta-defensin identified is active against predominantly intestinal pathogens. The mucosal barrier formed by cationic antimicrobial peptides (CAMPs) is believed to be crucial for host protection from pathogenic gut infection [25]. Daneshmand et al.[26] showed that the AMPs increased the population of *Lactobacillus* spp. and harmful bacteria challenged in the ileum of *E. coli*-chickens.

Immune-related cells in the intestinal mucosa are the first cells in contact with the body by pathogenic microorganisms and play a protective role during the infection, forming an epithelial mechanical cleaning barrier and mucosal immune barrier to resist the bacterial invasion. These cells are mainly composed of mast cells, intraepithelial lymphocytes, lamina propria lymphocytes and goblet cells. Lymphocytes have a role in protecting the intestinal mucosal immune system, and many auto-immune diseases and intestinal diseases in animals are associated with decreased lymphocyte numbers and dysfunction [27-29]. Goblet cells are glandular-type cells that can secrete glycoproteins and play an important role in intestinal immunity before passive immunity is established in neonatal animals [30,31]. The results showed that dietary supplementation of the antimicrobial peptides was able to significantly increase the number of intestinal intraepithelial lymphocytes and villus height in the duodenum and jejunum of chickens at 28, 42 and 56 days [32].

The results of this experiment revealed that dietary supplementation of the fly maggot antimicrobial peptide could significantly reduce the number of *E. coli* in the duodenum, jejunum, and ileum of the yellow-feathered broilers, while effectively increased the number of *Lactobacilli* spp. in each segment of the small intestine. The number of the lymphocytes and goblet cells in each segment of the small intestine in the AMPs group was significantly higher than that in the control group, which was basically consistent with the above reports, indicating that the fly maggot antimicrobial peptide could effectively regulate the number of beneficial and harmful bacteria in the intestine, improve the intestinal microecological environment, and facilitate the rapid establishment and maintenance of intestinal microbial balance in the yellow-feathered broilers, which is of great significance for body homeostasis.

In conclusion, the addition of the antimicrobial peptides of the fly maggots in the basal diet could significantly increases the serum globulin content, promotes the development of immune organs, reduces the number of intestinal harmful bacteria such as *E. coli,* increases the number of beneficial bacteria such as *Lactobacilli* spp., improves the intestinal epithelial cells, and improves the body's immunity in the yellow-feathered broilers. Therefore, future work and research should be tailored to a better understanding of the mechanisms of action of antimicrobial peptides to investigate their full potential in the poultry farming industry.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Author Contributions

HS and JW conceived and supervised this study. ZW completed the main experimental content. JY and SG collected and analyzed data. LD and XX wrote the first draft of the manuscript. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

References

1. Zasloff M: Antimicrobial peptides of multicellular organisms. *Nature,* 415 (6870): 389-395, 2002. DOI: 10.1038/415389a

2. Lazzaro BP, Zasloff M, Rolff J: Antimicrobial peptides: Application

informed by evolution. *Science*, 368 (6490):eaau5480, 2020. DOI: 10.1126/science.aau5480

3. Silveira RF., Roque-Borda CA, Vicente EF: Antimicrobial peptides as a feed additive alternative to animal production, food safety and public health implications: An overview. *Anim Nutr* 7 (3): 896-904, 2021. DOI: 10.1016/j.aninu.2021.01.004

4. Pal L, Brahmkhatri VP, Bera S, Bhattacharyya D, Quirishi Y, Bhunia A, Atreya HS: Enhanced stability and activity of an antimicrobial peptide in conjugation with silver nanoparticle. *J Colloid Interface Sci,* 483, 385-393, 2016. DOI: 10.1016/j.jcis.2016.08.043

5. Silva JP, Appelberg R, Gama FM: Antimicrobial peptides as novel anti-tuberculosis therapeutics. *Biotechnol Adv*, 34 (5): 924-940, 2016. DOI: 10.1016/j.biotechadv.2016.05.007

6. Fu J, Song J, Ren G, Zhu J, Feng X: Antimicrobial peptides from fly maggots and their application in animal production. *Chinese Feed*, 17, 11-17, 2018. DOI: 10.15906/j.cnki.Cn11-2975/s.20181702

7. Wang Z, Wang J, Zhang Y, Wang X, Zhang X, Liu Y, Xi J, Tong H, Wang Q, Jia B, Shen H: Antimicrobial peptides in housefly larvae *(Musca domestica)* affect intestinal *Lactobacillus acidophilus* and mucosal epithelial cells in *Salmonella pullorum*-infected chickens. *Kafkas Univ Vet Fak Derg*, 23 (3): 423-430, 2017. DOI: 10.9775/kvfd.2016.16901

8. Yacoub HA, Elazzazy AM, Mahmoud MM, Baeshen MN, Al-Maghrabi OA, Alkarim S, Ahmed ES, Almehdar HA, Uversky VN: Chicken cathelicidins as potent intrinsically disordered biocides with antimicrobial activity against infectious pathogens. *Dev Comp Immunol*, 65, 8-24, 2016. DOI: 10.1016/j.dci.2016.06.012

9. Dutta P, Das S: Mammalian antimicrobial peptides: Promising therapeutic targets against chronic infection and inflammation. *Curr Top Med Chem,* 16 (1): 99-129, 2016. DOI: 10.2174/1568026615666150703121819

10. Xia X, Cheng L, Zhang S, Wang L, Hu J: The role of natural antimicrobial peptides during infection and chronic inflammation. *Antonie Van Leeuwenhoek*, 111 (1): 5-26, 2018. DOI: 10.1007/s10482-017-0929-0

11. Wang G, Song Q, Huang S, Wang Y, Cai S, Yu H, Ding X, Zeng X, Zhang J: Effect of antimicrobial peptide microcin J25 on growth performance, immune regulation, and intestinal microbiota in broiler chickens challenged with *Escherichia coli* and *Salmonella*. *Animals (Basel),* 10 (2):345, 2020. DOI: 10.3390/ani10020345

12. Jozefiak A, Engberg RM: Insect proteins as a potential source of antimicrobial peptides in livestock production. A review. *J Anim Feed Sci,* 26, 87-99, 2017. DOI: 10.22358/jafs/69998/2017

13. Morsink MAJ, Willemen NGA, Leijten J, Bansal R, Shin SR: Immune organs and immune cells on a chip: An overview of biomedical applications. *Micromachines (Basel)*, 11 (9):849, 2020. DOI: 10.3390/mi11090849

14. Zhang X, Zhao Q, Wen L, Wu C, Yao Z, Yan Z, Li R, Chen L, Chen F, Xie Z, Chen F, Xie Q: The effect of the antimicrobial peptide plectasin on the growth performance, intestinal health, and immune function of yellow-feathered chickens. *Front Vet Sci,* 8:688611, 2021. DOI: 10.3389/fvets.2021.688611

15. Yang Y, Jiang Y, She R, Yin Q, Peng K, Bao H, Wang D, Liu T, Zhou X: Effects of chicken intestinal antimicrobial peptides on humoral immunity of chickens and titres after vaccination with chicken bursal disease virus in antibody. *Arch Anim Nutr*, 60 (5): 427-435, 2006. DOI: 10.1080/17450390600884484

16. Kogut MH: The effect of microbiome modulation on the intestinal health of poultry. *Animal Feed Sci Technol*, 250, 32-40, 2019. DOI: 10.1016/j.anifeedsci.2018.10.008

17. Yadav S, Jha R: Strategies to modulate the intestinal microbiota and their effects on nutrient utilization, performance, and health of poultry. *J Anim Sci Biotechnol* 10:2, 2019. DOI: 10.1186/s40104-018-0310-9

18. Hirsch R, Wiesner J, Bauer A, Marker A, Vogel H, Hammann PE, Vilcinskas A: Antimicrobial peptides from rat-tailed maggots of the drone fly *Eristalis tenax* show potent negative activity against gram-bacteria. *Microorganisms*, 8 (5):626, 2020. DOI: 10.3390/microorganisms8050626

19. Liu Y, Shi J, Tong Z, Jia Y, Yang K, Wang Z: Potent broad-spectrum antibacterial activity of amphiphilic peptides against multidrug-resistant bacteria. *Microorganisms*, 8 (9):1398, 2020. DOI: 10.3390/microorganisms8091398

20. Tanhaeian A, Ahmadi FS, Sekhavati MH, Mamarabadi M: Expression and purification of the main component contained in camel milk and its antimicrobial against plant pathogens. *Probiotics Antimicrob Proteins*, 10, 787-793, 2018. DOI: 10.1007/s12602-018-9416-9

21. Tanhaeian A, Azghandi M, Razmyar J, Mohammadi E, Sekhavati MH: Recombinant production of a chimeric antimicrobial peptide in *E. coli* and assessment of its activity against some clinically isolated avian chimeric pathogens. *Microb Pathog*, 122, 73-78, 2018. DOI: 10.1016/j.micpath.2018.06.012

22. Tanhaeian A, Jaafari MR, Ahmadi FS, Vakili-Ghartavol R, Sekhavati MH: Secretory expression of a chimeric peptide in *Lactococcus lactis:* Assessment of its cytotoxic activity and a deep view on interaction with cell-surface glycosaminans by molecular modeling. *Probiotics Antimicrob Proteins*, 11 (3): 1034-1041, 2019. DOI: 10.1007/s12602-018-9496-6

23. Daneshmand, A, Kermanshahi H, Sekhavati MH, Javadmanesh A, Ahmadian M, Alizadeh M, Aldawoodi A: Effects of cLFchimera peptide on intestinal morphology, integrity, microbiota, and immune cells in broiler chickens challenged with necrotic enteritis. *Sci Rep*, 10: 17704, 2020. DOI: 10.1038/s41598-020-74754-x

24. Higgs R, Lynn DJ, Gaines S, McMahon J, Tierney J, James T, Lloyd AT, Mulcahy G, O'Farrelly C: The synthetic form of a novel chicken beta-defensin identified in silico is predominantly active against intestinal pathogens. *Immunogenetics*, 57 (1-2): 90-98, 2005. DOI: 10.1007/s00251-005-0777-3

25. Goto R, Miki T, Nakamura N, Fujimoto M, Okada N: *Salmonella* Typhimurium PagP- and UgtL-dependent resistance to antimicrobial peptides contributes to thegut colonization. *PLoS One,* 12 (12):e0190095, 2017. DOI: 10.1371/journal.pone.0190095

26. Daneshmand A, Kermanshahi H, Sekhavati MH, Javadmanesh A, Ahmadian M: Antimicrobial peptide, cLF36, affects performance and intestinal morphology, microflora, junctional proteins, and immune cells in broilers challenged with *E. coli. Sci Rep,* 9:14176, 2019. DOI: 10.1038/s41598-019-50511-7

27. Luo X, Zheng Y, Wen R, Deng X, Zhou L, Liao H: Effects of ceftriaxone-induced intestinal dysbacteriosis on lymphocytes in different tissues in mice. *Immunobiol*, 221 (9): 994-1000, 2016. DOI: 10.1016/j.imbio.2016.04.003

28. Maglio M, Florian F, Vecchiet M, Auricchio R, Paparo F, Spadaro R, Zanzi D, Rapacciuolo L, Franzese A, Sblattero D, Marzari R, Troncone R: Majority of children with type1 diabetes produce and deposit anti-tissue transglutaminase antibodies in the small intestine. *Diabetes,* 58 (7): 1578-1584, 2009. DOI: 10.2337/db08-0962

29. Fuchs A, Vermi W, Lee JS, Lonardi S, Gilfillan S, Newberry RD, Cella M, Colonna M: Intraepithelial type-1 innate lymphoid cells are a unique responsive subset of IL-12 and IL-15 IFN-γ-producing cells. *Immunity*, 38 (4): 769-781, 2013. DOI: 10.1016/j.immuni.2013.02.010

30. Knoop KA, McDonald KG, McCrate S, Newberry RD: Microbial sensing by goblet cells controls immune surveillance of luminal antigens in the colon. *Mucosal Immunol*, 8 (1): 198-210, 2015. DOI: 10.1038/mi.2014.58

31. Ridler C: Sentinel goblet cells flush out bacteria from crypts. *Nat Rev Gastroenterol Hepatol,* 13 (8): 438, 2016. DOI: 10.1038/nrgastro.2016.117

32. Liu T, She R, Wang K, Bao H, Zhang Y, Luo D, Hu Y, Ding Y, Wang D, Peng K: Effects of rabbit sacculus rotundus antimicrobial peptides on the intestinal mucosal immunity in chickens. *Poult Sci,* 87 (2): 250-254, 2008. DOI: 10.3382/ps.2007-00353

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Research Article

Expression of Cytokeratin 8, 18 and 19 in the Period of Late Lactation and Involution in Cow Mammary Gland

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Abstract: Cytokeratins are intermediate filament proteins found in epithelial cells. Specific cytokeratin expression has been suggested to mark different epithelial cell line and also to associate with mammary stem/progenitor cells. However, a comparative analysis of the expression of cytokaratins in the mammary gland during the late lactation and involution periods was limited. Here, the aim of the present study is to evaluate CK8, CK18 and CK19 expressions of the mammary gland in cows during late lactation and involution periods, immunohistochemically. The lobe and lobule structure were prominent in the mammary tissue in the late lactation period, but the amount of connective tissue started to increase, and epithelial cells found on the walls of the alveoli had different appearances based on their secretion status. The walls of the ducts were covered by simple columnar epithelial cells during late lactation. CK8 and CK18 showed strong expressions in the epithelial cells of several alveoli and ducts inside the lobes and lobules in the late lactation and involution periods. In both late lactation and involution periods, there was no CK19 expression in the mammary gland tissue. In conclusion, it was demonstrated that CK8 and CK18 were expressed in the alveolar and ductal epithelial cells of the mammary gland in cows in the late lactation and involution periods, but CK19 was not expressed. Thus, our study findings revealed the role of CK8 and CK18 in mammary epithelial differentiation and maintenance of the normal mammary epithelial layer.

Keywords: *Mammary gland, Cytokeratin, Cow, Immunohistochemistry*

İnek Meme Bezinde Laktasyon Sonu ve İnvolusyon Döneminde Sitokeratin 8, 18 ve 19 Ekspresyonu

Öz: Sitokeratinler, epitel hücrelerinde bulunan ara filament proteinlerindendir. Ekspresyonları organ veya dokuya özgüdür ve epitel hücrelerinin tanımlanmasına izin verir, ayrıca meme kök/progenitor hücreler arasındaki ilişkiyi ortaya koyar. Bununla birlikte, geç laktasyon ve involüsyon dönemlerinde meme bezinde sitokaratinlerin ekspresyonunun karşılaştırmalı bir analizi sınırlıydı. Bu çalışmanın amacı, geç laktasyon ve involüsyon dönemlerinde ineklerde meme bezinde CK8, CK18 ve CK19 ekspresyonunu immünohistokimyasal olarak değerlendirmektir. Geç laktasyon döneminde meme bezi dokusunda lob veya lobül yapıları belirgindi, ancak bağ dokusu miktarı artmaya başlamıştı ve alveol duvarında bulunan epitel hücreleri salgı durumuna göre farklı görünümlere sahipti. Geç laktasyon süresince kanalların duvarları tek katlı kolumnar epitel hücreleri ile örtülmüştü. CK8 ve CK18, geç laktasyon ve involusyon dönemlerinde lob ve lobüllerin içindeki kalın epitel hüclerinde güçlü ekspresyonlar göstermiştir. Hem geç laktason döneminde hem de involusyon dönemlerinde meme bezi dokusunda CK 19 ekpresyonu yoktu. Sonuç olaral geç laktasyon ve involusyon döneminde ineklerde meme bezinin alveolar ve duktal epitel hücrelerinde CK8 ve CK18'in eksprese edildiği, ancak CK19'un eksprese edilmediği gösterildi. Böylece, çalışma bulgularımız CK8 ve CK18'in meme epitel farklılaşması ve normal meme epitel tabakasının korunmasındaki rolünü ortaya koydu.

Anahtar sözcükler: *Meme bezi, Sitokeratin, İnek, İmmunohistokimya*

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Introduction

The mammary gland is a complex organ that consists of various groups of tissues and cells, which undergo certain processes like growth differentiation as the organ develops in distinct stages. These physiological stages are regulated both systemically and locally, by means of steroid hormones of ovarian origin -like estrogen and progesterone-, as well as several other molecular factors. The sustainability of milk yield in farm animals depends on the reproduction strategies implemented for the herd at large, but the aforementioned cellular mechanisms that determine the numbers and activities of secretory cells in the mammary gland determine the final milk output and its sustainability [1]. The mammary gland of cows is composed of a network of alveoli and ducts localized in the stroma, which show a high degree of branching [2]. This system consists of epithelial and myoepithelial cells that cover the ducts and alveoli [2,3]. The epithelial cells are surrounded by the stroma at the perimeter, where some cell types responsible for the homeostasis of the mammary gland (adipocytes, fibroblasts, and immune cells) can be found. Alveolar epithelial cells synthesize and secrete milk. Myoepithelial cells surround alveolar epithelial cells, adhere to the base membrane, and contract to transfer the milk secreted in the alveolar lumen to the ducts. Myoepithelial cells in the mammary gland also mediate the proliferation, survival, and differentiation of alveolar cells, and contribute to breast morphogenesis by modulating stromal cells [4]. In dairy cows, the mammary tissue undergoes comprehensive morphological and functional transformation stages that occur concerning cellular and structural changes of the lactation period [5]. In the early lactation period, mammary cells multiply at a rate that exceeds the rate of apoptosis and increase their total number. With the decrease in milk yield in the late lactation period, the loss of mammary cells (apoptosis) starts. This situation leads to the involution process where the mammary alveoli are effaced [6].

Three types of filaments constitute the cytoskeleton of all mammalian tissues. These are microfilaments, microtubules, and intermediate filaments. Depending on both the function of the tissue and the type of epithelial cells, intermediate filaments regulate the growth status and differentiation of the tissue they are in [1]. Cytokeratins (CKs) are intermediate filament proteins that are present in most epithelial cells. Their expression is largely organ or tissue specific. The CK expressed by an epithelial cell is mainly dependent on the type of the epithelial cell, its state in the terminal differentiation process, and its developmental stage. Accordingly, determining CK expressions allow the identification of epithelial cells [3,7,8]. There are two types of CKs which are usually found in the form of heterodimers among acidic type I CKs (CK9–CK20) and basic or neutral type II CKs (CK1–CK8). Some CKs act as lineage markers in the mammary epithelium. While CK8 and CK18 are found characteristically in the luminal cells of a normal gland, CK5 and CK14 are localized in basal or myoepithelial cells. CK7 and CK19 are generally expressed in luminal cells, and sometimes basal cells [9]. Additionally, it has been accepted that CK6 is strongly expressed by mammary gland alveolar epithelial cells in mice, and it can be a marker for multipotent/bipotent mammary epithelial progenitor cells in the pregnancy, lactation, and involution periods [10-12]. CK14/CK8-positive suprabasal/luminal cells have rarely been detected in adult mouse mammary glands, and it was proposed that they are progenitor cells [13]. CK19 is known as a neutral CK, and its expression is accepted to be a lumen marker in human mammary glands [14]. Studies conducted in cows about the mammary gland have also reported that some CKs are expressed in various cell groups in the lactation or involution period [15].

Throughout the lifecycles of female mammals, the mammary gland goes through a set of periodical changes in pregnancy, lactation, and involution. These changes are regulated by the complex interaction of hormonal and molecular factors [16,17]. Cows are arguably the most significant animals for the dairy industry, and the cow mammary gland is a complex tissue with has various physiological, biochemical, and immunological functions [18]. The presence and amounts of molecular factors localized in the mammary gland may significantly influence the milk yield; therefore, understanding their physiological roles carries importance. This cellular and functional complexity make up for an interesting research topic regarding the contributions of different components in the functioning of the mammary gland. In this context, the aim of this study was to determine the localization of the CK8, CK18, and CK19 in the mammary gland cells of cows in the late lactation and involution periods, and to determine whether the localization and expression intensities of these locally expressed factors within the mammary glands are influenced by the lactation and involution periods.

Material and Methods

Experimental Animals and Samples Collection

The animal material of this study consisted of 10 Holstein cows between the ages of 2 to 8. The animals had been transported to local slaughterhouses in the province of Diyarbakır, Türkiye had normal reproductive performances, and did not show any noticeable mammary problems in their macroscopic examination. Through clinical observation, the mammary glands of the animals were examined to determine the presence or absence of diseases such as mastitis. The owners of the animals were questioned to obtain information regarding the

periods in which the mammary glands of the animals were [4,19]. The study animals were selected based on the statements of their owners and clinical inspections, and the animals were slaughtered. Samples were then collected from both the right frontal and left posterior lobes of the mammary glands of the animals. Tissue samples from different parts of mammary glands (upper, middle and lower parts) were also taken. The specimens were fixed in a 10% formaldehyde-alcohol solution after collection. Following fixation, the tissues were dehydrated, cleared and embedded in paraffin blocks. Five-µm-thick serial sections were then taken from these blocks at intervals of at least 100 µm. To help identify the periods of the mammary glands histologically, sections samples were stained with Crosman's triple stain and examined. For immunohistochemical (IHC) analyses, 3 preparations were made from each mammary gland specimen. The tissue sections were placed on slides coated with 3- aminopropyl-ethoxy silane (APES) (Sigma–Aldrich Chemicals, St. Louis, MO, USA) and were left to dry at room temperature for a day.

Determination of the Mammary Glands Periods

In the identification of the periods of the mammary glands of the animals, in addition to the clinical examinations and the statements of their owners, histological examination of the lobes and lobules in the mammary glands was used. The amount of connective tissue, formations of large vacuoles in epithelial cells (as a result of the intracellular accumulation of droplets of fat), and of secretory vesicles, the histological appearances of alveolar and ductal epithelial cells, and the presence of casein concretions (in the form of colloidal masses or a concentric structure in alveolar lumina) were used as determination criteria [4,19]. Based on the examinations, the animals were divided into two groups as late lactation (N:4) and involution (N:6) groups.

Immunohistochemical Staining

The Avidin-Biotin-Peroxidase Complex (ABC) procedure was performed for immunohistochemical staining. To inactivate endogenous peroxidase, the sections were kept in 3% hydrogen peroxide prepared with methanol for 30 min and washed with 0.01M PBS for 3 x 5 min. For antigen retrieval, the sections were boiled in citrate buffer solution (pH:6) for 20 min. Next, they were cooled and washed with PBS for 3 x 5 min. The sections were incubated with UV block for 10 min. The prepared specimens were incubated overnight at +4°C with 1/100-diluted CK8, CK18 and CK19 primary antibodies (Mouse Monoclonal Antibody, Cytokeratin 8, catalogue no: ab2530 Abcam, Mouse monoclonal Antibody Cytokeratins 18, catalogue no: ab668 Abcam, Mouse Monoclonal Antibody Cytokeratin 19, catalogue no: MA5-12663 Invitrogen). After incubation, the sections were washed with 0.01M PBS for 3 x 5 min. The samples were then kept at room temperature for 20 min with biotinylated secondary antibodies (Histostain Plus Bulk Kit, Zymed) and washed with 0.01M PBS for 3 x 5 min. Next, the sections were incubated with streptavidin-peroxidase (HRP- Histostain Plus Bulk Kit, Zymed) for 20 min, followed by washing with PBS for 3 x 5 min. Finally, the sections were incubated with 3,3-diaminobenzidine (DAB) chromogen solution for 5-15 min according to the manufacturer's protocol, counterstained with Harris Hematoxylin for 3 min, dehydrated through an alcohol series, cleared in xylene, and mounted in Entellan (Merck, Darmstadt, Germany, Cat. No:107960) under a coverslip.

The specificity of the immunohistochemical staining process was analyzed using negative and positive controls. Tongue and skin sections of the cows were utilized as the positive control. For the negative control, non-specific immune serum was dripped instead of primary antibodies, and the remaining staining steps were repeated as in the normal staining procedures. The same protocol was applied to all sections.

The immunoreactivities of CK8, CK18, and CK19 were examined and photographed using a Nikon Eclipse E400 (Nikon, Tokyo, Japan) microscope equipped with a DS-RI1 video camera (DS-U3, Nikon, Tokyo, Japan).

Semi-Quantitative Analysis

The immunohistochemical staining results were examined based on the intensity score. The cells that showed a positive reaction for the expressions of CK8, CK18, and CK19 were qualitatively analyzed. The positive cells were scored on four levels as (-) negative; (+) weak; (++) medium or (+++) strong, based on their intensity of staining. The assessments of the positively stained cells were made by two experiment-blind researchers (A.A.A. and U.T.), and average scores were calculated. In the mammary gland sections, the expression of CK8, CK18, and CK19 were examined at 40x, 100x and 400x magnifications. In each zone of the mammary gland specimen, three randomly selected areas were examined. The results were separately examined for alveolar and ductal epithelial, stromal, and myoepithelial cells. The results are presented in the results section *(Table 1)*.

Results

Histological Results

In all specimens, the mammary gland was surrounded by a capsule formed by connective tissue with compound tubule-alveolar structures, as described in previous studies. It was observed that the lobe-and-lobule structure was prominent in the mammary tissue during the late lactation period, but the amount of connective tissues was slightly elevated, and epithelial cells found on the walls

of the alveoli had different appearances based on their secretion status. Accordingly, in the alveoli, both simple columnar epithelial cells filled with secretion could be seen, along with empty, flatter cells that had released their secretion. Casein concretions in the form of colloidal masses were detected within the lumens of some of the alveoli. A thin layer of connective tissue surrounded the walls of the ducts, which in turn as covered by simple columnar epithelial cells. The ducts' lumens were still wide, the branching continued. Lumens of some ducts were filled with secretory material.

Table 1. *Semiquantitative evaluations of immunohistochemical staining intensities of CK8, CK18 and CK19*

Mammary	Cells	CK8	CK18	CK19
End of Lactation	Alveolar epithelium	(+++)	(+++)	(-)
	Channel Epithelium	(++) /(+++)	(+++)	(-)
	Stromal Cells	(-)	(-)	(-)
	Myoepithelial Cells	(-)	(-)	(-)
Involution	Alveolar epithelium	(++) /(+++)	(+++)	(-)
	Channel Epithelium	(++) /(+++)	(+++)	(-)
	Stromal Cells	(-)	(-)	(-)
	Myoepithelial Cells	(-)	(-)	(-)

In the mammary tissue in the involution period, as a result of the intracellular accumulation of droplets of fat and secretory vesicles, large vacuoles in the simple cuboidal epithelial cells were noticeable. The lumen of the alveoli had become narrower, and the inter-alveolar connective tissue amount was elevated. The lumens of many of the mammary alveoli were narrower than their counterparts in the late lactation period samples. The ducts were lined with simple cuboidal or simple squamous epithelium, and these ducts were filled with secretory material *(Fig. 1)*.

Immunohistochemical Results

Late lactation: It was revealed that CK8 and CK18 showed strong expressions in the epithelial cells of several alveoli and ducts within the lobes and lobules. These expressions were particularly localized in the cytoplasm. There were no CK8 or CK18 expressions in the myoepithelial cells. No CK8 or CK18 expression was detected in the cells and blood vessels of the connective tissues forming the lobes and lobules, either. Some individual cells localized in the connective tissue displayed positive reactions of CK8 and CK18 at varying intensities *(Fig. 2, Fig. 3-A)*. There was no CK19 expression in any tissue component constituting the mammary glands of the cows in our study *(Fig. 4-A)*.

Involution: CK8 and CK18 were found to show strong

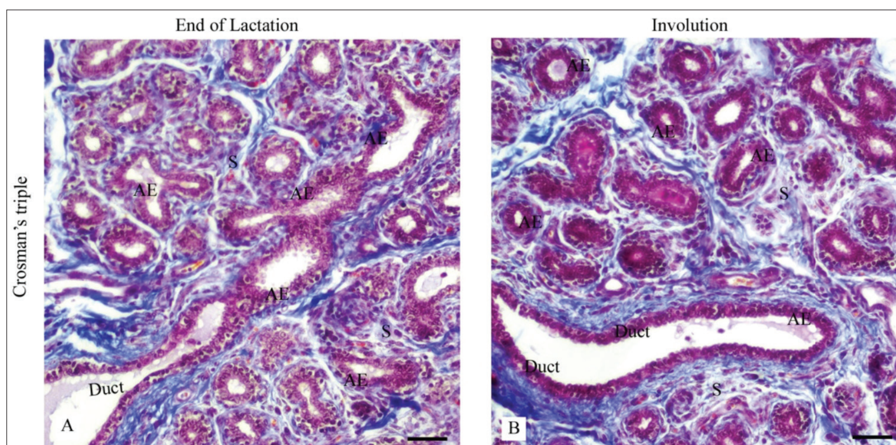

Fig 1. Crosman's triple stain. **A:** Late of lactation, **B:** Involution; **AE:** Alveolar epithelium, **S:** Stroma, **Duct:** Alevolar ductus. Bar: 50 µm (A,B)

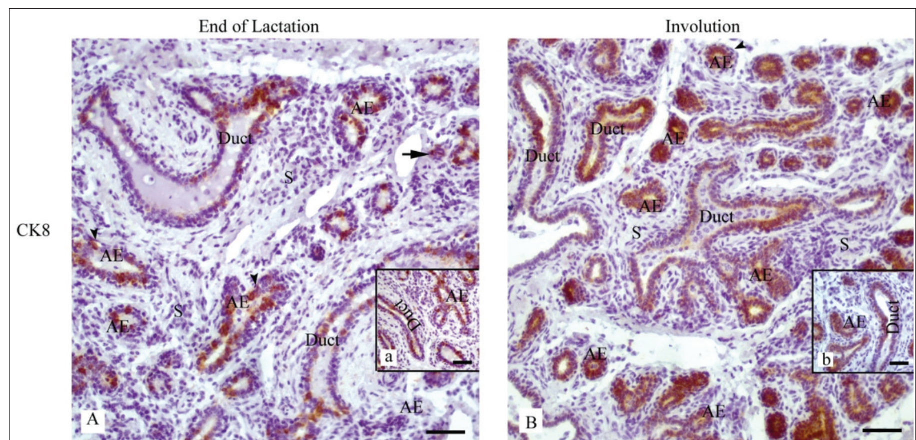

Fig 2. Immunohistochemical expression of CK 8. **A:** late of lactation, **B:** Involution; **AE:** Alveolar epithelium, **S:** Stroma, **Duct:** Alevolar ductus. *Arrow:* Alveolar epithel, *Arrow head:* Myoepithelial cells. Bar: 25 µm (a,b), 50 µm (A,B)

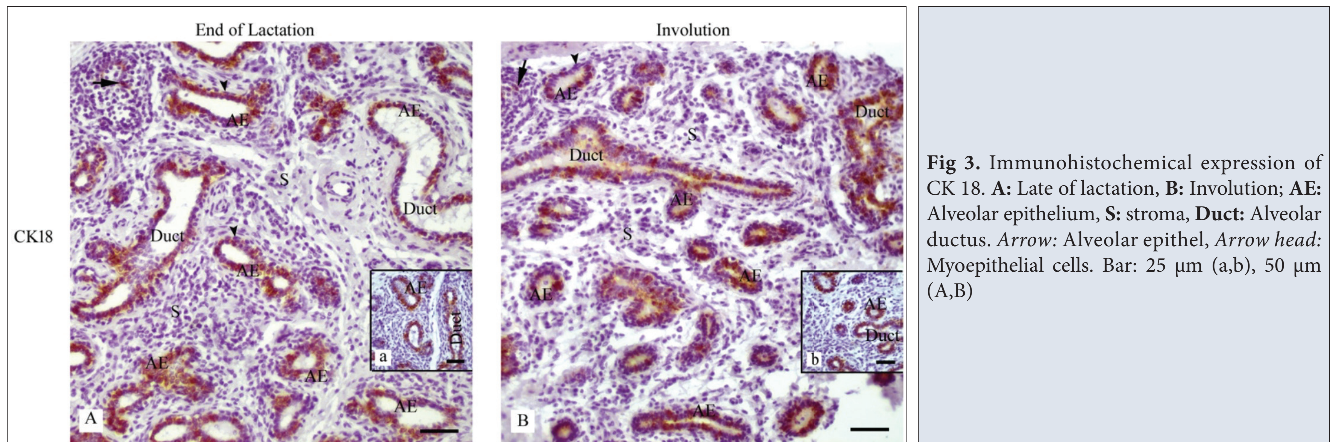

Fig 3. Immunohistochemical expression of CK 18. **A:** Late of lactation, **B:** Involution; **AE:** Alveolar epithelium, **S:** stroma, **Duct:** Alveolar ductus. *Arrow:* Alveolar epithel, *Arrow head:* Myoepithelial cells. Bar: 25 µm (a,b), 50 µm (A,B)

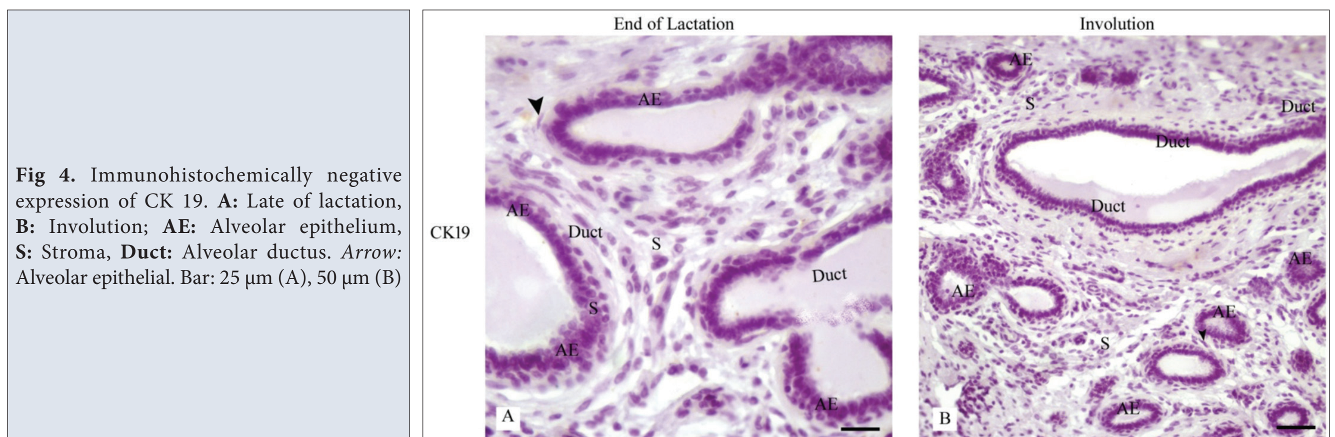

Fig 4. Immunohistochemically negative expression of CK 19. **A:** Late of lactation, **B:** Involution; **AE:** Alveolar epithelium, **S:** Stroma, **Duct:** Alveolar ductus. *Arrow:* Alveolar epithelial. Bar: 25 µm (A), 50 µm (B)

cytoplasmic expressions in the epithelial cells covering the alveoli and ducts. CK8 and CK18 had positive reactions in some individual cells belonging to the fibrocyte/fibroblast, macrophage (probably) in the septa of the connective tissue *(Fig. 2, Fig. 3-B)*. There was no expression in myoepithelial cells, connective tissue cells or blood vessels. There was no CK19 expression in the mammary gland tissue in this period either *(Fig. 4-B)*.

Discussion

In the mammary gland tissue, molecular regulations influence the process of lactation and involution periods. Expressions of genes that regulate hormonal and molecular factors in the mammary gland are important for milk production in ruminants. In the development (mammogenesis) and lactation (galactopoiesis) periods of the mammary gland in dairy cows, each group of cells has a certain property related to the plasticity of the mammary gland. As in most glandular tissues, the mammary glands of adults also contain several types of cells that interact to shape the organ and make it functional. This study revealed the expressions and localizations of CKs among the intermediate filaments taking part in the cytoskeleton, especially in groups of cells that play a role in mammary gland plasticity, as well as their potential roles in cell differentiation, in the late lactation and involution periods.

The ruminant mammary gland is organized in terminal duct-lobular units. A terminal duct lobular unit is a group, or lobule, of mammary acini with dead-ends, along with intralobular and extralobular portions of the subtending terminal ducts of the mammary gland [20]. The involution period has some dramatic changes occurring within the mammary gland at the end of lactation. Involution is initiated after sudden or gradual weaning among all species in both experimental and natural settings. Understanding the biological process of mammary gland regression is important as it supports livestock measures taken at weaning time to reduce the incidence of mastitis in all ruminants, including cows [21]. A typical lactation curve in cows is defined by a peak point in milk yield that lasts between the 30th and 90th days, followed by a decrease in milk yield, which is known as the late lactation period. In this study, it was determined that lobe formation was prominent in the late lactation and involution periods as stated by Holst et al.[19] and Hurley [22] for cows, but the amount of connective tissue was found to be elevated. The epithelial cells covering the walls of the alveoli and ducts also had different appearances based on their secretion status. Casein corpuscles were seen in the lumina of some

alveoli. The walls of the ducts were found to be covered by simple columnar epithelial cells, and the lumina were filled with secretory material. Furthermore, a large reduction in the volume of the mammary gland was observed with the onset of involution, and the residue of the collapsed alveolar structures was more prominent compared to that of the late lactation period. The alveoli and ducts also had narrower lumina [19,22].

A literature review performed as part of this study revealed that most studies about the localization of CKs have been conducted on mammary gland tumors [23,24]. Some studies have reported that alveolar and ductal epithelial cells and myoepithelial cells in the normal mammary tissue (in humans and mice) expressed CK8 and CK18 at different intensities and to a noticeable extent [16,25]. CK8 and CK18 were shown to be expressed in epithelial cells in the mammary glands of cows in the involution period [2]. Similarly, it was reported that CK18 was strongly expressed in mammary gland primary epithelial cells and cell cultures obtained from the mammary gland during the lactation period in cows. This was surmised as a potential characteristic marker for mammary epithelial cells in cows [26]. In other studies, performed on the human mammary gland in resting state, it was reported that CK8 and CK18 were expressed in alveolar and ductal epithelial cells, but no information was provided about the intensity of expression [3,27]. It was revealed that CK18 was strongly expressed in the *in vitro* cultures of cow and mouse mammary epithelial cells [21]. In agreement with the studies mentioned above, in this study, CK8 and CK18 were strongly expressed in the alveolar and ductal epithelial cells of the mammary gland in the late lactation and involution periods in cow mammary tissues. However, as opposed to reports on humans and mice, no CK8 or CK18 expression was detected in the myoepithelial cells of the cows in this study [16,25]. Additionally, in our study, no cellular localization of either of these two factors was observed in connective tissue cells and blood vessels in the late lactation period or the involution period.

The complex events that develop in the normal mammary gland throughout pregnancy, lactation and involution can be distinguished based on the expressions of CKs as markers of epithelial cell differentiation [28,29]. Mammary epithelial cells may also be recognized by their expression of intermediate filaments, although this may vary between species and between locations within the mammary gland. For example, the expression of specific CKs may be different between epithelial cells in ducts and within alveolar units [30]. In both normal and neoplastic mammary gland tissues, the biological importance of heterogeneous CK expressions has not been completely understood, and there is a need to shed light on the control mechanisms of these expressions in normal mammary growth and malignant transformation [31,32].

CK filaments take part in providing the mechanical scaffolding that is needed by cells in epithelial tissue [33]. In any case, they regulate morphogenesis and cell differentiation in tissues or organs. In particular, CKs show molecular and functional expressions in tissues depending on epithelium type and differentiation characteristics. These situations provide us with information about different cytogenetic changes in tissues [16,33]. Among intermediate filament in the CK family, CK18 is the most prevalently encountered member in tissues and organs, and it is usually found alongside CK8. Both are expressed in simple epithelial cells in the organism, and they are known as significant markers for alveolar and ductal epithelial cells [16,22]. Considering the information given above, the presence of CK8 and CK18 expressions in the late lactation and involution periods in our study suggested that these CKs may also perform similar functions in the mammary glands of cows.

CK19 is known as an important marker for alveolar epithelial cells [32]. CK19 expression is mostly seen in cells with an epithelial origin that has high plasticity such as stem cells, cells with a high capacity to differentiate, and tumoral cells [8]. Alveolar and ductal epithelial cells in human and mouse mammary glands were reported to express CK19 at varying intensities [16]. In their study on cows, Ontsouka et al.[32] demonstrated that CK7 and CK19 levels were either at very low or undetectable levels in the primary cells (epithelial cells) of the mammary gland. The localization of CK7 and CK19 expressions have been shown in cell cultures that were subcultured and made immortal after collection from the mammary gland, especially in the pregnancy, lactation or involution period of cows [32-34]. In our study, neither alveolar nor ductal epithelial cells in the cow mammary glands displayed CK19 expressions in either of the late lactation or involution periods. The absence of CK19 expressions in the mammary glands of cows in this study and clear contradictions between this study and other studies demonstrated the complex nature of cells with the mammary origin and suggested that analyses conducted based solely on the expressions of cell type markers may be risky. Moreover, as opposed to the results of this study, the information that CK19 is a marker for epithelial cells in both mammary gland epithelial cells and cell cultures obtained from the mammary gland still holds.

In conclusion, in this study, it was demonstrated that CK8 and CK18 were expressed in the alveolar and ductal epithelial cells of the mammary gland in cows in the late lactation and involution periods, but CK19 was not expressed in any of these scenarios. This gave rise to the idea that CK8 and CK18 expressions may be effective -rather than CK19- on the differentiation of epithelial cells for the cytoarchitecture in the mammary gland,

at the very least for cows. We humbly believe that by increasing our knowledge about the regulation of these molecular factors in the mammary gland, we will be able to develop methods for increasing milk yield and lactation sustainability, shorten the length of the dry period between two consecutive lactation periods, and increase milk productivity. Furthermore, data on cytokeratins in the mammary glands of cows may constitute an excellent alternative model for biochemical studies that investigate their potential roles in the physiological functions of the breast and carcinogenesis.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author (A. Arkaş Alklay) on reasonable request.

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Competing Interest

The authors declare that they no conflict of interest.

Author Contributors

AAA and UT planned the study, designed the experiments and helped manuscript writing; FÇ helped with data analyses and bioinformatics and wrote the manuscript; UT, FÇ and NA collected samples and conducted laboratory process; BB and ÖA analysed the statistics data. All authors read and approved the final manuscript.

Ethical Approval

The materials used in our study were collected from the slaughterhouses of the province of Diyarbakir, and in accordance with the regulation on the working procedures and principles of animal experimentation ethics committees in the official gazette published on February 15, 'Procedures with dead animals or tissues, slaughterhouse materials, waste fetuses' are not subject to HADYEK permission.

References

1. Pantschenko AG, Woodcock-Mitchell J, Bushmich SL, Yang TJ: Establishment and characterization of a caprine mammary epithelial cell line (CMEC). *In Vitro Cell Dev Biol Anim*, 36, 26-37, 2000.

2. Mujyambere B, Jayaraj R, Suja S: Cytokeratin 19 (CK19) as a marker for epithelial differentiation and malignant transformation: Its clinical relevance in diagnosis, prognosis and treatment response monitoring. *IRE J*, 2 (3): 51-61, 2018.

3. Marettova E, Maretta M: Immunohistochemical study of the stromal cells in the lactating bovine mammary gland. *Folia Vet*, 62 (3): 29-35, 2018. DOI: 10.2478/fv-2018-0024

4. Alkafafy M, Rashed R, Helal A: Immunohistochemical studies on the bovine lactating mammary gland *(Bos taurus)*. *Acta Histochem*, 114 (2): 87-93, 2012. DOI: 10.1016/j.acthis.2011.02.012

5. Finot L, Chanat E, Dessauge F: Molecular signature of the putative stem/progenitor cells committed to the development of the bovine mammary gland at puberty. *Sci Rep,* 8 (1):16194, 2018. DOI: 10.1038/s41598-018-34691-2

6. Bocchinfuso WP, Lindzey JK, Hewitt SC, Clark JA, Myers PH, Cooper R, Korach KS: Induction of mammary gland development in estrogen receptor-α knockout mice. *Endocrinology,* 141, 2982-2994, 2000. DOI: 10.1210/endo.141.8.7609

7. Jaiswal P, Yadav YK, Sinha SS, Singh MK: Expression of cytokeratin 8 in oral squamous cell cancer. *Indian J Pathol Oncol*, 5 (1): 55-60, 2018. DOI: 10.18231/2394-6792.2018.0011

8. Stasiak PC, Purkis PE, Leigh IM, Lane EB: Keratin 19: Predicted amino acid sequence and broad tissue distribution suggest it evolved from keratinocyte keratins. *J Invest Dermatol*, 92, 707-716, 1989. DOI: 10.1016/0022-202X(89)90186-3

9. Sun P, Yuan Y, Li A, Li B, Dai X: Cytokeratin expression during mouse embryonic and early postnatal mammary gland development. *Histochem Cell Biol*, 133, 213-221, 2010. DOI: 10.1007/s00418-009-0662-5

10. Grimm SL, Bu W, Longley MA, Roop DR, Li Y, Rosen JM: Keratin 6 is not essential for mammary gland development. *Breast Cancer Res*, 8:R29, 2006. DOI: 10.1186/bcr1504

11. Sapino A, Macri L, Gugliotta P, Pacchioni D, Liu YJ, Medina D, Bussolati G: Immunophenotypic properties and estrogen dependency of budding cell structures in the developing mouse mammary gland. *Differantiation*, 55, 13-18, 1993. DOI: 10.1111/j.1432-0436.1993.tb00028.x

12. Sonu S, Prasad GR, Lal JB, Deepika L, Ragini H: Pathomorphological studies and immunohistochemical expression of p53 and pan-cytokeratin in bovine epithelial tumors. *Indian J*, 44 (1): 1-6, 2020. DOI: 10.5958/0973-970X.2020.00001.2

13. Li Y, Welm B, Podsypanina K, Huang S, Chamorro M, Zhang X, Rowland T, Egeblad M, Cowin P, Werb Z, Tan LK, Rosen JM, Varmus HE: Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. *Proc Natl Acad Sci USA*, 100, 15853-15858, 2003. DOI: 10.1073/pnas.2136825100

14. Bartek J, Bartkova J, Taylor-Papadimitriou J: Keratin 19 expression in the adult and developing human mammary gland. *Histochem J,* 22, 537-544, 1990. DOI: 10.1007/BF01005976

15. Woelfle U, Sauter G, Santjer S, Brakenhoff R, Pantel K: Down-regulated expression of cytokeratin 18 promotes progression of human breast cancer. *Clin Cancer Res,* 10, 2670-2674, 2004. DOI: 10.1158/1078-0432.ccr-03-0114

16. Abd El-Rehim DM, Pinder SE, Paish CE, Bell J, Blamey RW, Robertson JFR, Nicholson RI, Ellis IO: Expression of luminal and basal cytokeratins in human breast carcinoma. *J Pathol,* 203, 661-671, 2004. DOI: 10.1002/path.1159

17.Wood CE, Hester JM, Cline JM: Mammary gland development in early pubertal female macaques. *Toxicol Pathol*, 35, 793-803, 2007. DOI: 10.1080/01926230701584213

18. Anand V, Dogra N, Singh S, Kumar SN, Jena MK, Malakar D, Dang AK, Mishra BP, Mukhopadhyay TK, Kaushik JK, Mohanty AK: Establishment and characterization of a buffalo *(Bubalus bubalis)* mammary epithelial cell line. *PLoS One*, 7 (7):e40469, 2012. DOI: 10.1371/journal.pone.0040469

19. Holst BD, Hurley WL, Nelson DR: Involution of the bovine mammary gland: Histological and ultrastructural changes. *J Dairy Sci*, 70, 935-944, 1987. DOI: 10.3168/jds.S0022-0302(87)80097-8

20. Capuco AV, Ellis S, Wood DL, Akers RM, Garrett W: Postnatal mammary ductal growth: Three-dimensional imaging of cell proliferation, effects of estrogen treatment, and expression of steroid receptors in prepubertal calves. *Tissue Cell,* 34 (3): 143-154, 2002. DOI: 10.1016/S0040-8166(02)00024-1

21. Petridis IG, Fthenakis GC: Mammary involution and relevant udder health management in sheep. *Small Ruminant Res,* 181, 66-75, 2019. DOI: 10.1016/jsmallrumres.2019.07.001

22. Hurley WL: Mammary gland function during involution. *J Dairy Sci,* 72,1637-1646, 1989. DOI: 10.3168/jds.S0022-0302(89)79276-6

23. Hu H, Wang J, Bu D, Wei H, Zhou L, Li F, Loor JJ: *In vitro* culture and characterization of a mammary epithelial cell line from Chinese Holstein dairy cow. *PLoS One*, 4 (11):e7636, 2009. DOI: 10.1371/JOURNAL.PONE.0007636

24. Böcker W, Moll R, Poremba C, Holland R, Van Diest PJ, Dervan P, Bürger H, Wai D, Diallo RI, Brandt B, Herbst H, Schmidt A, Lerch MM, Buchwallow IB: Common adult stem cells in the human breast give rise to glandular and myoepithelial cell lineages: A new cell biological concept. *Lab Invest*, 82 (6): 737-746, 2002. DOI: 10.1097/01.lab.0000017371.72714.c5

25. Boutinaud M, Herve L, Lollivier V: Mammary epithelial cells isolated from milk are a valuable, non-invasive source of mammary transcripts. *Front Genet*, 6:323, 2015. DOI: 10.3389/fgene.2015.00323

26. Choi I, Gudas LJ, Katzenellenbogen BS: Regulation of keratin 19 gene expression by estrogen in human breast cancer cells and identification of the estrogen responsive gene region. *Mol Cell Endocrinol*, 164 (1-2): 225-237, 2000. DOI: 10.1016/S0303-7207(00)00197-0

27. Taylor-Papadimitriou J, Lane EB: Keratin expression in the mammary gland. In, Neville MC, Daniel CW (Eds): The Mammary Gland. New York, NY, Plenum, 1987.

28. Taylor-Papadimitriou J, Stampfer M, Bartek J, Lewis A, Boshell M, Lane EB, Leigh IM: Keratin expression in human mammary epithelial cells cultured from normal and malignant tissue: Relation to *in vivo* phenotypes and influence of medium. *J Cell Sci*, 94 (Pt 3): 403-413, 1989. DOI: 10.1242/jcs.94.3.403

29. Gould VE, Koukoulis GK, Jansson DS, Nagle RB, Franke WW, Moll R: Coexpression patterns of vimentin and glial filament protein with cytokeratins in the normal, hyperplastic, and neoplastic breast. *Am J Pathol*, 137 (5): 1143-1155, 1990.

30. Dontu G, Ince TA: Of mice and women: A comparative tissue biology perspective of breast stem cells and differentiation. *J Mammary Gland Biol Neoplasia*, 20 (1-2): 51-62, 2015. DOI: 10.1007/s10911-015-9341-4

31. Coulombe PA, Omary MB: 'Hard' and 'soft' principles defining the structure, function and regulation of keratin intermediate filaments. *Curr Opin Cell Biol*, 14 (1): 110-22, 2002. DOI: 10.1016/S0955-0674(01)00301-5

32. Ontsouka EC, Bertschi JS, Huang X, Lüthi M, Müller S, Albrecht C: Can widely used cell type markers predict the suitability of immortalized or primary mammary epithelial cell models? *Biol Res,* 49:1, 2016. DOI: 10.1186/s40659-015-0063-2

33. Perruchot MH, Arevalo-Turrubiarte M, Dufreneix F, Finot L, Lollivier V, Chanat E, Mayeur F, Dessauge F: Mammary epithelial cell hierarchy in the dairy cow throughout lactation. *Stem Cells Dev*, 25 (19): 1407-1418, 2016. DOI: 10.1089/scd.2016.0098

34. Zavizion B, van Duffelen M, Schaeffer W, Politis I: Establishment and characterization of a bovine mammary epithelial cell line with unique properties. *In Vitro Cell Dev Biol Anim,* 32, 138-148, 1996. DOI: 10.1007/BF02723679

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RESEARCH ARTICLE

The Effects of Fast and Slow Thawing on Spermatological Parameters and Detect of Chromatin Condensation by Toluidine Blue Staining in Frozen-Thawed Bull Sperm

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Abstract: The purpose of this study was to observe the effect of different thawing methods on semen parameters such as motility and morphology and sperm chromatin integrity as assessed by toluidine blue (TB) staining. A total of 20 frozen sperm straws from the same Holstein bull were used. While the 30 sec thawing protocol at 37°C, which is used for thawing frozen sperm straws, constitutes our slow thawing group (n=10), the 6 sec thawing protocol at 70°C constitutes our fast-thawing group (n=10). The motility, viability, morphology, plasma membrane integrity, and sperm chromatin condensation parameters of all thawed sperm were investigated. There was a significant difference (P<0.05) in sperm plasma membrane integrity, head defect, and total abnormal sperm morphology. The chromatin decondensation rate detected by TB in bull semen thawed in the slow thaw system, and the decondensation rate in the fast thaw system, differed significantly from each other in line with the literature data (P<0.05). According to the evaluations made in terms of chromatin decondensation rate, the rate obtained in slow thawing (7±0.39) shows an increase up to two times compared to the fast-thawing rate (3.3±0.33) (P<0.05). The TB staining procedure can be used to evaluate infertility and chromatin integrity, especially in cases that are suspicious and require rapid evaluation.

Keywords: *Diagnostic test, Sperm chromatin, Sperm morphology, Toluidine blue*

Dondurulmuş-Çözdürülmüş Boğa Spermasında Hızlı ve Yavaş Çözdürmenin Spermatolojik Parametreler ve Toluidin Mavisi Boyama ile Kromatin Kondenzasyonunun Belirlenmesi Üzerine Etkisi

Öz: Bu çalışmanın amacı, farklı çözdürme yöntemlerinin motilite ve morfoloji gibi sperma parametreleri ve toluidin mavisi boyası kullanılarak sperm kromatin bütünlüğü üzerindeki etkisini gözlemlemekti. Aynı Holstein boğasından toplam 20 adet donmuş sperma payeti kullanıldı. Dondurulmuş sperma payetlerinin çözdürülmesinde kullanılan 37°C'de 30 saniyelik çözdürme protokolü yavaş çözdürme grubumuzu (n=10) oluştururken, 70°C'de 6 saniyelik çözdürme protokolü hızlı çözdürme grubumuzu (n=10) oluşturmaktadır. Tüm çözdürülmüş spermaların motilitesi, canlılığı, morfolojisi, plazma membran bütünlüğü ve sperm kromatin yoğunlaşma parametrelerinin analizleri yapıldı. Karşılaştırılan iki grup arasında spermatozoon plazma membran bütünlüğü, baş ve toplam anormal spermatozoon morfolojisinde önemli bir fark gözlendi (P<0.05). Mevcut literatür verileri doğrultusunda TB boyaması ile saptanan kromatin dekondenzasyonu yavaş çözdürülen spermalarda hızlı çözdürülen spermalara göre birbirinden önemli ölçüde farklılık göstermiştir (P<0.05). Kromatin yoğunlaşma hızı açısından yapılan değerlendirmelere göre yavaş çözdürme ile elde edilen oran (7±0.39) hızlı çözülme hızına (3.3±0.33) göre iki kata kadar artış göstermektedir (P<0.05). Böylelikle TB boyama prosedürü, özellikle şüpheli ve hızlı değerlendirme gerektiren durumlarda infertilite ve kromatin bütünlüğünü değerlendirmek için kullanılabilir.

Anahtar sözcükler: *Tanısal test, Sperm kromatin, Sperm morfolojisi, Toluidin mavisi*

INTRODUCTION

The use of frozen-thawed sperm to improve bovine population genetics is widely accepted and used worldwide [1]. Sperm cryopreservation is an effective method for managing and preserving male fertility in animals [2]. It is also important for livestock production because it facilitates the storage and transport of germplasm, reducing

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the spread of genetic diversity and increasing the global distribution of genetically superior animals [3]. In terms of spermatozoa survival, the thawing of sperm is as important as the freezing procedure [4]. The thawing rate significantly affects the survival of spermatozoa, and it is well known that the appropriate thawing rate is affected by many factors of cryopreservation procedures, including diluent type, glycerol concentration, and freezing speed [5]. Many studies have been conducted to determine the best thawing temperature and time to obtain the highest percentage of viable sperm after thawing [6,7].

Regardless of extender type and cooling rate, it is recommended to thaw frozen semen in a water bath at 33-35°C for 30-40 sec (slow thawing) in laboratory and farm applications [8]. However, many studies have shown that faster thawing at 60-80°C increases post-thaw motility and preserves acrosome integrity [4,5,9]. Rapid thawing of sperm reduces the harmful effects of recrystallization and hydration, preventing damage to the spermatozoon membrane and cytoplasm. As a result, the thawing rate and temperature of the sperm are critical for improving post-thaw parameters [10]. Motility, concentration, morphology, viability, and membrane function are the spermatological parameters that are routinely tested after thawing frozen sperm. However, DNA analysis has recently gained prominence in routine sperm examination [11]. The nucleus of a spermatozoon has a highly condensed chromatin formed by combining double-stranded DNA with proteins known as protamines and histones [1]. Protamine is an essential protein that replaces histones in the nucleus of mature spermatozoa. Protamine binds to multiple disulphide bonds, allowing chromatin to compact and crystallize [12]. Apoptosis, reactive oxygen species (ROS), and protamine deficiency, on the other hand, cause poor chromatin condensation. It causes spermatozoon DNA damage, infertility, and poor results in assisted reproductive techniques in this case [13].

In the last 20 years, many new techniques have been developed to evaluate sperm chromatin condensation. In the evaluation of sperm chromatin integrity, toluidine blue (TB) [14], Acridine orange [15], aniline blue [16], chromomycin A3 (CMA3) staining, and COMET test, TUNEL test, sperm chromatin structure assay (SCSA), sperm chromatin dispersion test (SCD) [17], DNA breakage detection-fluorescence *in situ* hybridization test (DBD-FISH) are used [18]. However, many of these techniques necessitate the use of equipment that is not readily available in laboratories or animal breeding facilities. In addition to these expensive and more infrastructure-requiring tests, TB is preferable because it is cheaper than the others. Toluidine blue is fast, simple, and inexpensive compared to all these methods [12,14]. Toluidine blue is a basic thiazine metachromatic dye that specifically binds to acidic cellular components of the tissue. It shows a high affinity for binding to the phosphate residue of immature spermatozoon DNA. The results of sperm chromatin analysis with toluidine blue are also correlated with other methods, indicating that its use is appropriate [19]. For these reasons, our study aimed to show that in the evaluation of thawed bull sperm at different temperatures, in addition to routine semen parameters, DNA damage can be detected by using a staining method (TB), which is easier to find and gives faster results.

Material and Methods

Ethical Statement

Ondokuz Mayis University Animal Experiments Local Ethics Committee provided an ethics report for this study (E-68489742-602.99-104799).

Sperm Samples and Experimental Design

In the study, a total of 20 frozen sperm straws from the semen of the same Holstein bull were used in order to eliminate the changes that may arise from individual differences. While the 30-sec thawing protocol at 37°C, which is used as the gold standard for thawing frozen sperm straws, constitutes our control group (n=10), the 6 sec thawing protocol at 70°C constitutes our other group (n=10). All thawed sperm were tested for motility, viability, morphology, membrane integrity, and sperm chromatin condensation parameters.

Sperm Motility

The Computer-Aided Sperm Analyzer (CASA), (SCA®, Microptic, Barcelona, Spain) was used to assess frozen-thawed sperm motility and movement characteristics. Total motility (0-100%), progressive motility (0-100%), VAP (mean path velocity, µm/s), VSL (straight-line velocity, µm/s), VCL (curvilinear velocity, µm/s) and ALH (lateral head change, m), BCF (Crossover frequency rhythm Hertz (Hz) values were measured and recorded in at least 5 microscope fields in the software system.

Sperm Morphology

Sperm morphology was determined in accordance with the protocol of the SpermBlue® test kit (Microptic, Spain). Ten µL of the sperm sample was taken and smeared on the slide and left to dry at a 45-degree angle. After drying, the slide was dipped in the jar containing SpermBlue staining. The slide was kept in the dye for 2 min. Then the slide was left to dry at 60-80 degrees. After the drying process, the slide immersed twice into the jar containing distilled water was slowly removed, and the slide was allowed to dry. Following the staining procedure, at least 100 spermatozoa were tested in the CASA system. Morphological disorders of the head, acrosome, middle and tail regions of spermatozoa were evaluated.

Sperm Viability

According to Gilmore et al.[20], the eosin-nigrosin stain was used to test sperm viability. The slides were stained according to the protocols, dried, and coated with a cover slide before being examined with CASA at 60x magnification. The rates (percentages) of live (white sperm heads) and dead (pink sperm heads) sperm were determined by counting 200 spermatozoa per stained slide.

Hypoosmotic Swelling Test

The HOS test was used to evaluate the functional membrane integrity of sperm. One mL of the HOST solution (7.35 g sodium citrate and 13.51 g fructose per 1:1, v/v of distilled water) was collected and placed in an eppendorf tube at 37°C [21]. It was incubated at 37°C for 30 min after adding 10 µL of the semen sample to the HOST solution. After incubating the mixture, one drop was placed on the slide and a smear was obtained. After drying the slide at a 45-degrees angle, this slide was examined at 40x magnification under the microscope, and 200 sperm per slide were counted. The percentage of HOS-positive sperm was calculated with those with a coiling tail.

Determination of Sperm Chromatin Condensation by TB Staining

According to the study by Beletti and Mello [22], sperm samples were thawed in two ways before being subjected to smear preparation. The smears were first fixed in ethanol acetic acid (3:1, v/v) for 1 min before being fixed in 70% ethanol (3 min). The smears were then hydrolyzed for 25 min in 4N hydrochloric acid, washed in distilled water and dried at room temperature. The slides were stained with a droplet of TB 0.025% (w/v) in a sodium citric acid-phosphate buffer (McIlvaine buffer) produced at pH 4.0 and then covered with a coverslip. After 3 min, the images were captured using a light microscope at a magnification of 100x (Nikon, Eclipse, Tokyo, Japan) and CASA. A total of 100 spermatozoon were counted in different areas of each slide using oil immersion and 100x magnification under light microscopic examination. Sperm cell heads with good chromatin integrity were light blue, while those with poor chromatin integrity were deep violet (purple). Deep violet sperms were considered abnormal, and the percentage of sperms with this color was calculated.

Statistical Analyses

Ten semen straws (n=10) were used for each group. Mann Whitney U was used for mean comparisons. The SPSS software (Version 21, SPSS, IBM) was used for all statistical analyses, and differences were considered significant at the P<0.05 level. The results are shown as the Mean±SE.

Results

Sperm Characteristics

Values of sperm characteristics (motility, progressive motility, kinematic parameters, viability, membrane function, and morphology) can be observed in *Table 1*. There was a significant difference (P<0.05) in sperm plasma

Table 1. *Mean and standard error for the post-thaw sperm analysis and the probability values in the slow thawing and fast thawing groups*

Variable	Slow Thawing (n=10)	Fast Thawing (n=10)
Total sperm motility (%)	63.62±3.70	63.49±1.67
Sperm progressive motility (%)	49.68±3.63	48.37±1.35
Sperm curvilinear velocity (µm/s)	111.28±3.37	112.57±2.99
Sperm velocity average pathway (µm/s)	61.91±2.24	58.45±1.86
Sperm velocity straight line (µm/s)	46.17±2.21	42.55±2.03
Sperm straightness (%)	68.09±1.39	66.60±1.55
Sperm linearity (%)	40.42±1.51	36.58±1.89
Wobble (%)	55.82±1.34	52.21±1.64
Amplitude of lateral head displacement (µm)	4.32±0.19	4.63±0.20
Sperm beat cross-frequency (Hz)	9.75±0.38	9.02±0.42
Viability (%)	66.10±2.54	71.40±0.77
Membrane Integrity (%)	63.20±2.78*	74.10±1.71*
Acrosomal defect (%)	1.50±0.26	1.90±0.37
Head defect (%)	5.40±0.30*	3.40±0.37*
Mid-piece Defect (%)	2.50±0.47	2.10±0.23
Tail defect (%)	6.20±0.48	3.70±0.39
Total abnormal sperm morphology (%)	15.60±0.61	11.10±0.40

** Superscripts within the same line differ significantly at P<0.05*

Table 2. Percentages of chromatin alterations identified by toluidine blue in bull sperm from different thawed (Mean±SE)

Variable	Slow Thawing (n= 10)	Fast Thawing (n= 10)
Normal chromatin condensation	93.0±0.39[b]	96.7±0.33[a]
Chromatin decondensation	7.0±0.39[b]	3.3±0.33[a]

a,b Same letter in the same row indicate that significance (P<0.05)

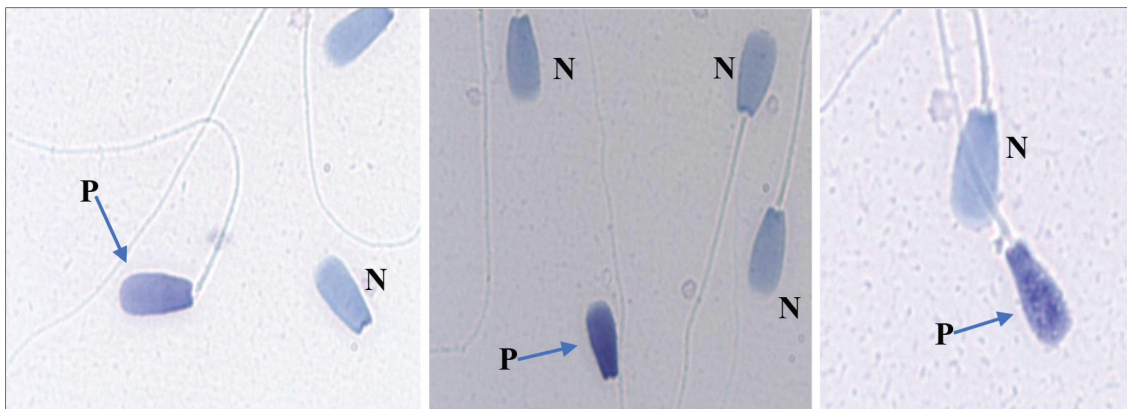

Fig 1. Sperm chromatin structure assessed by toluidine blue staining in bull semen. Sperm cell heads with good chromatin structure were light blue (N); those of abnormal chromatin structure were deep violet (P). The photographs were obtained with an image analyzer using the CASA system

membrane integrity, head defect, and total abnormal sperm morphology *(Table 1)*. However, no difference was found in sperm motility, progressive motility, sperm kinematic parameters, viability, tail defect, sperm acrosome defect, and sperm mid-piece defect between the samples for the slow thawing (37°C for 30 sec) and fast thawing (70°C for 6 sec) *(Table 1)* (P>0.05).

Changes in Chromatin Condensation

The percentages of abnormal sperm chromatin structure and condensation were compared between the two thawing protocols for sperm. Significant differences (P<0.05) were observed for the TB patterns between the samples stained for slow thawing (37°C for 30 sec) and fast thawing (70°C for 6 sec) when the two thawing protocols were compared *(Table 2)*. The TB staining patterns observed in bull semen were as follows: light blue (TB negative, normal chromatin condensation), and dark blue-violet (TB positive, a high degree of chromatin decondensation; *Fig. 1*). The rate obtained in slow thawing (7±0.39) is up to two times higher than the rate obtained in fast thawing (3.3±0.33) (P<0.05), according to the evaluations made in terms of chromatin decondensation rate. Rapid thawing, according to the literature, produces a successful outcome in this situation. It is critical to use TB staining to clearly show these changes in sperm chromatin condensation and decondensation.

Discussion

In this study, besides the analysis of routine semen parameters of frozen-thawed bull semen, the effectiveness and success of the Toluidine Blue staining procedure, which is an inexpensive and easily applicable staining method and used to determine the condensation/decondensation change of sperm chromatin, was determined. According to the findings obtained from the study data, membrane integrity, head defect, tail defect, total abnormal sperm morphology, TB negative, and TB positive were statistically significantly different after thawing at 70°C for 6 sec compared with 37°C for 30 sec in cryopreserved bull semen.

Rapid thawing of sperm reduces the negative effects of recrystallization and hydration, preventing damage to the sperm membrane and cytoplasm [23]. Increased thawing rate has been shown to reduce intracellular ice recrystallization, which can result in the formation of larger and more stable ice crystals that can damage mitochondria. However, it should be noted that, while temperatures above 35°C appear to have more positive effects, the thawing time should be shortened and carefully timed. Because protein denaturation causes spermatozoa to lose vitality when exposed to high temperatures for an extended period of time [4]. The plasma membrane is the principal location of freezing injury in spermatozoa and is critical for freeze-thaw survival [24]. HOST has been shown to be effective in detecting minor changes in bull sperm membranes [25]. Membrane proteins denature during the thawing process due to osmotic and mechanical stress, causing membrane integrity to be disrupted. Rapid thawing, according to Mazur [23] and Holt [26], prevents water molecules from recrystallizing, which may be harmful to

cell membranes. The increased osmotic pressure changes are the main disadvantage of the slow thawing method [27]. According to Foote [28], the thawing process must be completed quickly to limit the harm caused by changes in ice crystals during the unfreezing of the sperm and preserve membrane integrity and potential fertility to the greatest extent possible. Confirming this information, our study found that rapid thawing at 70°C resulted in significant reductions in membrane integrity (74.10±1.71 versus 63.20±2.78) and sperm abnormal morphology (11.10±0.40 versus 15.60±0.61), particularly in the rate of chromatin decondensation (7±0.39 versus 3.3±0.33). While previous studies [6,29] found that temperatures above 35°C result in higher motility values, our results revealed no statistically significant difference between motility parameters. This could be due to differences in the composition of the extenders used, changes in the freezing procedure, and, most importantly, differences depending on the semen analysis system used. Our findings show that when the straws were thawed at 70°C for 6 sec, total abnormal sperm morphology, as well as other head defect, were significantly lower than when they were thawed at 37°C for 30 sec. When the methods used and the results obtained are compared, our findings are consistent with those of Nur et al.[4] and Yilmaz et al.[30]. The cause of tail-related abnormalities in spermatozoa is mainly unknown [31]. According to research, coiled and coiled tailed spermatozoa significantly reduce ejaculate motility and may lower male fertility potential [32].

The critical temperature range for thawing sperm is between -60°C and -10°C, and the temperature should be changed as soon as possible during freezing and thawing [30]. Rapid thawing of semen was found to be beneficial in preventing harm during rewarming by Vishwanath and Shannon [33]. It is well known that the cold shock that occurs between these temperatures increases morphological defects [6]. The temperature of the straw in a 37°C water bath reaches 0°C in the first 5 sec and 30°C in the 15th sec, but at 70°C, these temperatures are reached in a fraction of the time. While the short transition time minimizes the rate of morphological defects, the exposure period to temperatures must also be attending. If the temperature within the straw rises above 41°C, the spermatozoa will be permanently damaged [30].

The integrity of the paternal genome is linked to reproductive success, which includes fertilization and embryonic development. As a result, research that identifies the chromatin compaction pattern throughout the reproductive tract, as well as its implications for possible sperm aberrations, is critical for understanding the mechanisms underlying male fertility [34]. Several factors can produce chromatin abnormalities in sperm: disturbances during histone to protamine exchange, a lack of protamines, disturbances at the level of sperm maturation in the epididymis, or maintenance of chromatin stability during ejaculation [35]. Environmental factors such as increased body temperature, toxic chemicals, components of the extender in which semen is stored, storage conditions are known to cause sperm chromatin damage/abnormal structure [36]. For this reason, several methodologies have been used to study abnormal chromatin forms throughout the years [37]. The purpose of this study was to put to the test a TB staining that was simple, inexpensive, and reliable for determining how two different thawing temperatures affect sperm chromatin condensation in the bull. Souza et al.[15] investigated various chromatin assessment methods (TB, 6-diamidino-2-phenylindole (DAPI) and anti-protamine 1 antibody (anti-PR1)). Other methods allow us to see the areas where sperm chromatin condensation occurs in greater detail, but TB cannot distinguish between different types of chromatin changes. Indeed, DAPI and anti-PRI are not routine procedures because they require immunocytochemistry and fluorescence microscopy. However, the condensation of sperm chromatin as positive and negative in sperm samples could still be detected. Despite being consistent with our findings, we were able to identify positive and negative sperm chromatin condensation differences when comparing two different thawing methods.

Banaszewska et al.[38] compared Acridine orange (AO), Aniline blue (AB) and chromomycin (CMA3) stains to evaluate sperm quality in terms of chromatin abnormalities in the sperm nucleus. Staining with AO identifies the abnormal, single-stranded DNA structure in the sperm cell. The use of AB enables the identification of abnormal histone retention, while CMA3 identifies sperm cells with protamination disorders. TB was not included in the study. However, the examination of nuclear proteins in terms of infertility shows the importance of normal chromatin structure on the functioning of sperm cells [38].

In a study on human sperm, a negative correlation was found between sperm chromatin integrity with TB and AB staining and sperm count, normal morphology, and cut-off value in progressive motility and specificity. AB and TB staining sensitivity were accepted as an indicator of male infertility. Therefore, AB and TB staining has been reported to be helpful for the assessment of male fertility potential. In addition, the study shows a negative relationship between the lack of chromatin material and the fertility potential of spermatozoa [39]. Chromatin integrity assessment using AB and TB is a reliable indicator of pregnancy [38].

Erenpreiss et al.[19] compared toluidine blue (TB), sperm chromatin structure test (SCSA) and Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) test in a study evaluating sperm DNA integrity.

They reported that the proportion of sperm cells with abnormal DNA integrity obtained from the TB test was strongly correlated with the proportion of abnormal cells detected by the SCSA and TUNEL. They also stated that while AO-based assays (SCSA) are less sensitive to DNA-protein interactions due to limited external staining, TB is sensitive to both DNA strand breaks and chromatin packaging changes. TB has also been evaluated as a cheaper and easier to administer test than the more popular SCSA (sperm chromatin structure test) and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) tests [40].

Evaluating the presence of chromatin damage in a quick and low-cost manner can make significant contributions to the relevant field, particularly in laboratories that perform routine sperm analysis. Besides, the TB procedures provided have the advantage of not requiring expensive computer-assisted sperm analysis systems, which are not available at most insemination stations or reproduction laboratories [38].

The degree of staining of spermatozoa ranges from light blue to dark blue, and the differentially stained sperm population is thought to represent chromatin changes and to show that this method is capable of identifying alterations in the DNA-protein complex caused by heat exchange in bull spermatozoa [22]. The thawing rate influences sperm chromatin condensation rate and previous studies have demonstrated that increasing the thawing rate increases sperm chromatin condensation [14]. Furthermore, sperm DNA is compacted during spermatogenesis by replacing histones with protamines [41]. Because chromatin is an important component of the sperm head, changes in its compaction process may affect the morphometric features of the sperm head [42]. In bulls, there has previously been reported a relationship between sperm head morphometry and chromatin [14]. The results of our study's 37°C thawing group are consistent with these findings, and there is a positive relationship between head disorders and primary chromatin decondensation.

In conclusion, increasing the temperature from 37°C to 70°C resulted in a decrease in sperm chromatin condensation, consistent with previous studies. Improved sperm chromatin condensation can be attributed to thawing at 70°C, which preserves the chromatin structure better than thawing at 37°C. In addition, sperm chromatin condensation test was found to be highly correlated with sperm morphology. If CASA and morphology assessments are routinely used to evaluate stud bulls with suspected infertility/subfertility, additional TB and chromatin damage may be determined quickly and inexpensively.

Availability of Data and Materials

The datasets and analyzed during the current study available from the corresponding author (M. Çevik) on reasonable request.

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Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Contributions of Authors

BE, MA, MDT, CK and MÇ are listed according to the determination of the subject, experimental design and writing stages, their contribution rates to laboratory studies and literature review, and corrections. All authors contributed to the revision and final proofreading of the article.

Ethical Statement

The Local Animal Ethics Committee provided an ethics report for this study (E-68489742-602.99-104799).

References

1. Rodriguez-Martinez H: Laboratory semen assessment and prediction of fertility: Still utopi? *Reprod Domest Anim,* 38 (4): 312-318, 2003. DOI: 10.1046/j.1439-0531.2003.00436.x

2. Sharma V: Sperm storage for cancer patients in the UK: A review of current practice. *Hum Reprod*, 26 (11): 2935-2943, 2011. DOI: 10.1093/humrep/der281

3. Grötter LG, Cattaneo L, Marini PE, Kjelland ME, Ferré LB: Recent advances in bovine sperm cryopreservation techniques with a focus on sperm post-thaw quality optimization. *Reprod Domest Anim,* 54 (4): 655-665, 2019. DOI: 10.1111/rda.13409

4. Nur Z, Dogan I, Soylu MK, Ak K: Effect of different thawing procedures on the quality of bull semen. *Rev Med Vet*, 154 (7): 487-490, 2003.

5. Rodriguez OL, Berndtson WE, Ennen BD, Pickett BW: Effect of rates of freezing, thawing and level of glycerol on the survival of bovine spermatozoa in straws. *J Anim Sci,* 41 (1): 129-136, 1975. DOI: 10.2527/jas1975.411129x

6. Correa JR, Rodriguez MC, Patterson DJ, Zavos PM: Thawing and processing of cryopreserved bovine spermatozoa at various temperatures and their effects on sperm viability, osmotic shock and sperm membrane functional integrity. *Theriogenology,* 46 (3): 413-420, 1996. DOI: 10.1016/0093-691X(96)00163-X

7. Dhami AJ, Sahni KL: Evaluation of different cooling rates, equilibration periods and diluents for effects on deep-freezing, enzyme leakage and fertility of taurine bull spermatozoa. *Theriogenology,* 40 (6): 1269-1280, 1993. DOI: 10.1016/0093-691X(93)90297-I

8. DeJarnette JM, Barnes DA, Marshall CE: Effects of pre-and post-thaw thermal insults on viability characteristics of cryopreserved bovine semen. *Theriogenology,* 53 (6): 1225-1238, 2000. DOI: 10.1016/S0093-691X(00)00267-3

9. Hernández M, Roca J, Gil MA, Vázquez JM, Martínez EA: Adjustments on the cryopreservation conditions reduce the incidence of boar ejaculates with poor sperm freezability. *Theriogenology*, 67 (9): 1436-1445, 2007. DOI: 10.1016/j.theriogenology.2007.02.012

10. Lyashenko A: Effect of different thawing procedures on the quality and fertility of the bull spermatozoa. *Asian Pac J Reprod*, 4 (1): 17-21, 2015. DOI: 10.1016/S2305-0500(14)60051-8

11. Agarwal A, Allamaneni SS: The effect of sperm DNA damage on assisted

reproduction outcomes. *Minerva Gynecol*, 56 (3): 235-245, 2004.

12. Monachesi NE, Neild D, Carretero M: Dog sperm DNA: Raw semen evaluation with Toluidine blue stain. *Reprod Domest Anim*, 54 (8): 1078-1084, 2019. DOI: 10.1111/rda.13490

13. Oliva R: Protamines and male infertility. *Hum Reprod Update*, 12 (4): 417-435, 2006. DOI: 10.1093/humupd/dml009

14. Córdova-Izquierdo A, Oliva JH, Lleó B, García-Artiga C, Corcuera BD, Perez-Gutierrez JF: Effect of different thawing temperatures on the viability, *in vitro* fertilizing capacity and chromatin condensation of frozen boar semen packaged in 5 ml straws. *Anim Reprod Sci*, 92 (1-2): 145-154, 2006. DOI: 10.1016/j.anireprosci.2005.05.011

15. Souza ET, Silva CV, Travençolo BAN, Alves BG, Beletti ME: Sperm chromatin alterations in fertile and subfertile bulls. *Reprod Biol*, 18 (2): 177-181, 2018. DOI: 10.1016/j.repbio.2018.04.001

16. Hammadeh ME, Zeginiadov T, Rosenbaum P, Georg T, Schmidt W, Strehler E: Predictive value of sperm chromatin condensation (aniline blue staining) in the assessment of male fertility. *Arch Androl*, 46 (2): 99-104, 2001. DOI: 10.1080/01485010117363

17. Ribas-Maynou J, García-Peiró A, Fernández-Encinas A, Abad C, Amengual MJ, Prada E, Navarro J, Benet J: Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay. *Andrology*, 1 (5): 715-722, 2013. DOI: 10.1111/j.2047-2927.2013.00111.x

18. Cortés-Gutiérrez EI, Dávila-Rodríguez MI, Cerda-Flores RM, Fernández JL, López-Fernández C, Aragón Tovar AR, Gosálvez J: Localisation and quantification of alkali-labile sites in human spermatozoa by DNA breakage detection-fluorescence *in situ* hybridisation. *Andrologia*, 47 (2): 221-227, 2015. DOI: 10.1111/and.12250

19. Erenpreiss J, Jepson K, Giwercman A, Tsarev I, Erenpreisa J, Spano M: Toluidine blue cytometry test for sperm DNA conformation: Comparison with the flow cytometric sperm chromatin structure and TUNEL assays. *Hum Reprod*, 19 (10): 2277-2282, 2004. DOI: 10.1093/humrep/deh417

20. Gilmore A, Hitit M, Ugur MR, Dinh TTN, Tan W, Jousan D, Nicodemus M, Topper E, Kaya A, Memili E: Functional variables of bull sperm associated with cryotolerance. *Kafkas Univ Vet Fak Derg*, 27 (3): 371-379, 2021. DOI: 10.9775/kvfd.2021.25653

21. Padrik P, Hallap T, Kaart T, Bulitko T, Jaakma Ü: Relationships between the results of hypo-osmotic swelling tests, sperm motility, and fertility in Estonian Holstein dairy bulls. *Czech J Anim Sci*, 57 (10): 490-497, 2012. DOI: 10.17221/6349-CJAS

22. Beletti ME, Mello MLS: Comparison between the toluidine blue stain and the Feulgen reaction for evaluation of rabbit sperm chromatin condensation and their relationship with sperm morphology. *Theriogenology*, 62 (3-4): 398-402, 2004. DOI: 10.1016/j.theriogenology.2003.10.016

23. Mazur P: Freezing of living cells: Mechanisms and implications. *Am J Physiol*, 247 (3 Pt 1): C125-C142, 1984. DOI: 10.1152/ajpcell.1984.247.3.C125

24. Watson PF: Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod Fertil Dev*, 7 (4): 871-891, 1995. DOI: 10.1071/rd9950871

25. Correa JR, Heersche G, Zavos PM: Sperm membrane functional integrity and response of frozen-thawed bovine spermatozoa during the hypoosmotic swelling test incubation at varying temperatures. *Theriogenology*, 47 (3): 715-721, 1997. DOI: 10.1016/S0093-691X(97)00029-0

26. Holt WV: Basic aspects of frozen storage of semen. *Anim Reprod Sci*, 62 (1-3): 3-22, 2000. DOI: 10.1016/S0378-4320(00)00152-4

27. Curry MR, Watson PF: Osmotic effects on ram and human sperm membranes in relation to thawing injury. *Cryobiology*, 31 (1): 39-46, 1994. DOI: 10.1006/cryo.1994.1005

28. Foote RH: Artificial insemination to cloning: Tracing 50 years of research. *Internet-First University Press*, 1998.

29. Nur Z, Ileri IK, Ak K: Effects of different temperature treatments applied to deep stored bull semen on post-thaw cold shocked spermatozoa. *Bull Vet Inst Pulawy*, 50 (1): 79, 2006.

30. Yilmaz E, Ak K, Baran A: Effect of different thawing time and high temperature on frozen thawed bull semen traits. *J Anim Vet Adv*, 18 (7): 239-245, 2019. DOI: 10.36478/javaa.2019.239.245

31. Rota A, Manuali E, Caire S, Appino S: Severe tail defects in the spermatozoa ejaculated by an English bulldog. *J Vet Med Sci*, 70 (1): 123-125, 2008. DOI: 10.1292/jvms.70.123

32. Molnár A, Sarlós P, Fáncsi G, Rátky J, Nagy S, Kovács A: A sperm tail defect associated with infertility in a goat - Case report. *Acta Vet Hung*, 49 (3): 341-348, 2001. DOI: 10.1556/004.49.2001.3.11

33. Vishwanath R, Shannon P: Storage of bovine semen in liquid and frozen state. *Anim Reprod Sci*, 62 (1-3): 23-53, 2000. DOI: 10.1016/S0378-4320(00)00153-6

34. Martins MC, Gonçalves LM, Nonato A, Travençolo BAN, Alves BG, Beletti ME: Sperm head morphometry and chromatin condensation are in constant change at seminiferous tubules, epididymis, and ductus deferens in bulls. *Theriogenology*, 161, 200-209, 2021. DOI: 10.1016/j.theriogenology.2020.12.004

35. Kazerooni T, Asadi N, Jadid L, Kazerooni M, Ghanadi A, Ghaffarpasand F, Zolghadr J: Evaluation of sperm's chromatin quality with acridine orange test, chromomycin A3 and aniline blue staining in couples with unexplained recurrent abortion. *J Assist Reprod Genet,* 26 (11): 591-596, 2009. DOI: 10.1007/s10815-009-9361-3

36. Rastegarnia A, Shahverdi A, Topraggaleh TR, Ebrahimi B, Shafipour V: Effect of different thawing rates on post-thaw viability, kinematic parameters and chromatin structure of buffalo *(Bubalus bubalis)* spermatozoa. *Cell J*, 14 (4): 306-313, 2013.

37. Dutta S, Henkel R, Agarwal A: Comparative analysis of tests used to assess sperm chromatin integrity and DNA fragmentation. *Andrologia*, 53 (2):e13718, 2021. DOI: 10.1111/and.13718

38. Banaszewska D, Andraszek K, Biesiada-Drzazga B: Evaluation of sperm chromatin structure in boar semen. *Bull Vet Inst Pulawy*, 59 (2): 271-277, 2015. DOI: 10.1015/bvip-2015-0040

39. Muratori M, Tamburrino L, Marchiani S, Cambi M, Olivito B, Azzari C, Forti G, Baldi E: Investigation on the origin of sperm DNA fragmentation: Role of apoptosis, immaturity and oxidative stress. *Mol Med,* 21 (1): 109-122, 2015. DOI: 10.2119/molmed.2014.00158

40. Ajina T, Ammar O, Haouas Z, Sallem A, Ezzi L, Grissa I, Sakly W, Jlali A, Mehdi M: Assessment of human sperm DNA integrity using two cytochemical tests: Acridine orange test and toluidine blue assay. *Andrologia,* 49 (10):e12765, 2017. DOI: 10.1111/and.12765

41. Hao SL, Ni FD, Yang WX: The dynamics and regulation of chromatin remodelling during spermiogenesis. *Gene*, 706, 201-210, 2019. DOI: 10.1016/j.gene.2019.05.027

42. Lange-Consiglio A, Antonucci N, Manes S, Corradetti B, Cremonesi F, Bizzaro D: Morphometric characteristics and chromatin integrity of spermatozoa in three Italian dog breeds. *J Small Anim Pract*, 51 (12): 624-627, 2010. DOI: 10.1111/j.1748-5827.2010.01001.x

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RESEARCH ARTICLE

Etiological and Predisposing Factors in Calves with Neonatal Diarrhea: A Clinical Study in 270 Case Series [1]

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Abstract: This study was carried out to find out the etiology and predisposing factors of calves having diarrhea from Kayseri province and its neighboring cities between January 2016 and September 2019. A total of 270 neonatal diarrheic calves were included to this study. Comprehensive information was obtained by face-to-face interviews with the animal owners about administrative practices such as the vaccination status of the dams, farm type, colostrum intake status. The etiological agents were determined using the lateral flow immunochromatographic test kits. As a result of this investigation, out of 270 diarrheic cases; 21.9% (59) *Cryptosporidium* spp., 15.6% (42) *E. coli* K99+, 14.1% (38) bovine coronavirus (BCoV), 10.4% (28) bovine rotavirus (BRV), 9.3% (25) *Cryptosporidium* spp.+BRV, 8.5% (23) BRV+BCoV were found. Interms of shelter type; 85.2% (230) were traditional and 14.8% (40) were modern type. Regarding the colostrum intake situation; 7.4% (20) received no colostrum, 11.1% (30) received insufficient colostrum and 81.5% (220) received colostrum adequately and on time. Additionally, 36.7% (99) calf mothers were vaccinated and 63.3% (171) were unvaccinated. Compared to those born in autumn, calves born in winter; 6.5-fold, in the spring season; 3.6-fold and in summer; 5.2-fold more likely to develop diarrhea caused by *E. coli* K99+. These findings may generate valuable information not only for the clinicians and researchers but also animal health experts, policy makers, farmer etc.

Keywords: *Calf, Diarrhea, Etiology, Predisposing factor*

Neonatal İshalli Buzağılarda Etiyolojik ve Predispoze Faktörler: 270 Olgu Serisinde Klinik Bir Çalışma

Öz: Bu çalışma, Ocak 2016-Eylül 2019 tarihleri arasında Kayseri ili ve çevre illerinden ishal olan buzağıların etiyolojisi ve predispozan faktörlerinin belirlenmesi amacıyla yapılmıştır. Bu çalışmaya toplam 270 yenidoğan ishalli buzağı dâhil edildi. Hayvan sahipleri ile yüz yüze görüşme yapılarak anaların aşılanma durumu, çiftlik tipi, kolostrum alma durumu ve şekli gibi yönetimsel uygulamalar hakkında kapsamlı bilgi alındı. Etiyolojik ajanlar, lateral flow immünokromatografik test kitleri kullanılarak belirlendi. Bu inceleme sonucunda 270 ishal olgusundan; %21.9 (59) *Cryptosporidium* spp., %15.6 (42) *E. coli* K99+, %14.1 (38) bovine koronavirus (BCoV), %10.4 (28) bovine rotavirus (BRV), %9.3 (25) *Cryptosporidium* spp.+BRV, %8.5 (23) BRV+BCoV bulundu. Barınak türü açısından; %85,2'si (230) geleneksel, %14.8'i (40) modern tipti. Kolostrum alma durumu ile ilgili olarak; %7.4'ü (20) hiç kolostrum almamış, %11.1'i (30) yetersiz kolostrum ve %81.5'i (220) kolostrumu yeterli ve zamanında almıştır. Kolostrum alma durumu açısından; sahiplerinin verdiği bilgilere göre %7.4'ü (20) hiç kolostrum almamış, %11.1'i (30) yetersiz kolostrum, %81.5'i (220) ise kolostrumu yeterli ve zamanında aldığı kaydedildi. Ayrıca annelerin %36.7'si (99) aşılı, %63.3'ü (171) aşısızdı. Sonbahar mevsiminde doğanlara kıyasla, kış mevsiminde doğan buzağıların; 6.5 kat, bahar mevsiminde; 3.6 kat ve yaz mevsiminde; 5.2 kat daha fazla *E. coli* K99+'un neden olduğu ishal geliştirme olasılığı olduğu görüldü. Bu bulgular sadece klinisyenler ve araştırmacılar için değil, aynı zamanda hayvan sağlığı uzmanları, politikacılar, çiftçiler vb. için de değerli bilgiler üretebilir.

Anahtar sözcükler: *Buzağı, İshal, Etiyoloji, Predispoze faktör*

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Introduction

Neonatal calf diarrhea (NCD) is one of the most important problems in calf rearing [1]. In the etiology of NCD; *Cryptosporidium* spp., bovine rotavirus (BRV), bovine coronavirus (BCoV), enterotoxigenic *Eschericia coli* (ETEC) K99+ and *Giardia* spp. are commonly reported endemic microorganisms [2,3]. These infectious agents cause diarrhea in calves alone or as mixed infections [2]. Two of these, *Cryptosporidium* spp. and BRV are the most abundant enteropathogens in the feces of calves with diarrhea [4].

Many factors are known to contribute to calf diarrhea. In most cases, there is interaction between environmental conditions, management practices and microorganisms [5]. It is stated that many factors such as the number of animals in the farm, colostrum intake problems such as not giving colostrum in time, adequately or not at all, vaccination problems such as not vaccinating pregnant mothers against infectious diarrhea agents (BRV, BCoV and ETEC K99+), umblical cord problems such as not performing umbilical cord disinfection are all effective in the formation of diarrhea in newborn calves [6,7]. In addition, farm type, shelter structure, season and age are the predisposing factors affecting the emergence and severity of the disease [8]. Furthermore, it is stated that many administrative factors such as unsuitable shelter conditions (crowding, ventilation, lighting, temperature and relative humidity) and poor cleaning and disinfection of vehicles used in collective breeding shelters are also effective in the formation of diarrhea [5].

Various laboratory methods [virus isolation, bacterial culture, polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), direct microscopy of fecal smear (acid-fast stain)] have been used to detect entero-pathogens from stool samples [8,9]. These procedures are reliable; however, they are time consuming, expensive and require specialized knowledge. Lateral flow immuno-chromatographic (LFI) diagnostic kits; It is widely used in clinics, animal hospitals and in the field to detect major enteropathogens from stool samples taken from calves with diarrhea in a fast, easy and inexpensive way [10,11]. Since highly selective antigen-antibody reaction and mono-clonal antibodies as detector antibody used mainly in these tests, their specificity and sensitivity are generally over 98% [12].

The incidence of major enteropathogen that cause neonatal calf diarrhea may vary according to countries, regions, farm types, and sampling locations [3,4,8,11,13]. The incidence and distribution of enteropathogens that cause diarrhea in calves have been extensively studied in stool samples collected from farms or barns and have been reported by many researchers [3,4,12,14]. However, in animal hospitals where sick calves are taken for diagnosis, treatment and improvement of their general condition, there is still a lack of epidemiological data on the etiology and predisposing factors of diarrhea in calves brought especially from traditional and modern farms.

In this study, we conducted a cross-sectional and questionnaire-based study using multivariate analyzes to determine the factors that predispose to diarrhea in calves brought to the animal hospital from different parts of Kayseri and its surrounding provinces. So, it was aimed to investigate the prevalence of major enteropathogens that play role in the etiology of neonatal calf diarrhea. In addition, risk factors that predispose to diarrhea and affects on general condition was another aim of the study. Furthermore, data obtained from traditional and modern farms were also compared.

Material and Methods

Ethical Statement

This study was approved by the committee of HADYEK-Local Ethics Committee for Animal Experiments Office of Erciyes University (Approval no: 13/10).

Study Design

A cross-sectional study was performed to determine major etiological and predisposing factors on neonatal calves having diarrhea brought from Kayseri (n=194) and the neighbouring cities [Sivas (n=14), Nevşehir (n=28), Yozgat (n=15), Niğde (n=7), Kırşehir (n=12)] to the Erciyes University, Faculty of Veterinary Medicine, Animal Hospital between January 2016 and September 2019.

Target Population and Sampling

The target population was 1-35 days old diarrheic calves brought from traditional and modern farms. Regions of the farms where the calves are brought, accounted for approximately 11.2% of national large animal registries [15]. Each calf in the present study represents a different farm. Calves included in the study; were randomly selected from the calves brought to the animal hospital for the diagnosis. Only clinical signs (abnormally frequent, soft or watery consistency and bad odor) of diarrhea were determined as inclusion criteria. No other inclusion criteria were established. The number of samples used for questionnaire in the study was determined according to Krejcie and Morgon table [16].

Animals

A total of 270 diarrheic neonatal calves from 270 different farms aged between 1-35 days (116 calves were aged between 1-7 days, 84 calves were aged between 8-14 days, 49 calves were aged between 15-21 days, 5 calves were aged between 22-28 days and 16 calves were aged between

29-35 days), in different breeds (187 Simmental, 53 Holstein, 21 Brown Swiss, 5 Cross-breed, 2 Charolais, 1 Belgian Blue, 1 Limousin), from both sex (153 male, 117 female) were the animal materials of the present study. Only one animal from each farm was included to the study.

Data Collection

Comprehensive information was obtained by face-to-face interviews with the animal owners about administrative practices such as demographic, managerial and health factors presumed to be associated with diarrhea in calves. Demographic data contained; race, age and sex of the calves. Among the administrative factors; there were questions such as the type of farm (modern or traditional), the status of receiving colostrum, the number of animals in the farm, the way colostrum was given, umbilical cord disinfection applied or not, vaccination status of mothers, starting time of diarrhea. Information was taken from a total of 270 persons (ranchers or business executives, animal owners) whom animals used in this study. Those who did not responded to our notification or gave false information, discarded from this study which are not within 270 animal owners.

The data of the date (month and year) when the calves were obtained retrospectively from the patient registration system of the Erciyes University (Patient Registration System, ERUVetO; V.15042019/2015, Kayseri, Türkiye).

Clinical Examination

Calves included in the study were subjected to a complete physical examination including rectal temperature (°C), pulsation (bpm), respiratory rate, hydration status, suckling reflex, general condition assessment, stool consistency and color. Hydration status of calves were evaluated according to demeanor, recession of the globe into the orbit and skin tent duration (sec) described by Smith [17].

The general clinical conditions of the calves were categorized according to the non-invasive five-point sequential scale clinical evaluation scoring table developed by Sayers et al.[18]. According to this table, diarrheic calves in the present study were categorized as clinically normal, mild, moderate, severe, and comatose.

In the present study, fresh feces taken from a calf was considered diarrheic if it is abnormally frequent, soft and watery consistency and had bad odor. According to the consistency of the stool and presence of blood in its content; it was also classified as muddy (pasty, faeces spreading across the bottom of the container, but not liquid), loose (but stays on the top of floor), watery (liquid faeces) and hemorrhagic (stools that contain mostly blood and are nearly red in color). Stool colors were also recorded (yellow and its tones, white and its tones, green, brown, red, black and gray).

Pathogen Detection

Samples were taken from diarrheic calves into sterile stool containers by rectal stimulation. From these stool samples, lateral flow immunochromatographic (LFI) test kits (Anigen Rapid BoviD-5 Ag Test Kit, Bionote, Inc. Korea) were performed to detect antigens against *E. coli* K99^{+} [Sensitivity (sen); 97.8%, specificity (spe); 99.0%], BRV (sen; 99.0%, spe; 98.0%), BCoV (sen; 98.4%, spe; 98.0%), *Cryptosporidium* spp. (sen; 98.2%, spe; 99.0%) and *Giardia* spp. (sen; 92.1%, spe; 99.1%). During the analysis, the instructions in the user manual of the test kit were followed and the results were evaluated qualitatively (positive or negative). Samples with negative results for the above 5 antigens *(E. coli* K99^{+}, BRV, BCoV, *Cryptosporidium* spp. and *Giardia* spp.) were classified as "undiagnosed".

Data Management and Analysis

Descriptive statistics and frequency distribution variables were performed using SPSS for Windows Release 25.0 (SPSS Inc, Chicago, IL, USA). In order to determine the distribution of etiological agents by age range, calves were divided into five different age groups as 1-7 days, 8-14 days, 15-21 days, 22-28 days and 29-35 days. Calving seasons were defined as winter (December-February), spring (March-May), summer (June to August), autumn (September-November). Data were coded into variables using uniform definitions. The relationship between categorical variables was evaluated using the Pearson's chi-square ($\chi 2$) test (and Fisher's exact test). For variables with more than two categories, row (r) x column (c) (r>2 or c>2) chi-square test was used. This analysis included preliminary explorations, including pairwise analyses for relationship of binary variables using the chi-square test. This was followed by multivariable modelling using mixed effects logistic regression. Three main research questions were addressed. The first was to determine the predisposing factors associated with neonatal calf diarrhea caused by major enteropathogens (at least one or more; ETEC K99^{+}, BRV, BCoV, *Cryptosporidium* spp., *Giardia* spp.). The association between the predisposing factors and the presence/absence of major pathogen induced diarrhea were also analysed using the logistic regression model. The second was to identify the predisposing factors associated with neonatal calf diarrhea caused by each of the enteropathogens. The association between the predisposing factors and the presence/absence of each pathogen was analyzed using the same method. The third was to determine the predisposing factors that affect the general condition categories (normal, mildly affected, moderately affected, severely affected) of calves with diarrhea. Predisposing factors associated with general condition of diarrheic calves were analyzed using ordinal logistic regression model.

The predisposing factor with logistic regression analysis was achieved using three steps. Initially, the interrelationships of all variables taken individually with the occurrence of diarrhea were tested in a univariate model. Then, any variable with a p value <0.2 was considered eligible for the next step. In the third step, a final multivariate model was fitted with all the variables that had remained significant during the two previous steps. Odds ratios (ORs) with 95% confidence intervals were calculated to assess the likelihood of association. The graph showing the intersections of different etiological agents was created using the online Venn Diagram software (UGent, Genomics, & 927, 2020). For all analyses, a p value of <0.05 was considered to be statistically significant.

Results

Animal Population

A total of 4389 calves were registered to the Veterinary Teaching Hospital between at January 2016 and September 2019. The total number of diarrheic neonatal calves were 2545 (58%) (Patient Registration System, ERUVetO; V.15042019/2015, Kayseri, Turkey). The number of diarrheic calves included in this study were 270 (10.6%).

Descriptive Data Analysis

One or more than one etiological agent was determined positive in 238 out of 270 diarrheic calves used in the present study. A single etiological agent in 63.7% (172/270), two etiological agents in 23.3% (63/270), three etiological agents in 1.1% (3/270) were detected in the diarrheic calves *(Table 1)*. However, in 11.9% (32/270) diarrheic calves, major antigens (BRV, BCoV, *E. coli* K99^{+}, *Cryptosporidium* spp., *Giardia* spp.) could not be detected.

It was seen that 56.7% (153/270) of diarrheic calves in this study were male and 43.3% (117/270) were female. Diarrheic calves investigated in the present study were 66.7% (187/270) Simmental, 19.6% (53/270) Holstein, 7.8% (21/270) Swiss Brown, 1.9% (5/270) cross-breed, 0.74% (2/270) Charolais, 0.4% (1/270) Belgian Blue and 0.4% (1/270) Limousin race. There was no statistically significant relationship between the categories of variables of breed and etiological agent ($\chi 2$= 53.115, P=0.986).

It was noted that 81.5% (220/270) of the calves included in the study received colostrum fully and in time, 11.1% (30/270) received less, and 7.4% (20/270) did not receive it at all. In diarrheic calves, which were stated to have never received colostrum; mostly *E. coli* K99^{+} (35.0%), in diarrheic calves, which were stated to have received less colostrum; mostly *Cryptosporidium* spp. (20.0%), in diarrheic calves, which were stated to receive colostrum fully and in time, mostly *Cryptosporidium* spp. (18.6%) were diagnosed.

In calves given colostrum with a feeding bottle; at most, *Cryptosporidium* spp. (18.5%), then respectively; *E. coli* K99^{+} (14.8%), BCoV (13.6%), BRV (13.0%), *Cryptosporidium* spp. +BRV (9.9%), BRV+BCoV (8.0%) were diagnosed. In calves given colostrum by suckling; at most, *Cryptosporidium* spp. (19.0%), then respectively; *E. coli* K99^{+} (18.0%), BCoV (16.0%), BCoV+BRV (10.0%), *Cryptosporidium* spp. +diarrhea cases due to BRV (9.0%) were observed.

Considering the number of calves brought to the hospital according to the seasons; In the winter season (december to february) 85, spring season (march to may) 133, summer season (june to august) 34, autumn season (September to November) 18 calves with diarrhea were included to the study.

Considering the stool colors of the calves with diarrhea in the present study; 60.4% (163/270) were yellow, 11.9% (32/270) white and its tones, 9.3% (25/270) green, 6.7% (18/270) brown, 4.5% (12/270) red, 3.7% (10/270) black and 3.7% (10/270) were gray. There was no statistically significant correlation between stool color of the calves with diarrhea and categories of etiological diagnosis variables.

The general conditions of the calves with diarrhea included in the study; 6.7% (18/270) were normal, 25.2% (68/270) were mild, 42.2% (114/270) were moderate and 25.9% (70/270) were severe. *Cryptosporidium* spp. were the mostly (44.4%, 8/18) detected patogen in calves having a healthy general condition, coronavirus was the mostly (19.1%, 13/68) detected patogen in the calves having mild general condition, *Cryptosporidium* spp. were the mostly (21.1%, 24/114) detected pathogen in calves having moderate general condition and *E. coli* were the mostly (27.1%, 19/70) detected patogen in the calves having severe general condition. Mean body temperatures, respiratory rate and heart rate of diarrheic calves were 38.3°C (32.8-41.6), 40/min (IQR; 28-48, min-max;10-160) and 107.9±24.9 (IQR; 93.5-120, min-max; 36-180) bpm, respectively.

Etiologic Agents and Age Ranges

As a single etiological agent from stool samples taken from diarrheic calves; at most, *Cryptosporidium* spp. (21.9%, 59/270), more than one etiological agent; at most, *Cryptosporidium* spp.+rotavirus (9.3%, 25/270) were detected *(Table 1, Fig. 1)*.

When etiological agents are examined according to age range, in 1-7 days-old calves; mostly, *E. coli* K99^{+} (33.6%, 39/116) cases of diarrhea were observed. In 8-14, 15-21 and 22-28 days-old calves, it was mostly; *Cryptosporidium* spp. [27.4% (23/84), 38.8% (19/49) and 40% (2/5), respectively] cases of diarrhea were determined. In 29-35 days-old calves, at most; cases of diarrhea caused by coronavirus (31.3%, 5/16) were detected *(Table 1, Fig. 2)*. Conversely, *Giardia* spp. were observed only sporadically (1.9%).

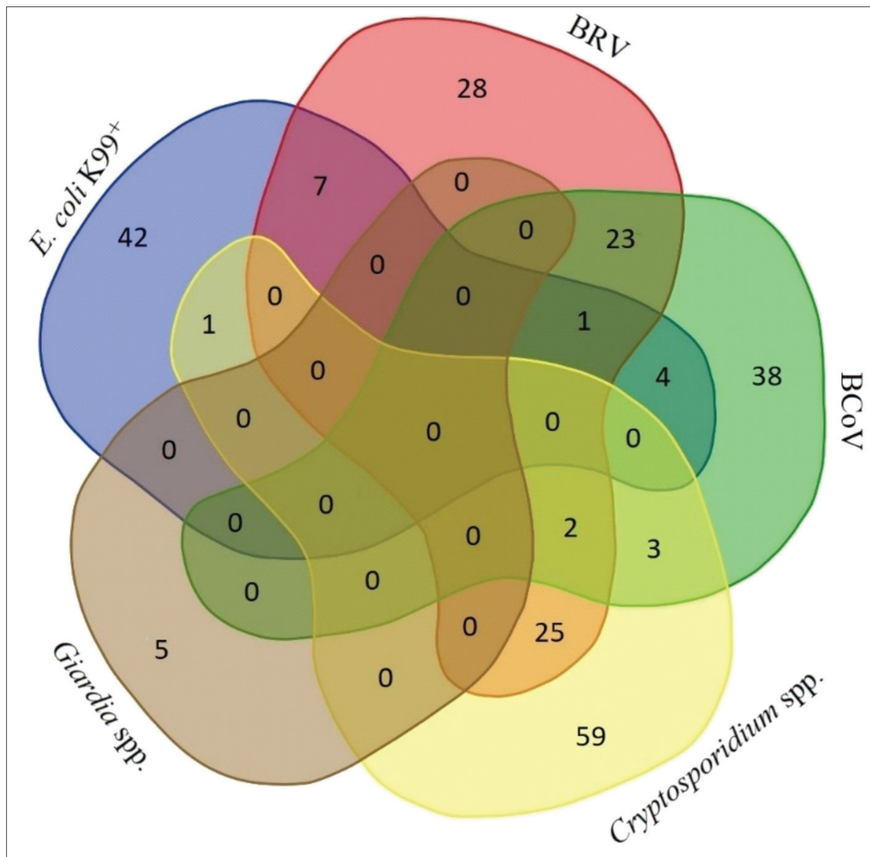

Fig 1. The intersections of major etiological agents that cause diarrhea in calves using Venn Diagram. BCoV; bovine coronavirus, BRV; bovine rotavirus

Table 1. Distribution of the major etiological agents in diarrheic calves in accordance to age groups and farm types

Calf Diarrhea Agent and Co-infections	Total, (n=270) % (n)	Farm Type	Agent Frequency and the Occurrence in Age Groups				
		TF/MF % (n)	**1-7 d (n=116) % (n)**	**8-14 d (n=84) % (n)**	**15-21 d (n=49) % (n)**	**22-28 d (n=5) % (n)**	**29-35 d (n=16) % (n)**
	100 (270)	**85.2 (230/270)/ 14.8 (40/270)**	**43.0 (116/270)**	**31.1 (84/270)**	**18.1 (49/270)**	**1.9 (5/270)**	**5.9 (16/270)**
Single-infected							
Cryptosporidium spp.	21.9 (59)	22.6 (52) /17.5 (7)	10.3 (12)	27.4 (23)	38.8 (19)	40 (2)	18.8 (3)
ETEC K99⁺	15.6 (42)	16.5 (38) /10.0 (4)	33.6 (39)	1.2 (1)	4.1 (2)	0 (0)	0 (0)
BCoV	14.1 (38)	15.7 (36) /5.0 (2)	13.8 (16)	14.3 (12)	10.2 (5)	20 (1)	31.3 (5)
BRV	10.4 (28)	8.7 (20) /20.0 (8)	10.3 (12)	11.9 (10)	10.2 (5)	0 (0)	0 (0)
Giardia spp.	1.9 (5)	1.7 (4) /2.5 (1)	1.7 (2)	2.4 (2)	2.0 (1)	0 (0)	0 (0)
Dual-infected							
Cryptosporidium spp.+BRV	9.3 (25)	8.3 (19)/15.0 (6)	5.2 (6)	15.5 (13)	10.2 (5)	0 (0)	6.3 (1)
BRV+BCoV	8.5 (23)	9.1 (21) /5.0 (2)	10.3 (12)	9.5 (8)	4.1 (2)	0 (0)	6.3 (1)
ETEC K99⁺+BRV	2.6 (7)	3.0 (7) /0 (0)	4.3 (5)	2.4 (2)	0 (0)	0 (0)	0 (0)
ETEC K99⁺+BCoV	1.5 (4)	1.7 (4) /0 (0)	2.6 (3)	1.2 (1)	0 (0)	0 (0)	0 (0)
Cryptosporidium spp.+BCoV	1.1 (3)	0.9 (2) /2.5 (1)	0 (0)	1.2 (1)	2.0 (1)	0 (0)	6.3 (1)
Cryptosporidium spp.+ETEC	0.4 (1)	0.4 (1) /0 (0)	0 (0)	0 (0)	2.0 (1)	0 (0)	0 (0)
Multi-infected							
Cryptosporidium spp.+BCoV+BRV	0.7 (2)	0.9 (2) /0 (0)	1.7 (2)	0 (0)	0 (0)	0 (0)	0 (0)
BRV+BCoV+ETEC K99⁺	0.4 (1)	0 (0) /2.5 (1)	0.9 (1)	0 (0)	0 (0)	0 (0)	0 (0)
Undiagnosed	11.9 (32)	10.4 (24) /20.0 (8)	5.2 (6)	13.1 (11)	16.3 (8)	40 (2)	31.3 (5)

*Data were expressed as % positive (number of positive/number of total cases), undiagnosed, those are not positive for enterotoxigenic E. coli (ETEC) K99⁺, bovine rotavirus (BRV), bovine coronavirus (BCoV), Cryptosporidium spp., and Giardia spp.; **TF:** traditional farm (n=230); **MF:** modern farm (n=40); d, days*

Occurrence of Etiological Agents According to Farm Type (Traditional/Modern)

It was determined that 85.2% (230/270) of the diarrheic calves came from traditional farm and 14.8% (40/270) of them came from modern farms. One or more antigens of *Cryptosporidium* spp., BRV, BCoV, *E. coli* K99$^+$ and *Giardia* spp. were determined as positive in 206 out of 230 diarrheic calves brought from traditional farms. In calves with diarrhea brought from traditional farms; at most, *Cryptosporidium* spp. 22.6% (52/230) cases of diarrhea were detected *(Table 1)*. One or more antigens of *Cryptosporidium* spp., BRV, BCoV, *E. coli* K99$^+$ and *Giardia* spp. were determined as positive in 32 out of 40 diarrheic calves brought from modern farms. In calves with diarrhea brought from modern farms; mostly BRV 20.0% (8/40) cases of diarrhea were seen *(Table 1)*. Moreover, to compared to those brought from traditional farms (undiagnosed; 10.4%, 24/270), it was seen to be more diarrhea cases classified as 'undiagnosed' (20%, 8/40) in diarrheic calves brought from modern farms. In the present study, in terms of animal numbers kept in the traditional farms were as follow: 1-10 animal (n=21 farms), 11-25 animal (n=59 farms), 26-50 animal (n=71 farms), 51-100 animal (n=52 farms), 101-250 (n=24 farms), 251-500 (n=3 farms). Furthermore, in the modern farms, number of animals kept in the farms were as 1-50 animal (n=13 farms), 51-100 animal (n=11 farms), 101-250 animal (n=12 farms), 250-1000 animal (n=4 farms).

Logistic Regression Model Results for Calves with Major Pathogen-Induced Neonatal Diarrhea

With the univariate logistic regression model, 4 predisposing factors (umbilical cord disinfection status, farm type, dam vaccination status, age group) associated with neonatal diarrhea (P<0.2) caused by major enteric pathogens in calves were determined. At the last stage, 2 variables showed a significant relationship with neonatal diarrhea originating from the major enteric pathogen. According to the final model results; compared to calves born from mothers that were vaccinated with *E. coli* K99$^+$, BRV and BCoV antigens in the last period of pregnancy, the probability of developing major pathogen-induced diarrhea in calves born from unvaccinated mothers increased by 3.5-fold. In terms of age groups, it was determined that 15-21, 22-28, 29-35-days-old calves probability of developing major enteric pathogen-induced neonatal diarrhea increased 4.7-fold, 14.4-fold, 8.7-fold respectively compared to the 1-7-days-old age group.

Predisposing Factors According to the Logistic Regression Model Significantly Associated with Calf Diarrhea Caused by Each Pathogen

With the univariate logistic regression model, the predisposing factors associated with each of the major enteropathogens [*Cryptosporidium* spp., (7 variables), *E. coli* K99$^+$ (7 variables), BRV (4 variables), BCoV (3 variables)] causing neonatal diarrhea (P<0.2) in calves were determined separately. These variables were included in the final multivariate logistic regression model. At the last stage, variables (P<0.05) showing a significant relationship with neonatal diarrhea caused by each of these pathogens are as follows: for *Criptosporidium*; the onset of diarrhea, months of birth, the consistency of feces and dam vaccination status, for *E. coli* K99$^+$ onset of diarrhea, season of birth, and colostrum intake status, for BRV; age groups and the number of animals in the farms, for BCoV; the farm type and age groups were found important.

Predisposing Factors According to the Logistic Regression Model Significantly Associated with Calf Diarrhea Caused by *Cryptosporidium* spp., *E. coli*, BRV and BCoV

It was observed that the probability of diagnosing

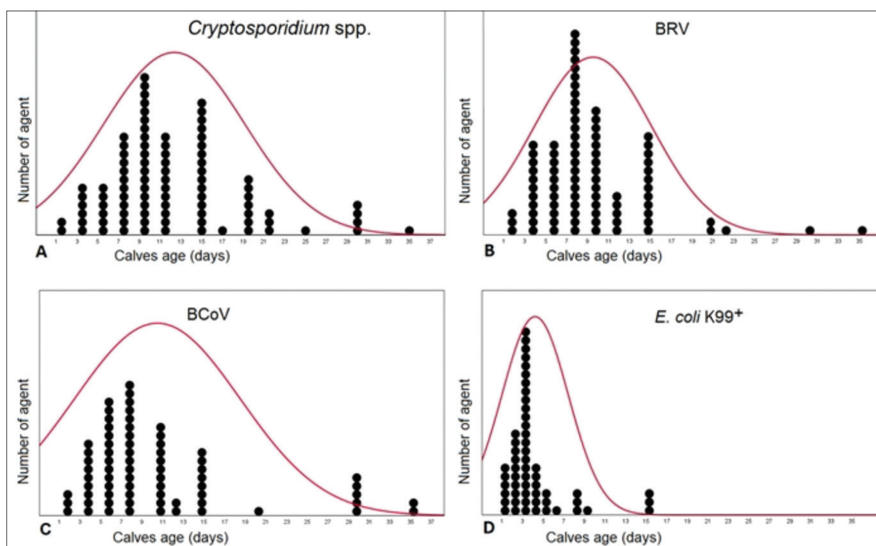

Fig 2. (A); *Cryptosporidium* spp. (n=90), **(B)**; bovine rotavirus (n=86) (BRV), **(C)**; bovine coronavirus (BCoV) (n=71), **(D)**; *E. coli* K99$^+$ (n=55) distribution with concern to age in neonatal calves with diarrhea. Black dots (•) show number of positive cases for each antigen. Red line shows distribution curve

Cryptosporidium spp-induced diarrhea increased by 0.4-fold in calves with a diarrhea onset time of "72 h and more" (B:-1.051, OR: 0.350, 95% CI, 0.177 to 0.689, Wald $\chi2$= 9.234, P= 0.002) compared to calves with a diarrhea onset time of "24 h and before". Compared to the calves born from mothers that were vaccinated, the probability of *Cryptosporidium* spp-induced diarrhea increased by 1.9-fold in calves born from unvaccinated mothers. Futhermore, It was determined that the probability of diarrhea caused by *Cryptosporidium* spp. increased 0.3-fold in calves with sludge-like consistency compared to those with watery stools. Additionally, it was also determined that the probability of getting diarrhea caused by *Cryptosporidium* spp. in calves born in the winter season was 14-fold increased in 8-28 days old calves compared to the autumn season.

It was determined that calves did not receive colostrum at all were 0.1-fold more likely to develop *E. coli* $K99^{+}$-induced diarrhea, compared to those who received full and on-time colostrum. The probability of developing diarrhea caused by *E. coli* $K99^{+}$ from calves with a diarrhea onset time of "72 h and more" compared to a diarrhea onset time "<24 h and previous", was found to be; 0.1-fold higher. It was determined that calves born in winter season; 6.5-fold, spring season; 3.6-fold and summer season; 5.2-fold more likely to develop *E. coli* $K99^{+}$-induced diarrhea, compared to those born in the autumn season.

Compared to the 29-35-days-old age group, 7-14-days-old calves were 0.2-fold more likely to develop rotavirus-induced neonatal diarrhea. It was determined that every 1 animal increase in the total number of animals in the farm increased the probability of calves getting rota-virus 1.0-fold.

Calves from traditional farms were 0.3-fold more likely to be diagnosed with coronavirus-induced neonatal diarrhea compared to calves brought from modern farms. Compared to the 29-35-days-old age group, 15-21-days-old calves were 4.0-fold more likely to develop coronavirus-induced neonatal diarrhea.

Predisposing Factors According to the Ordinal Logistic Regression Model Significantly Associated with General Conditions (Normal, Slightly Affected, Moderately Affected, Severely Affected) of Diarrheic Calves

As a result of this analysis, it was determined that especially absence of suckling reflex, rectal temperature, season of birth, starting time of diarrhea were effective on the general condition of the calf (P<0.05) *(Table 2)*. When compared with the calves having "good suckling reflex", each increase in the number of calves 'without suckling reflex' was found to worsen the general condition of the calves by 6.8-fold (P<0.001) and in calves having 'poor suckling reflex" by 2.7-fold (P<0.001). Compared to autumn, calves born in summer had a 3.0-fold higher risk of being severe in the general condition categories (P<0.05). An increase in rectal temperature (for each °C) was associated with decrease in the odds of general condition, with an odds ratio of 0.765 (P=0.001). An increase in *E. coli* $K99^{+}$ positive case (expressed in numbers) was associated with an increase in the odds of general condition, with an odds ratio of 1.846 (P=0.069, without statistically significance).

Chi-square ($\chi2$) Test Results

There was no statistically significant relationship between the dam vaccination status and the type of farms. It was observed that the vaccination rates of pregnant animals

Table 2. *Predisposing factors associated with general conditions of diarrheic calves in ordinal logistic regression model*

Variable Description		Estimate	SE	Sig.	OR	95% CI	
Suckling reflex	No reflex	1.971	0.393	<0.001	6.801	3.261	14.182
	There is but not good	1.059	0.278	<0.001	2.721	1.603	4.619
	Good (Reference category)						
Season of birth	Winter	0.673	0.506	0.184	1.960	0.727	5.284
	Spring	0.425	0.485	0.381	1.530	0.591	3.957
	Summer	1.113	0.568	0.050	3.043	1.000	9.265
	Autumn (Reference category)						
Starting time of diarrhea	<3 h	-1.405	0.992	0.157	0.245	0.035	1.715
	12 h	0.194	0.409	0.636	1.214	0.545	2.706
	24 h	1.209	0.411	0.003	3.350	1.497	7.497
	48 h	-0.201	0.325	0.536	0.818	0.433	1.547
	72 h and more (Reference category)						
Rectal temperature (°C)		-0.268	0.083	0.001	0.765	0.655	0.893
E. coli $K99^{+}$ Yes/No		0.613	0.337	0.049	1.846	0.954	3.573

SE: *Standart Error,* ***Sig:*** *significance,* ***OR:*** *odds ratio, 95%* ***CI:*** *95% confidence interval, R^2= 0.246 (Cox & Snell), R^2= 0.269 (Nagelkerke). Model: $\chi2$ (11)= 76.288*

in traditional (36.5%) and modern farms (37.5%) were similar. In calves born from mothers vaccinated (36.7%); at most, *Cryptosporidium* spp. (22%), then respectively; *Cryptosporidium* spp.+rotavirus (14.1%), *E. coli* K99+ (13.1%), coronavirus+rotavirus (11.1%), rotavirus (10.1%), coronavirus (10.1%) diarrhea were determined. In calves born from unvaccinated mothers (63.3%); mostly *E. coli* K99+ (17%), then respectively; Coronavirus (16.4%), *Cryptosporidium* spp. (15.8%), rotavirus (9.9%), rotavirus + coronavirus (7%), *Cryptosporidium* spp. + rotavirus (6.4%) diarrhea were observed.

It was noted that 75.6% of the calves included in the study had umbilical cord disinfection and 24.4% were not. In calves undergoing umbilical cord disinfection; at most, *Cryptosporidium* spp. (16.7%), then respectively; *E. coli* K99+ (14.7%), coronavirus (11.8%), *Cryptosporidium* spp., rotavirus (11.3%) infections were seen. For those who did not have umbilical cord disinfection; at most, *Cryptosporidium* spp. (22.7%), then respectively; coronavirus (21.2%), *E. coli* K99+ (18.2%), rotavirus (10.6%), *E. coli* K99+ +rotavirus (6.1%) infections were recorded. A statistically significant relationship was observed between the type of farms and the application of umbilical cord disinfection (P=0.007). The rate of umbilical cord disinfection in modern farms (92.5%) was higher than in traditional farms (72.6%).

When looking at the way of taking colostrum, it was noted that 60% of calves received colostrum by feeding bottle and 40% of them received colostrum by suckling. A statistically significant relationship was observed between colostrum intake (suckling, feeding bottle) and colostrum intake status [(did not receive, received less, received fully and on time) (P=0.021)]. It was stated that 85.8% of the calves given colostrum with a feeding bottle received the colostrum fully and on time. In calves given colostrum by suckling, the rate of taking colostrum fully and on time was 75.0%. The proportion of calves "received less" colostrum with a feeding bottle was 6.8%, while the same ratio of those who received colostrum by suckling was recorded as 17.6%. In calves receiving colostrum with a feeding bottle; at most, *Cryptosporidium* spp. (18.5%), then respectively; *E. coli* K99+ (14.8%), coronavirus (13.6%), rotavirus (13.0%), *Cryptosporidium* spp.+rotavirus (9.9%), rotavirus+coronavirus (8.0%) infections were seen. In calves received colostrum through suckling; at most, *Cryptosporidium* spp. (19.0%), *E. coli* (18.0%), coronavirus (16.0%), coronavirus + rotavirus (10.0%), *Cryptosporidium* spp. + rotavirus (9.0%) infections were seen.

A statistically significant relationship was observed between the suckling reflex and the general condition of the calves (P<0.001). A statistically significant relationship was observed between the general condition of the calves and the type of farm where the calves were brought (P=0.014). It was observed that the proportion of calves with mild general condition was lower in calves from traditional farms (21.7%) than those from modern farms (45.0%). There was no statistically significant relationship between the onset of diarrhea and the type of farm (P=0.079). A statistically significant relationship was observed between the seasons and the type of farm where calves were brought (P=0.05). It was observed that the ratio of calves with diarrhea brought to our clinic in the winter season was higher in modern farms (47%) than in traditional farms (28.7). In the spring season, the ratio of calves with diarrhea brought to our clinic in traditional farms (51.7%) was higher than in modern farms (35.0%).

Discussion

In this study, the incidence of major enteropathogens and the distribution of these pathogens according to 5 different age groups in neonatal diarrheic calves brought from two different farm types (traditional/modern farm) in Kayseri and its surrounding provinces were determined. Predisposing factors associated with neonatal calf diarrhea caused by each pathogen were recorded. In addition, predisposing factors affecting the general condition of calves with diarrhea were also revealed.

Considering the fact that in the present study, the animal owners applied to the animal hospital in order to determine the etiology, treatment and improvement of the general condition of the diarrheic cases that did not improve and/or had a severe course, the underrepresentation of treatment-responsive or cured diarrheic cases from the main population could be a potential source of error. The same disadvantage is often present in other studies investigating the etiology of neonatal calf diarrhea [3,8]. In addition, 270 stool samples from calves with diarrhea were analyzed with lateral flow immunochromatographic (LFI) test kits in the present study. As a result of these analyses, 5 etiological agents causing diarrhea (BRV, BCoV, *E. coli* K99+, *Cryptosporidium* spp., *Giardia* spp.) could not be determined in 32 calves with diarrhea. Failure to confirm the accuracy of stool samples determined as positive or negative by LFI test kits with more sensitive diagnostic methods such as "virus isolation, conventional and/or real-time PCR, bacterial culture, etc" and toxin isolation [heat] for ETEC K99+-stable enterotoxin-a (STa), heat-labile enterotoxin-IIc (LTIIc)] is another weakness of the present study. Recent studies have focused on the toxins (Sta, LTIIc) secreted from this bacterium which is responsible for the main pathogenic effect, rather than the diagnosis of *E. coli* in the feces of calves with diarrhea [19,20]. In the current study, these sensitive diagnostic methods were not preferred because they were expensive and take a long time between examination and diagnosis of sick calves. Thus, the reason choosing LFI test kits was that they are faster (<5 min), inexpensive and have the advantage

of starting early treatment against the agent. Once the enteropathogens are diagnosed in a short time, measures such as segregation of affected animals and disinfection of contaminated clinics, animal hospitals, farms and/or shelters can be implemented quickly to prevent the spread of infection to other animals. In addition, it is a fact that this diagnostic method has high specificity and sensitivity rates due to the use of monoclonal antibodies as detector antibodies in these tests [12]. Another weakness of the present study is that the accuracy of the answers given to the questions that asked face to face could not be confirmed. The farms where the calves were brought from could not be visited. Because of the economic or other concerns of the calf owners, there may be a possibility of giving wrong answers to the questions posed to hide the current situation.

Cryptosporidium spp. (21.9%, 59/270) induced diarrhea cases were observed mostly as a single etiological agent in diarrheic calves, in the current study *(Table 1)*. While the results of the present study were similar to the values reported by many researchers [8,21,22], but higher than the values reported by some other researchers [3,10,23]. This situation can be explaned by the age of calves, number of samples, difference in farm type, differences in the hygiene and management practices in farms, climate and geografical differences in which the study was conducted.

When more than one etiological agent taken into consideration, at most; *Cryptosporidium* spp. + BRV (9.3%) combination were seen, in the present study *(Table 1)*. In calves less than 30 days old, BRV was the most reported etiologic agent in addition to *Cryptosporidium* [1,4]. When antigen positivity rates examined (including mix infections) in stool samples, the most common antigen was *Cryptosporidium* spp. (33.3%), followed by BRV with 31.9% *(Table 1)* in our study. As a matter of fact, it is stated that there is a positive correlation between *Cryptosporidium* spp. and BRV infections [24]. In addition, it has been recently reported that *Cryptosporidium* spp. is the major causative factor in neonatal calf diarrhea and is a risk factor for the occurrence of BRV [24,25].

In this study, the diagnosed *Giardia* spp. ratio (1.8%) were lower than studies conducted in our country [26] and other countries [27,28]. The prevalence of enteropathogens may vary depending on the countries, regions, climates, farms and the application of management and hygiene measures. For this reason, it may be more beneficial for veterinarians to evaluate enteropathogens such as BRV, BCoV, *E. coli* K99$^+$ and *Cryptosporidium* spp. rather than *Giardia* spp. in the etiology of neonatal diarrhea in the said region.

The incidence of diarrhea caused by *Cryptosporidium* spp. was 1.9-fold higher in calves born from unvaccinated dams compared to calves born from vaccinated dams ($P<0.05$) in the present study. This may be related to the reduction in diarrhea caused by ETEC K99$^+$ and BCoV due to vaccination. Diarrhea cases due to *E. coli* K99$^+$ (13.1%) and BCoV (10.1%) in calves born from vaccinated dams compared to calves born to unvaccinated dams [*E. coli* K99$^+$ (17%) and BCoV (16.4%)] were found to be lower. BRV incidence rates in calves born from vaccinated and unvaccinated dams were very close to each other. Frequent mutation and antigenic variation of rotaviruses due to recombination may also occur. Thus, BRV vaccines may require frequent surveillance and further characterization of circulating rotaviruses in the field [29,30]. Additionally, the high prevalence of BRV-induced diarrhea in vaccinated patients may be associated with the higher incidence of *Cryptosporidium* spp. related diarrhea. Because *Cryptosporidium* spp. and BRV are risk factors for the formation of each other [1,24]. Furthermore, *Cryptosporidium* spp. can increase the proliferation of viral agents, especially rotaviruses, in the digestive tract by causing malabsorptive diarrhea in calves [31].

The probability of catching *Cryptosporidium* spp.-induced diarrhea was higher in calves born in the winter season compared to the autumn season in calves aged 8-28 days in this study. This result is in agreement with the results reported by Hamnes et al.[32]. On the other hand, in the current study, the majority of calves were brought to the hospital in winter (31.5%) and spring (49.2%) seasons. Therefore, the reason for the high incidence of diarrhea caused by *Cryptosporidium* spp. in winter observed in this study, may be due to patient load in winter season [33]. Similarly, Sanford et al.[33] reported high patient load during the winter months. In contrast, some researchers speculated higher in other seasons than winter [34,35]. A possible explanation for our findings is that during the winter months, reduced cleaning routines may result in a heavier pathogen load in the farms [32]. As a matter of fact, crowding, lower temperature and higher humidity in winter months increase the level of infectious agents that elevate the risk of developing diarrhea [32,36,37].

Consistent with the literature in the current study, diarrhea cases due to ETEC K99$^+$ (33.62%) were the most common in calves aged 1-7 days. This result is compatible with the results of other studies conducted in our country (22.58%-32.1%) [11,21] but, higher than the values reported from different parts of the world (1.4-17.4%) [1,38]. The reason for our results can be attributed to the type of farm (mostly traditional farms; 85.2%), inadequate shelter, hygiene and management practices in the farms, as stated by Cho and Yoon [39]. In addition, it was determined that preventive vaccination against enteropathogens *(E. coli*, rotavirus, coronavirus) in these farms was low (36.7%). So, *E. coli* K99$^+$ (17%) related diarrhea were seen at most in calves

born from unvaccinated dams which can be atributed to low vaccination rate.

Colostrum management is one of the most important preventive measures in reducing infectious calf diarrhea [40]. In the current study, it was determined that the probability of diarrhea caused by *E. coli* K99+ was higher in calves that colostrum was not given compared to calves given full and timely colostrum. According to the results of this study, *E. coli* K99+ (35.0%) related diarrhea was the most common diarrhea in calves that colostrum was not given. Inadequate quality and quantity of colostrum given in the first colostrum feeding is an important reason for the failure of passive immunity transfer [41].

In the present study, it was determined that calves born in the winter season were 6.5-fold more likely to develop diarrhea caused by *E. coli* K99+ compared to the autumn season. Similar findings were also reported by other researchers [38,42]. During the winter months, more crowded shelters, lower ambient temperature and higher indoor air humidity may increase the level of infectious agents such as *E. coli* [38,43].

In our study, compared to the 29-35-days-old age group, 7-14-days-old calves were 0.2-fold more likely to develop rotavirus-induced neonatal diarrhea. Similar results were also reported by other researchers [1]. It was determined that every 1 animal increase in the total number of animals in the farms increased the probability of calves getting rotavirus 1.0-fold, in the present study. The increase in the number of animals on farms may increase transmission of infectious agents by adult cattle or healthy-looking calves [6,44], and could be the reason for above results. In addition, it is a fact that vaccination rates against BRV were low in the study population. This is due to the prevailing belief that vaccination causes an increase in costs.

In this study, calves from traditional farms were 0.3-fold more likely to be diagnosed with coronavirus-induced neonatal diarrhea compared to calves brought from modern farms. In another study, it was reported that coronaviruses are more common in group housing systems compared to individual housing systems [44]. It has been reported that the spread of coronavirus in adult cattle increases during birth, therefore newborns are susceptible to infections and their mothers plays a major role in the exposure of calves to the agent in the first days of their lives. In addition, this disease is more common in animals raised or housed indoors for a long time, especially in winter months [45]. The reason of getting high rate of coronavirus infection in the present study, could be due to keeping calves at the same environment with adult animals especially in traditional farms.

When compared with the calves having "good suckling reflex", each increase in the number of calves "without suckling reflex" was found to worsen the general condition of the calves by 6.8-fold and in calves having 'poor suckling reflex" by 2.7-fold. It has been reported that the suckling reflex in calves with diarrhea is closely related to base excess (BE) values [31,46]. Furthermore, dehydration, metabolic acidosis and increased serum D-lactate concentration are common findings in calves with diarrhea with or without a reduced suckling reflex [31].

In the present study, an increase in rectal temperature (for each °C) in the diarrheic calves was associated with decrease in the odds of general condition, with an odds ratio of 0.765. Boccardo et al.[47] reported that a 1-unit increase in rectal temperature (°C) in calves with diarrhea increased the calf survival rate 1.2-fold. In calves with diarrhea with poor general condition, the prognosis can still be considered favorable if the body temperature is above 38°C. Lower values indicate a poor prognosis [6].

Calves born in summer had 3.0-fold higher risk of being severe in the general condition categories compared to autumn in this study. Indeed, Windyer et al.[48] reported that calves born in summer are 2.0-fold less likely to respond to treatment for neonatal diarrhea compared to calves born in autumn. Similarly, in another study, bovine viral diarrhea virus related diarrheic calves born in summer show 2.7-fold more severe symptoms than calves born in autumn [49]. It has been speculated that many factors such as heat stress and suitable environmental conditions for bacterial growth in summer may play role [49]. Another reason may be related to the workload of animal owners in the summer season.

It was determined that 60% of the calves received colostrum by feeding bottle and 40% by suckling, in this study. Suckling colostrum is the least preferred approach. Because, this approach in the end belived to cause higher rates of passive transfer failure [2,6]. Moreover, in the present study, *E. coli* related diarrhea was seen at higher rates in calves received colostrum by suckling (18.0%) than those received colostrum by feeding bottle (14.8%). In contrast, Mohammed et al.[50] reported that the prevalence of *E. coli* was higher in calves received colostrum by feeding bottle than those received colostrum by suckling. Unlike the present study, Mohammed et al.[50] were carried out their study entirely on modern farms with concern to careless management systems during bottle feeding.

Contrary to the fact that the rate of calves with diarrhea brought from traditional farms in the winter season is lower than that of modern farms, this result can be attributed to various reasons. One of these can be explained by the inadequacy of traditional business owners in transporting their patients to veterinarians due to transportation, distance and adverse weather conditions in winter. On the other hand, it can be concluded that modern business

owners are more sensitive to veterinary consultation. Another reason can be explained by the fact that the total number of calves (40) brought from modern processing is lower than the total number of calves (230) brought from traditional farms.

As a result of above findings, etiological and predisposing factors of calf diarrhea have been put forward. These findings may generate valuable information not only for the clinicians and researchers but also animal health experts, policy makers, farmer etc. Investigating the subtypes of the identified etiological agents in future studies will also contribute to the development of vaccines, especially against different serotypes. Further studies with concern to etiology and predisposing factors at different locations in our country and in the world should be performed in future, because, locations and animal movements may also affect such factors. So, precautions specific to each area can be taken.

Availability of Data and Materials

The data given in this study may be obtained from the corresponding outher on reasonable request.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Statement

This study was approved by the committee of HADYEK-Local Ethics Committee for Animal Experiments Office of Erciyes University (Approval no: 13/10).

Authors' Contributions

İK, MÇ, VG, ÖA and ACO supervised the study. GE, ET, İKB, KV and ÖD collected the data. GE made the statistics. The first draft of the manuscript was written by GE and İK and all authors contributed to the critical revision of the manuscript and have read and approved the final version.

References

1. Bartels CJM, Holzhauer M, Jorritsma R, Swart WAJM, Lam TJGM: Prevalence, prediction and risk factors of enteropathogens in normal and non-normal faeces of young Dutch dairy calves. *Prev Vet Med*, 93 (2-3): 162-169, 2010. DOI: 10.1016/j.prevetmed.2009.09.020

2. Blanchard PC: Diagnostics of dairy and beef cattle diarrhea. *Vet Clin North Am Food Anim Pract*, 28 (3): 443-464, 2012. DOI: 10.1016/j.cvfa.2012.07.002

3. Altuğ N, Yüksek N, Özkan C, Keleş I, Başbuğan Y, Ağaoğlu ZT, Kaya A, Akgül Y: Neonatal buzağı ishallerinin immunokromotografik test kitleri ile hızlı etiyolojik teşhisi. *Van Vet J*, 24 (3): 123-128, 2013.

4. Delgado-González RA, Meza-Herrera CA, González-Álvarez VH, Alvarado-Espino AS, Contreras-Villareal V, Gaytán-Alemán LR, Arellano-Rodríguez G, Véliz-Deras FG: Enteropathogens in Holstein calves with diarrhea during the first five weeks of age in Mexico. *Indian J Anim Res*, 53 (8): 1085-1089, 2019. DOI: 10.18805/ijar.B-875

5. Tilling O: Neonatal calf diarrhoea: A case study. *Livestock*, 18 (3): 64-69, 2013. DOI: 10.12968/live.2013.18.3.64

6. Gül Y: Sindirim sistemi hastalıkları. **In,** Gül Y (Ed): Geviş Getiren Hayvanların İç Hastalıkları (Sığır, Koyun-Keçi). 4th ed., 148-149, Medipres Matbaacılık, Malatya, 2012.

7. Bulut O: Buzağı kayıplarına neden olan viral hastalıklar. **In,** Erdem H, Ciftci E, Isık K, Yorgancılar MU, Yaralı C (Eds): Buzağı Kayıplarının Önlenmesinde Buzağı Sağlığı ve Yetiştiriciliği. 74-75, Medisan Yayınevi, Ankara, Türkiye, 2020.

8. Berber E, Çanakoğlu N, Sözdutmaz İ, Simsek E, Sursal N, Ekinci G, Kökkaya S, Arıkan E, Ambarcıoğlu P, Göksu AG, Keleş İ: Seasonal and age-associated pathogen distribution in newborn calves with diarrhea admitted to ICU. *Vet Sci*, 8 (7): 128, 2021. DOI: 10.3390/vetsci8070128

9. Cho YI, Kim WI, Liu S, Kinyon JM, Yoon KJ: Development of a panel of multiplex real-time polymerase chain reaction assays for simultaneous detection of major agents causing calf diarrhea in feces. *J Vet Diagn Invest*, 22 (4): 509-517, 2010. DOI: 10.1177/104063871002200403

10. Choe C, Jung YH, Do YC, Cho A, Kim SB, Kang HS, Yoo JG, Park J: Use of rapid diagnostic kit for the diagnosis of Korean native calf diarrhea. *Korean J Vet Serv*, 40 (1): 61-66, 2017. DOI: 10.7853/kjvs.2017.40.1.61

11. Küliğ CC, Coşkun A: Sivas ve ilçelerindeki neonatal ishalli buzağılarda *E. coli*, *Cryptosporidium, Clostridium perfringens*, Rotavirüs ve Coronavirüs prevalansı. *Turk Vet J*, 1 (2): 69-73, 2019.

12. Uyunmaz Saklı G, Bulut O, Hasöksüz M, Hadimli HH: Investigation of bovine coronavirus and bovine rotavirus by rapid diagnosis kit and RT-PCR in diarrheic calf feces. *J Istanbul Vet Sci*, 3 (3): 57-63, 2019. DOI: 10.30704/http-www-jivs-net.601639

13. Lichtmannsperger K, Hinney B, Joachim A, Wittek T: Molecular characterization of *Giardia intestinalis* and *Cryptosporidium parvum* from calves with diarrhoea in Austria and evaluation of point-of-care tests. *Comp Immunol Microbiol Infect Dis*, 66:101333, 2019. DOI: 10.1016/j.cimid.2019.101333

14. Al Mawly J, Grinberg A, Prattley D, Moffat J, Marshall J, French N: Risk factors for neonatal calf diarrhoea and enteropathogen shedding in New Zealand dairy farms. *Vet J*, 203 (2): 155-160, 2015. DOI: 10.1016/j.tvjl.2015.01.010

15. Turkish Statistical Institute (TUIK): Animal Production Statistics. https://data.tuik.gov.tr/Bulten/Index?p=Animal-Production-Statistics-December-2020-37207. *Accessed*: 15.11.2021

16. Krejcie RV, Morgan DW: Determining sample size for research activities. *Educ Psychol Meas,* 30: 607-610, 1970. DOI: 10.1177/001316447003000308

17. Smith GW: Treatment of calf diarrhea: Oral fluid therapy. *Vet Clin North Am Food Anim Pract*, 25 (1): 55-72, 2009. DOI: 10.1016/j.cvfa.2008.10.006

18. Sayers RG, Kennedy A, Krump L, Sayers GP, Kennedy E: An observational study using blood gas analysis to assess neonatal calf diarrhoea and subsequent recovery with a European Commission-compliant oral electrolyte solution. *J Dairy Sci*, 99 (6): 4647-4655, 2016. DOI: 10.3168/jds.2015-10600

19. Wang H, Zhong Z, Luo Y, Cox E, Devriendt B: Heat-stable enterotoxins of enterotoxigenic *Escherichia coli* and their impact on host immunity. *Toxins (Basel)*, 11 (1): 24-35, 2019. DOI: 10.3390/toxins11010024

20. Cengiz S, Adiguzel MC: Determination of virulence factors and antimicrobial resistance of *E. coli* isolated from calf diarrhea, part of eastern Turkey. *Ankara Univ Vet Fak Derg*, 67 (4): 365-371, 2020. DOI: 10.33988/auvfd.640990

21. Içen H, Arserim NB, Işık N, Özkan C, Kaya A: Prevalence of four

enteropathogens with immunochromatographic rapid test in the feces of diarrheic calves in east and southeast of Turkey. *Pak Vet J*, 33 (4): 496-499, 2013.

22. Şimşek AT, İnci A, Yıldırım A, Çiloğlu A, Bişkin Z, Düzlü Ö: Nevşehir yöresindeki yeni doğan ishalli buzağılarda Cryptosporidiosis'in Real Time PCR ve Nested PCR yöntemleri ile saptanması. *Erciyes Üniv Vet Fak Derg,* 9 (2): 79-87, 2012.

23. Aydın F, Umur Ş, Gökçe G, Genç O, Güler MA: Kars yöresindeki ishalli buzağılardan bakteriyel ve paraziter etkenlerin izolasyonu ve identifikasyonu. *Kafkas Univ Vet Fak Derg*, 7 (1): 7-14, 2001.

24. Cruvinel LB, Ayres H, Zapa DMB, Nicaretta JE, Couto LFM, Heller LM, Bastos TSA, Cruz BC, Soares VE, Teixeira WF, de Oliveira JS, Fritzen JT, Alfieri AA, Freire RL, Lopes WDZ: Prevalence and risk factors for agents causing diarrhea (Coronavirus, Rotavirus, *Cryptosporidium* spp., *Eimeria* spp., and nematodes helminthes) according to age in dairy calves from Brazil. *Trop Anim Health Prod*, 52 (2): 777-791, 2020. DOI: 10.1007/s11250-019-02069-9

25. Garro CJ, Morici GE, Tomazic ML, Vilte L, Encinas M, Vega C, Bok M, Parreño V, Schnittger L: Occurrence of *Cryptosporidium* and other enteropathogens and their association with diarrhea in dairy calves of Buenos Aires province, Argentina. *Vet Parasitol Reg Stud Reports,* 24: 100567, 2021. DOI: 10.1016/j.vprsr.2021.100567

26. Göz Y, Altuğ N, Yüksek N, Özkan **C:** Parasites detected in neonatal and young calves with diarrhoea. *Bull Vet Inst Pulawy,* 50 (3): 345-348, 2006.

27. Taminelli V, Eckert J: The frequency and geographic distribution of *Giardia* infections in ruminants in Switzerland. *Schweiz Arch Tierheilkd*, 131 (5): 251-258, 1989.

28. O'Handley RM, Cockwill C, McAllister TA, Jelinski M, Morck DW, Olson ME: Duration of naturally acquired giardiosis and cryptosporidiosis in dairy calves and their association with diarrhea. *J Am Vet Med Assoc*, 214 (3): 391-396, 1999.

29. Cho YI, Han JL, Wang C, Cooper V, Schwartz K, Engelken T, Yoon KJ: Case-control study of microbiological etiology associated with calf diarrhea. *Vet Microbiol,* 166 (3-4): 375-385, 2013. DOI: 10.1016/j.vetmic.2013.07.001

30. Martella V, Banyai K, Matthijnssens J, Buonavoglia C, Ciarlet M: Zoonotic aspects of rotaviruses. *Vet Microbiol,* 140 (3-4): 246-255, 2010. DOI: 10.1016/j.vetmic.2009.08.028

31. Naylor JM: Neonatal Calf Diarrhea. *Food Anim Pract,* 70-77, 2009. DOI: 10.1016/B978-141603591-6.10021-1

32. Hamnes IS, Gjerde B, Robertson L: Prevalence of *Giardia* and *Cryptosporidium* in dairy calves in three areas of Norway. *Vet Parasitol,* 140 (3-4): 204-216, 2006; DOI: 10.1016/j.vetpar.2006.03.024

33. Sanford SE, Josephson GKA: Bovine Cryptosporidiosis: Clinical and pathological findings in forty-two scouring neonatal calves. *Can Vet J*, 23 (12): 343-347, 1982. PubMed PMID: 17422204

34. Mohammed HO, Wade SE, Schaaf S: Risk factors associated with *Cryptosporidium parvum* infection in dairy cattle in southeastern New York State. *Vet Parasitol*, 83 (1): 1-13, 1999. DOI: 10.1016/s0304-4017(99)00032-1

35. Urie NJ, Lombard JE, Shivley CB, Adams AE, Kopral CA, Santin M: Preweaned heifer management on US dairy operations: Part III. Factors associated with *Cryptosporidium* and *Giardia* in preweaned dairy heifer calves. *J Dairy Sci,* 101 (10): 9199-9213, 2018. DOI: 10.3168/jds.2017-14060

36. Gebregiorgis A, Tessema TS: Characterization of *Escherichia coli* isolated from calf diarrhea in and around Kombolcha, South Wollo, Amhara Region, Ethiopia. *Trop Anim Health Prod,* 48 (2): 273-281, 2016. DOI: 10.1007/s11250-015-0946-9

37. Arseopoulos K, Theodoridis A, Papadopoulos E: Effect of colostrum quantity and quality on neonatal calf diarrhoea due to *Cryptosporidium* spp. infection. *Comp Immunol Microbiol Infect Dis*, 53: 50-55, 2017. DOI: 10.1016/j.cimid.2017.07.005

38. Bendali F, Bichet H, Schelcher F, Sanaa M: Pattern of diarrhea in newborn beef calves in south-west France. *Vet Res*, 30 (1): 61-74, 1999.

39. Cho YI, Yoon KJ: An overview of calf diarrhea-infectious etiology, diagnosis, and intervention. *J Vet Sci*, 15 (1): 1-17, 2014. DOI: 10.4142/jvs.2014.15.1.1

40. Berge AC, Moore DA, Besser TE, Sischo WM: Targeting therapy to minimize antimicrobial use in preweaned calves: effects on health, growth, and treatment costs. *J Dairy Sci,* 92 (9): 4707-4714, 2009. DOI: 10.3168/jds.2009-2199

41. Keith PP, Andrea LF, Michael TC, Sheila MM: Comparison of passive transfer of immunity in neonatal dairy calves fed colostrum or bovine serum-based colostrum replacement and colostrum supplement products. *J Am Vet Med Assoc*, 237 (8): 949-954, 2010. DOI: 10.2460/javma.237.8.949

42. Frank NA, Kaneene JB: Management risk factors associated with calf diarrhea in Michigan dairy herds. *J Dairy Sci,* 76 (5): 1313-1323, 1993. DOI: 10.3168/jds.S0022-0302(93)77462-7

43. Çitil M, Aslan MÖ, Güneş V, Erdoğan HM: Neonatal buzağı ishallerinde *Cryptosporidium* ve *Eimeria* enfeksiyonlarının rolü. *Kafkas Üniv Vet Fak Derg,* 10 (1): 59-64, 2004.

44. Bertoni EA, Bok M, Vega C, Martinez GM, Cimino R, Parreño V: Influence of individual or group housing of newborn calves on rotavirus and coronavirus infection during the first 2 months of life. *Trop Anim Health Prod,* 53 (1): 62, 2021. DOI: 10.1007/s11250-020-02540-y

45. Clark MA: Bovine coronavirus. *Br Vet J*, 149 (1): 51-70, 1993. DOI: 10.1016/S0007-1935(05)80210-6

46. Sen I, Constable PD: General overview to treatment of strong ion (metabolic) acidosis in neonatal calves with diarrhea. *Eurasian J Vet Sci*, 29 (3): 114-120, 2013.

47. Boccardo A, Sala G, Ferrulli V, Pravettoni D: Cut-off values for predictors associated with outcome in dairy calves suffering from neonatal calf diarrhea. A retrospective study of 605 cases. *Livest Sci*, 245 (3): 104407, 2021. DOI: 10.1016/j.livsci.2021.104407

48. Windeyer MC, Leslie KE, Godden SM, Hodgins DC, Lissemore KD, LeBlanc SJ: Factors associated with morbidity, mortality, and growth of dairy heifer calves up to 3 months of age. *Prev Vet Med*, 113 (2): 231-240, 2014. DOI: 10.1016/j.prevetmed.2013.10.019

49. Muñoz-Zanzi CA, Hietala SK, Thurmond MC, Johnson WO: Quantification, risk factors, and health impact of natural congenital infection with bovine viral diarrhea virus in dairy calves. *Am J Vet Res*, 64 (3): 358-365, 2003. DOI: 10.2460/ajvr.2003.64.358

50. Mohammed SAEM, Marouf SAEM, Erfana AM, El-Jakee JKAEH, Hessain AM, Dawoud TM, Kabli SA, Moussa IM: Risk factors associated with *E. coli* causing neonatal calf diarrhea. *Saudi J Biol Sci*, 26 (5): 1084-1088, 2019. DOI: 10.1016/j.sjbs.2018.07.008

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Research Article

Differential Expression of Proteins in Tibetan Sheep Ovary and Relation to Litter Size Traits at Different Follicular Development Stages

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Abstract: Reproductive performance of Tibetan sheep is the most important factor affecting the sheep industry's production efficiency in Qinghai-Tibet Plateau. To identify differences in lambing traits of Tibetan ewes and the ovarian proteome at different stages of follicular development during the reproductive cycle, the ovarian proteome of Tibetan sheep with clear lambing records and different litter sizes were screened and analyzed using label-free mass spectrometry. The results showed that of 2508 proteins detected, there were 57 differentially expressed proteins during the anestrus season, and compared with single ewes, in twin lambing ewes expression was up-regulated in 43 proteins and down-regulated in 14 proteins; 2664 proteins, with 69 differentially expressed proteins, were detected on the 1st day of the ewe estrus period. Expression of 39 proteins in twin ewes was up-regulated and down-regulated in 30 proteins when compared with single ewes; 2704 proteins and 96 differentially expressed proteins were detected in 11th day of the ewe estrus period. Expression of 16 proteins in twin ewes was up-regulated and expression of 80 proteins was down-regulated compared with single ewes. The pathways in twin sheep that were significantly up-regulated were oxidative phosphorylation (phagosome and endocytosis), oocyte meiosis, extracellular matrix receptor interaction, and cell adhesion. The amino acids (lysine, valine, leucine, isoleucine) and fatty acid degradation, TGF-β signal and GnRH signal pathways were significantly down-regulated in twin sheep. GDF9, BMPR-1B, MTHFR and Cu/Zn-SOD were relatively active in different stages of follicular development during the reproductive cycle. This study contributes to the research of twin production in Tibetan sheep, the in-depth study of Tibetan sheep fertility, and promotes the development of animal husbandry in the Qinghai-Tibet Plateau.

Keywords: *Tibetan sheep, Twin trait, Reproductive cycles, Follicular development, Label-free Mass Spectrometry*

Tibet Koyun Ovaryumlarında Proteinlerin Diferansiyel Ekspresyonu ve Farklı Foliküler Gelişim Aşamalarında Batın Büyüklüğü Özellikleriyle İlişkisi

Öz: Tibet koyunlarının üreme performansı, Qinghai-Tibet Platosu'ndaki koyun endüstrisinin üretim verimliliğini etkileyen en önemli faktördür. Üreme döngüsü sırasında foliküler gelişimin farklı aşamalarında bulunan Tibet koyunlarının kuzulama özelliklerindeki ve yumurtalık proteomundaki farklılıkları belirlemek için net kuzulama kayıtları ve farklı batın büyüklükleri olan yumurtalık proteomları, etiketsiz kütle spektrometrisi kullanılarak tarandı ve analiz edildi. Sonuçlar, tespit edilen 2508 proteinden, anöstrus sezonu boyunca 57'sinin farklı şekilde eksprese edildiğini ve tek kuzu doğuran koyunlarla karşılaştırıldığında, ikiz kuzulayanlarda 43 proteinin daha fazla ve 14 proteinin daha az eksprese edildiğini gösterdi. Koyunlarda östrus periyodunun 1. gününde farklı şekilde eksprese edilmiş 69 protein dahil 2664 protein tespit edildi. Tek kuzu doğuran koyunlarla karşılaştırıldığında ikiz kuzulayan koyunlarda 39 protein daha fazla eksprese edilirken, 30 protein daha az eksprese edildi ve östrusun 11. gününde 96'sı farklı şekilde eksprese edilmiş 2704 protein tespit edildi. Tek kuzu doğuran koyunlarla karşılaştırıldığında, ikiz kuzulayan koyunlarda 16 proteinin ekspresyonu daha fazla iken, 80 proteinin ekspresyonu ise daha azdı. İkiz kuzulayan koyunlarda önemli ölçüde fazla eksprese edilen yolaklar, oksidatif fosforilasyon (fagozom ve endositoz), oosit mayozu, hücre dışı matris reseptör etkileşimi ve hücre adezyonuydu. Amino asitler (lizin, valin, lösin, izolösin) ve yağ asidi yıkımı, TGF-β sinyali ve GnRH sinyal yolaklarının ikiz kuzulayan koyunlarda önemli ölçüde ekspresyonu azalmıştı. GDF9, BMPR-1B, MTHFR ve Cu/Zn-SOD, üreme döngüsü sırasında foliküler gelişimin farklı aşamalarında nispeten aktifti. Bu çalışma, Tibet koyunlarında ikizlik ve doğurganlığının derinlemesine incelenmesine katkıda bulunmakta ve Qinghai-Tibet Platosu'nda hayvancılığın gelişimini teşvik etmektedir.

Anahtar sözcükler: *Tibet koyunu, İkizlik, Üreme döngüleri, Foliküler gelişim, Etiketsiz Kütle Spektrometrisi*

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Introduction

Tibetan sheep is the most common breed in Qinghai province where sheep cultivation is an important industry [1]. With the development of modern animal husbandry, improving lambing rate and reducing stocking density, while increasing economic benefits is occurring in Qinghai province's sheep cultivation industry. The ideal lambing performance of ewes is to produce twins; however, Tibetan sheep have a low reproductive rate with a twin rate of approximately 5% [2]. Sheep reproductive traits are usually regulated by many related genes in complex mechanisms. Improving lambing rate and reducing feed and management costs are an important area of research in Tibetan sheep production.

In recent years, the inherent reproductive performance of Tibetan sheep can no longer meet the current production needs as the sheep farming industry in Qinghai Province, the main production area in China, has grown rapidly. How to improve sheep reproductive performance has been the main puzzle of sheep industry in Qinghai and even the whole country for a long time. Over the past decade, many studies have shown that polymorphisms of fecundity genes, BMPR-1B, BMP15, and GDF9, would affect amino acids of the translated protein, increase ovulation rate, and ultimately affect the lambing characteristics of specific breeds [3-5]. Some studies have indicated that BMPR-1B, one of the oocyte-derived members of transforming growth factor-β family, has played an imperative role in follicular growth and ovulation [6-8]. Mutation at locus 746 in the coding region of the BMPR-1B gene (FecB mutation) increases the signal strength to downstream receptors during BMPR-1B signaling, promotes steroid production, alters SMAD expression and phosphorylation status, accelerates follicle maturation, and causes an additive effect on ovulation number in sheep [9]. During follicle development, the BMP system increased average ovulation in Booroola Merino sheep, Garole sheep, and Hu sheep [10]. The reproductive performance of sheep is not only controlled by small effects of multiple genes but is also affected and regulated by one or more quantitative trait loci (QTL). It has a non-additive dominant gene action mode, and its heritability (H^2) is very low. The heritability of sheep double lambs is only 0.126 [11].

Mammalian follicle development and ovulation are regulated by the downstream action of GnRH in the hypothalamus and FSH and LH in pituitary gonadotropins on follicular granulosa cells and/or membrane cells, as well as intraovarian effects of growth factors and steroids [12]. Research confirmed that various intraovarian factors could control the release of these gonadotropin hormones (FSH and LH) and extraovarian feedback effects on hypothalamic and/or pituitary gonadotropins. Genes affecting the development and control of the reproductive axis are potential candidate genes for twin production [13]. From long-term studies and observations of animal twins, it is clear that twins are mainly controlled by genetic factors. There are great differences in the rate of twin lambs among different sheep breeds, and the traits of twin lambs are greatly affected by their parents. In addition, they are also affected by parity, season and nutrition [14,15]. Therefore, the most effective way to improve the rate of sheep production is to further our understanding of the genetic basis of twin production in Tibetan sheep, find the main genes and molecular markers of twin production, and carry out molecular breeding. Here, we identified and analyzed different proteins in ovarian tissue of Tibetan ewes, with a history of producing single and twin ewes, at different stages of follicular development using label-free mass spectrometry and identified signal pathways and molecular mechanisms that have an impact on twin production in Tibetan sheep.

Material and Methods

Approval was granted by the Animal Care Committee of Qilu Normal University, China, for all the animal related protocols (QLNU-2019-10-0014). Furthermore, for slaughtering the animals, approval was granted by the National Administration of Mutton Sheep Slaughtering and Quarantine (Qinghai, China, 20200407).

Study Location

This work was carried out in the Hainan Tibetan Autonomous Prefecture, Qinghai Province, China, located at an altitude of more than 3200 meters from sea level in southeastern Qinghai-Tibetan Plateau, which has a desiccated cool climate.

Animals and Sample Collection

Twenty-four healthy Tibetan ewes (3-4 years old, 2 lambing records) from Wayu town, Qinghai province, China, were chosen. Twelve were sheep that produced twins (T group) and 12 produced singletons (S group). All experimental sheep were healthy, free of reproductive diseases, and without hormone treatment. All ewes were not pregnant. The experimental sheep were slaughtered during estrus (October 2020, Day1 and Day 11 of estrus cycle) and anestrus (April 2020), respectively. Three ewes each of different lambing types slaughtered at each stage. The reproductive state of the ewes (estrus) was identified based on the combination of ram test and vulvar observation. The ewes were deemed in estrus when they accepted mounting by the rams aged 2 to 3 years with high serving capacity as teasers with canvas apron conducted by leash. Experimental ewes were killed by bloodletting via the carotid artery at 7 a.m. before feeding and watering. The ovaries of Tibetan ewes were collected in accordance with

approved guidelines [16], and ovary samples were peeled and washed to remove all surface fat and ligaments. The ovaries were then transferred to sterile plastic tubes and stored at -80°C.

Ovary Samples Proteomics Collection and Measurement

The surface contaminants from the Tibetan sheep ovary were washed away with the help of phosphate buffer saline solution before their transfer to 1.5 mL centrifugal tubes. Prior to proteomics analysis, samples were kept at -80°C. Proteomics analysis was carried out by SMBPT Co., Ltd (China, Shanghai). Each group had 3 replicates. First, the lysis buffer method was used for protein extraction [17]. Next, 250 µg of protein was digested by complying with the described FASP procedure. Q Exactive TM mass spectrometer and Easy nLC system (Thermo Fisher Scientific, MA, USA) was used to perform label-free mass spectrometry (MS). For the analysis of MS data, we used Max Quant software while the UniProt database *(Ovis aries https://www.uniprot.org/uniprot/?query=Ovis+aries&sort=score)* was accessed for reference. Relative quantitative real-time PCR (qPCR) was performed to determine the copy number of target genes. RT-PCR system was 20 µL: 2×PerfectStart@Green qPCR SuperMix 10 µL, 1 µL of primers (0.5 µL each for F primer and R primer), 1.5 µL of 50 mg/µL template cDNA, and RNase-free ddH2O 8 µL. Reaction conditions: pre-denaturation 94°C for 30 s; denaturation 94°C for 5 s, annealing 60°C for 30 s, 72°C for 40 cycles. The primers were synthesized at Shanghai Biological Engineering Ltd., China *(Table 1)*.

Statistical Analysis

Differential proteomics analysis was conducted by Applied Protein Technology (Shanghai, China). 5800 MALDI-TOF/TOF (AB Sciex, USA) was used for mass spectrometer (MS) data analyses. For quantitative analysis, a protein must have at minimum of one unique peptide match with the MS ratios. A ≥1.5 or ≤1.5-fold cutoff value was used to identify up-regulated and down-regulated proteins with a P-value <0.05. We passed all the identified proteins through the Blast2GO program for functional annotation as well as classification against the UniProt database which contains cell components along with the biological process and molecular function. Furthermore, we applied the search pathway tool from the KEGG Mapper platform. Fisher's exact test was used to test for significant pathway enrichment. Pathways that had an adjusted P value of <0.05 were deemed significant. DPEs were used for cluster analysis. GeneMANIA was used to predict both physical, as well as functional, interactions among genes/proteins. Finally, the PPIs were analyzed.

Table 1. *Primer sequence of target genes*

Gene Name	Primer Sequences (5'-3')
GDF9	F: ACTGAATGAATAGGGTGTTG R: ATCTGTACCATATCTAAGTCC
BMPR1B	F: CCGCTCGAGAACATGCTTTTGCGAAGTTCAG R: CGCGCGATCCCAGAGCTTAATGTCCGGGACT
MTHFR-P1	F: AAGCTGCGTGATGATGAAATCG R: CTCCCGCAGACACCTTCTCC
MTHFR-P2	F: AACGAAGACTTCAAAGACACTT R: CTTCACTGGTCAGCTCCTCCCC
Cu/Zn-SOD	F: CTCTGCGGCATTATCACA R: GGAAAAGCCATAGAAGGT

Results

Changes in Proteome Profiles During Anestrus and Estrus Season

Single and twins producing Tibetan ewes of similar age exhibit differences in protein composition of ovarian tissue during different stages of follicular development. 2508 proteins and 57 differential proteins were detected during the anestrus season. In twin ewes expression of 43 proteins were up-regulated and 14 proteins were down-regulated compared with single ewes; 2664 proteins, with 69 differentially expressed proteins, were detected in the 1st day of ewe estrus period. In twin ewes 39 proteins were up-regulated and 30 proteins were down-regulated compared with single ewes; 2704 proteins and 96 differentially expressed proteins were detected during the 11th day of ewe estrus *(Fig. 1)*. The expression of 16 proteins in twin ewes were up-regulated and 80 proteins were down-regulated compared with single ewes. On the 11th day of the ewe estrus period, there were significantly more differentially expressed protein compared to during the ewe anestrus season and the 1st day of ewe estrus period, indicating that on the 11th day of estrus *(Fig. 2)*. There was an increase in ovarian activity. The protein expression of GDF9, BMPR-1B and MTHFR in twin producing ewes was lower than that of single lamb producing ewes during estrus period, but the expression trend of these genes during estrus period was opposite to that of the anestrus season.; The expression trend of Cu/Zn-SOD was opposite on the 1st day and 11th day of the ewe's estrus period.

Gene Ontology (GO) Analyses

To explore biological functions associated with differentially expressed proteins, enrichment analysis in the Gene Ontology (GO) was used *(Fig. 3)*. Using GO, we identified cellular components, molecular function and biological process. There were 21 terms in cellular components, which were mainly related to the following terms: enriched in cytoplasm, cytoplasmic matrix, nucleus and calcium complex. A total of 31 molecular function terms were enriched. These included ATP binding, signal transduction, calcium binding, oxygen transport activity, growth factor activity, DNA binding and carbohydrate binding. There

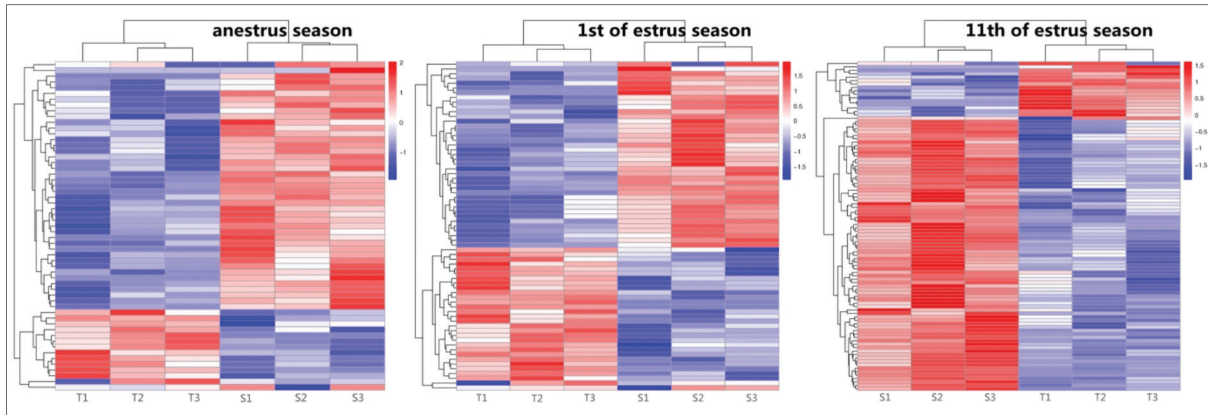

Fig 1. Differentially expressed proteins in three follicular development stage of Tibetan ewes. Three replicates for the double lambing group (T) and three replicates for the single lambing group (S). The image presents the relative abundance of proteins using different colors, where deeper red represents higher intensity and blue represents lower intensity

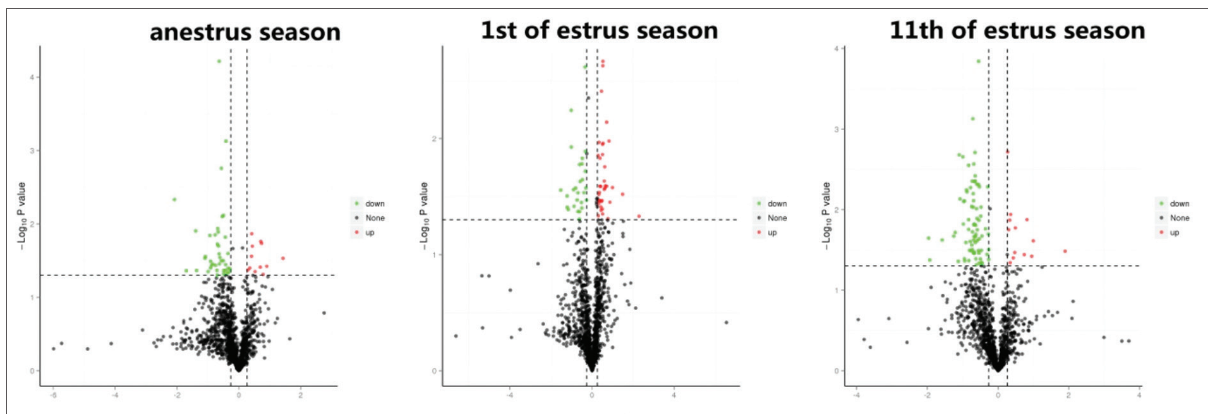

Fig 2. Differentially Expressed Proteins (DEPs) Cluster Evaluation in three follicular development stage of Tibetan ewes. Down was the DEPs of twins lambing group (T) were less than single lambing group (S); Up was the DEPs of twins lambing group (T) were more than single lambing group (S); None was the DEPs of twins lambing group (T) were no significant differences than single lambing group (S)

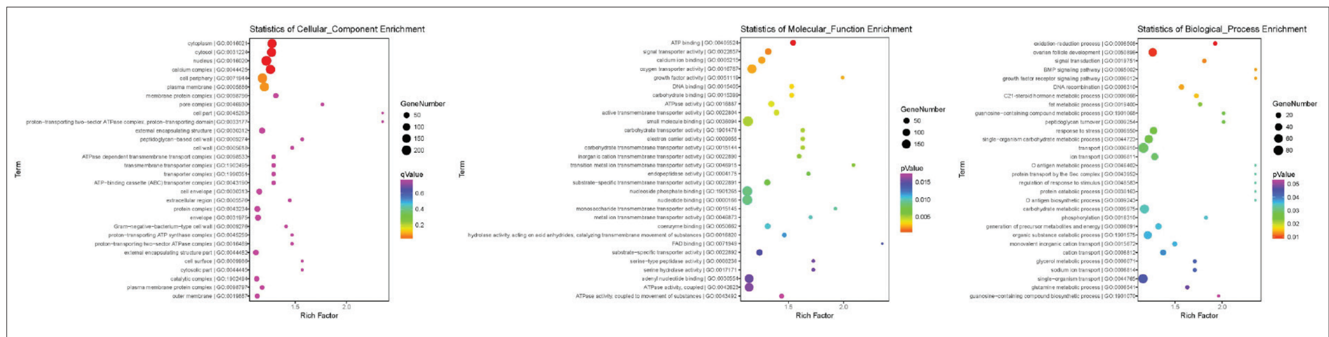

Fig 3. Differentially Expressed Proteins (DEPs) GO Evaluation in Tibetan ewes

were 35 enriched terms related to biological processes. These include oxidative phosphorylation, oocyte meiosis, signal transduction, BMP signal pathway, growth factor receptor signal pathway, C21 steroid hormone metabolism and fatty acids β oxidation process. Enriched biological processes in twin producing sheep are ovarian specific proteins including oxidative phosphorylation, oocyte meiosis, signal transduction and C21 steroid hormone metabolism which were all were up-regulated. These accounted for about 54% of the total number of up-regulated proteins in the ovary. Some proteins related to transcription regulation, protein translation and protein modification. These proteins were also up-regulated (accounting for 7% of the total up-regulated proteins); proteins related to intracellular pyrimidine synthesis accounted for about 4% of the up-regulated proteins. The

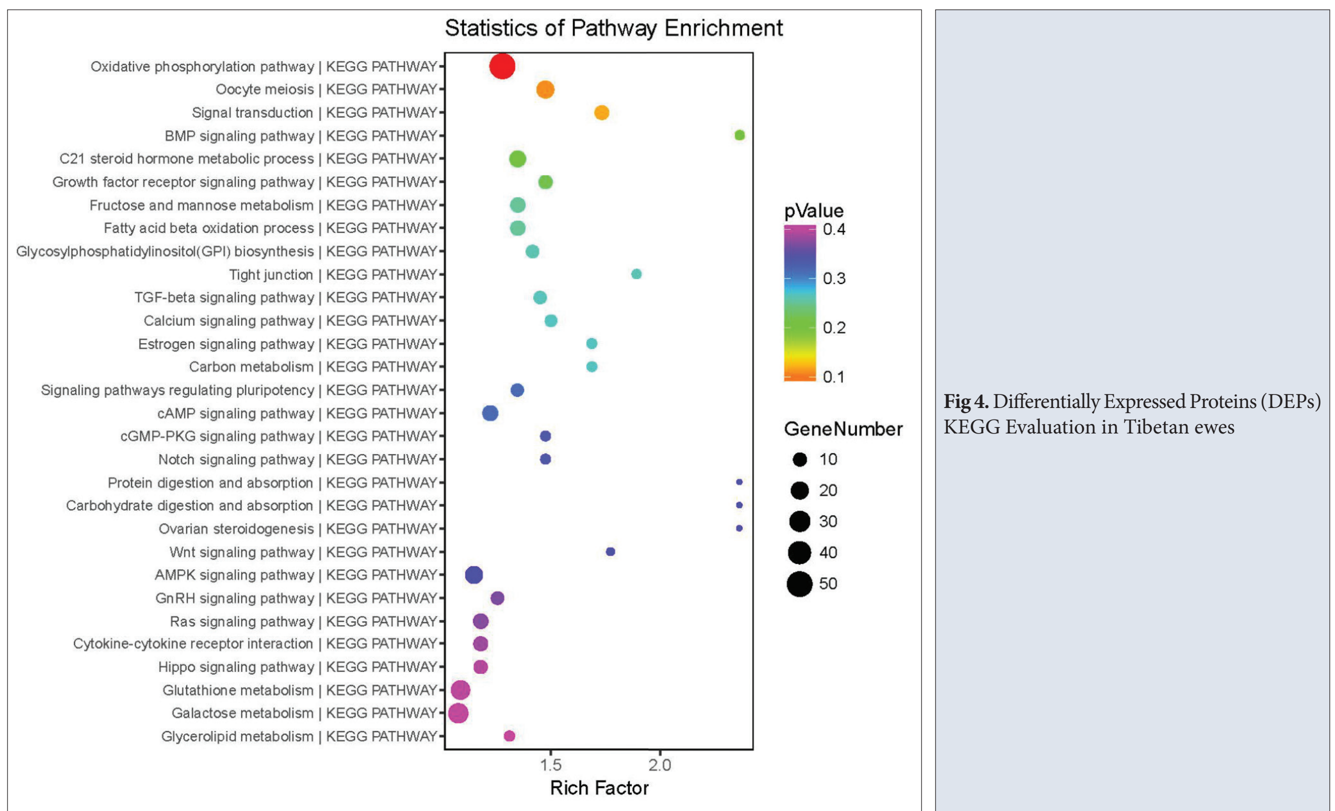

Fig 4. Differentially Expressed Proteins (DEPs) KEGG Evaluation in Tibetan ewes

synthesis, processing and transportation rate of protein in the ovaries of twin producing sheep were all accelerated, intracellular and extracellular signal transduction were enhanced, and the level of anaerobic respiration and metabolism was improved. BMP signaling and growth factor receptor signaling pathways were down-regulated, accounting for 26% of the total down-regulated proteins in the ovary. Fatty acids, β Oxidation, lipid, and small molecule transport were also partially down-regulated (accounting for 13% of the down-regulated proteins), indicating that the rate of transport and metabolism of small organic molecules such as amino acids and biotin decreased in the cells of twin producing sheep, and the aerobic respiration in the ovarian cells decreased.

Kyoto Encyclopedia of Genes and Genomes (KEGG) Analyses

The major pathways associated with differentially expressed proteins were identified using KEGG pathway analysis *(Fig. 4)*. A total of 99 differentially expressed proteins across 63 pathways were significantly enriched ($P<0.05$). Of these, 34.6% of the up-regulated protein and 48.3% of the down-regulated protein were enriched. The significantly up-regulated pathways in twin producing sheep were oxidative phosphorylation (phagosome and endocytosis), oocyte meiosis, extracellular matrix receptor interaction, and cell adhesion, indicating that the activity of ovarian cells in twin producing Tibetan sheep was increased and the material exchange between cells was more frequent. Amino acid (lysine, valine, leucine, isoleucine) and fatty acid degradation, TGF-β signalling pathway and GnRH signalling pathway were significantly down-regulated in twin producing sheep. The degradation of related amino acids synthesizing Smads family proteins was significantly inhibited in the ovarian cells of twin producing sheep. Intracellular metabolic efficiency decreased, and the degradation process of amino acids and fatty acids appeared.

Protein-Protein Interaction Network (PPI Network) Analyses

Protein networks among the 99 proteins were analyzed via STRING *https://string-db.org/cgi/input.pl* *(Fig. 5)*. Among the screened up-regulated differential proteins, ribosomal proteins interacted with glycolysis related proteins. Among the down-regulated differential proteins, lipid metabolism related proteins interacted. These differentially expressed proteins created a complex network of interactions. Interestingly, GDF9, BMPR-1B, MTHFR and Cu/Zn-SOD were at the intersection of this interaction network, indicating that they were relatively active during different stages of follicular development. Protein expression of GDF9, BMPR-1B and MTHFR in twin producing sheep was higher than that of single lamb producing sheep during anestrus season, but the expression trend is opposite during estrous season. Expression trends of Cu/Zn-SOD were opposite during the 1st day of anestrus, estrus and during the 11th day of estrus.

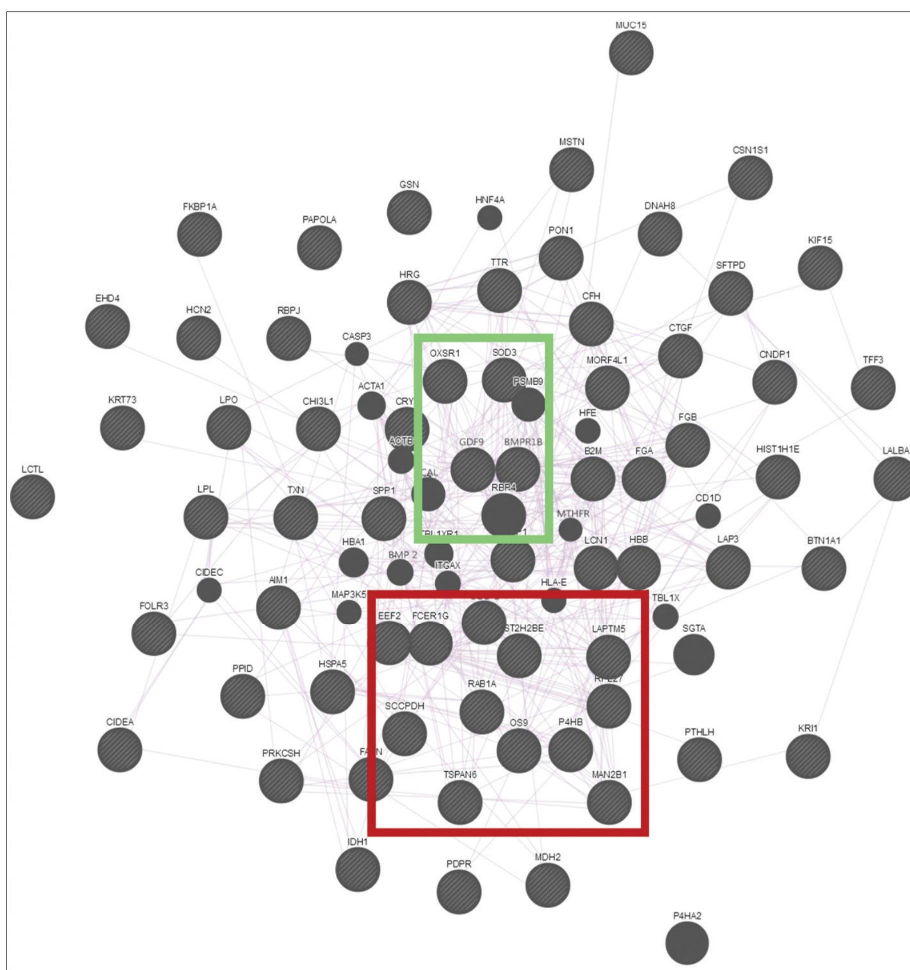

Fig 5. Differentially Expressed Proteins (DEPs) PPIs Evaluation in two follicular development stage of Tibetan ewes. The green zone was the differentially expressed proteins in twins lambing sheep higher than that in single lambing sheep; The red zone was the differentially expressed proteins in twins lambing sheep lower than that in single lambing sheep

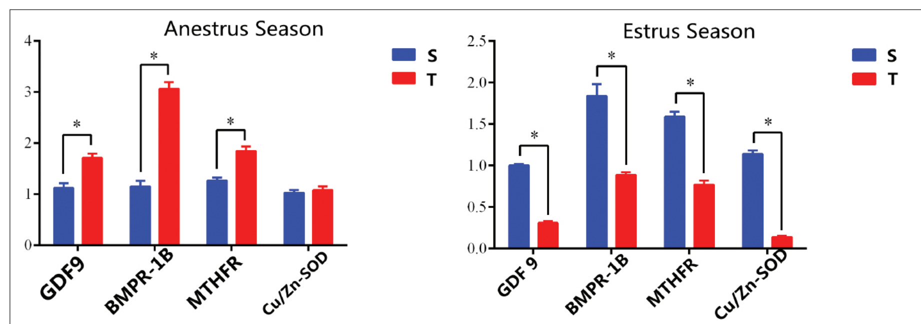

Fig 6. Effects of different litter size on the differentially expressed proteins coding genes in Tibetan sheep ovary in aneustrus and estrus seasons

Validation of Differentially Expressed Proteins Coding Genes by qPCR

The gene expression of GDF9, BMPR-1B and MTHFR in twins trait sheep was higher than that in single trait sheep at anestrus season, but the expression trend is opposite at estrous season (P<0.05) via RT-PCR, however, the gene expression of Cu/Zn-SOD were opposite at the 1st of anestrus, estrus and at 11th of estrus (P<0.05). In summary, the results of selected differentially expressed proteins coding genes by RT-PCR were the same expression tendency of the label-free analysis *(Fig. 6)*. The RT-PCR assay illustrated that the label-free results were reliable for further analyses.

Discussion

Tibetan sheep production has been the main revenue source of Tibet plateau residents. Sheep reproductive performance is economically important for the sheep industry and plays a key role in the development of the sheep industry [18]. Sheep fertility has complex molecular regulatory mechanisms and the regulatory mechanism controlling sheep reproductive performance is an exciting research area. At present, research on lambing traits of sheep at home and abroad mainly focuses on gene polymorphism [19,20]. There are few reports on sheep proteins from the perspective of proteomics. Studies show that molecular regulation of tissues and cells depends on many

cytokines and catalytic enzymes. Most cytokines and catalytic enzymes are essentially proteins [21]. Therefore, proteins drive cellular activities. Twin production in sheep is related to many factors including the development of ovarian follicles and ovulation frequency. Seasonal estrus, which results from changes in both ovarian function and hormonal secretions during the different seasons, is a critical factor limiting sheep fecundity and productivity [22]. Expression regulation in sheep ovarian tissue is important for understanding follicular development and ovulation regulation. The ovulation number of sheep controls lambing traits, and the ovary is the main site of follicular development and ovulation in animals. In the ovary, the morphology and function of the follicles change continuously with the different stages of development, during which the cells need to synthesize sufficient amounts of biomolecules to ensure normal cell proliferation and differentiation [23,24]. This may be an important physiological mechanism, explaining differences of the ovarian proteome between twin- and singleton-producing Tibetan ewes during different stages of follicular development.

Follicular development depends on the mutual regulation of oocytes, surrounding granulosa cells, and theca cells. The initiation of primordial follicle development is related to various factors secreted inside and outside the follicle, and neurotransmitters or signal transmission between oocyte granulosa cells [25]. Basic fibroblast growth factor, stem cell factor, leukemia inhibitory factor, nerve growth factor, bone morphogenetic protein, insulin and keratinocyte growth factor can induce the initial recruitment of primordial follicles and develop into mature follicles [26]. The ovarian proteome of Tibetan sheep with recorded differences in twin production were screened and analyzed by label-free Mass Spectrometry at different stages of follicular development. Results showed that of 2706 proteins 99 were differentially expressed. Of 2508 proteins, 57 were differentially expressed during ewe anestrus. Expression of 43 proteins in twin-producing ewes was up-regulated and expression of 14 proteins was down-regulated compared with single ewes; 2664 proteins and 69 differentially expressed proteins were detected in the 1st day of ewe estrus period. Expression of 39 proteins was up-regulated and expression of 30 proteins was down-regulated in twin-producing ewes compared with single ewes. On the 11th day of ewe estrus, 2704 proteins and 96 differentially expressed proteins were detected. The expression of 16 proteins in twin-producing ewes was up-regulated while expression of 80 proteins was down-regulated compared with single ewes. Differentially expressed proteins in twin-producing sheep were related to oxidative phosphorylation, follicular growth and development, signal transduction, growth factor receptor signal pathway, fatty acid degradation, tricarboxylic acid cycle, lipid metabolism, BMP signal pathway, TGF-β signaling pathway, metabolism of C21 steroids, and fatty acids β. Expression decreased significantly during oxidation. GDF9, BMPR-1B, MTHFR and Cu/Zn-SOD are relatively active during different stages of follicular development in ewe reproductive cycle. Our study showed that the protein expression of GDF9, BMPR-1B and MTHFR in twin-producing sheep was higher than in singleton-producing sheep during anestrus season. The expression trend is opposite during estrous season. Expression trends of Cu/Zn-SOD were opposite at the 1st of anestrus, estrus and at 11th of estrus. BMPR-1B, a member of BMPs, is a major gene affecting ovine ovulation rate, and plays a pivotal role in follicle development and litter size [27]. During anestrus, ovarian physiological activity is reduced with the follicular stage not developing. During the selection of the dominant follicle, the expression of BMPR-1B protein in the follicular granule cells declined followed by an increase in follicle size. Granular cells in atretic cells continued to demonstrate high expression of BMPR-1B mRNA [28]. Studies of the FecB gene have shown that the signaling pathway in FecB mutant ewes leads to increased intensity of signaling to downstream receptors during follicular development, promotes steroid production, and alters SMAD expression and phosphorylation status, leading to reduced follicular granulosa cell apoptosis, early follicular maturation, and increased ovulation numbers [29]. This is the same result as the enrichment of the KEGG pathway. cAMP, as the second messenger of hormones, activates protein kinase, enhances the activity of metabolic enzymes, strengthens the synthesis of proteins *in vivo*, and induces hormones (such as growth hormone, follicle stimulating hormone, etc.) or enzyme synthesis to promote the body's anabolism. In the ovary, follicle stimulating hormone increases the synthesis of estradiol and progesterone through the cAMP pathway [30,31]. Growth differentiation factor 9 (GDF9) belongs to the growth differentiation factor-β superfamily, which is only expressed in ovaries or oocytes, and has an important impact on follicular growth and development, and reproductive function [32]. GDF9 is an oocyte derived growth factor that affects ovine ovulation in a dose-dependent manner and is expressed at all stages of ovine follicular development. GDF9 can affect the distribution of organelles in oocytes, the integrity of the zona pellucida, stimulate granulosa cell proliferation, inhibit the differentiation of granulosa cells induced by FSH, regulate the expansion of cumulus, stimulate the formation of follicular membrane, and affect the synthesis of hormones in ovary [33]. A mutation in the methylenetetrahydrofolate reductase (MTHFR) gene will lead to a decrease in folate metabolism, DNA methylation, and increase concentration of homocysteine in plasma, resulting in disorders of biological processes such as cell

cycle regulation, DNA replication, and DNA and protein modification [34]. Superoxide dismutase (SOD) is an important biological antioxidant enzyme and plays a very important role in the immune system of organisms. Cu/Zn-SOD can inhibit the mitosis and meiotic maturation of oocytes [35]. Transgelin in the ovary will prevent the formation of ara54 dimers, thus blocking the combination of androgen receptors and ara54, resulting in the retention of androgen receptors in the cytoplasm, changing the transmission pathway of downstream signals, and then affecting the reproductive performance of animals [36]. The regulation and expression of GDF9, BMPR-1B, MTHFR and Cu/Zn-SOD were significantly different in ovarian tissues of Tibetan sheep of different lambing types. The regulation of these differentially expressed proteins and their protein pathways can significantly affect follicle development and ovulation, and a more in-depth study of this as a candidate target protein for Tibetan sheep double lambing can effectively elucidate the intrinsic molecular mechanisms of Tibetan sheep double lambing traits and provide a basis for subsequent studies of Tibetan sheep double lambing production. This study is not only beneficial to the research and utilization work on the double lamb trait of Tibetan sheep, but also to the in-depth research on the breeding power of Tibetan sheep and to promote the development of animal husbandry in the Tibetan Plateau region.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author (J. Jia).

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Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

YC and LZ: the hypothesis of this study; YC and JL: work management, article writing; YC, QC and LR: experimental procedure follow-up, statistical analysis; YC, JL, QC and LR: literature review, review of results; JJ: final decision, funding support.

References

1. Cui X, Wang Z, Yan T, Chang S, Wang H, Hou F: Rumen bacterial diversity of Tibetan sheep *(Ovis aries)* associated with different forage types on the Qinghai-Tibetan Plateau. *Can J Microbiol,* 65 (12): 859-869, 2019. DOI: 10.1139/cjm-2019-0154

2. Jia J, Xie D, Zhang Y, Zhang H, Zhang L, Hou S, Chen Q: Association of *BMPR-1B* Gene 3'-UTR region polymorphism with litter size in Tibetan sheep. *Kafkas Univ Vet Fak Derg,* 27 (3): 285-289, 2021. DOI: 10.9775/kvfd.2020.25064

3. Jia J, Chen Q, Gui L, Jin J, Li Y, Ru Q, Hou S: Association of polymorphisms in bone morphogenetic protein receptor-1B gene exon-9 with litter size in Dorset, Mongolian, and Small Tail Han ewes. *Asian-Australas J Anim Sci,* 32 (7): 949-955, 2019. DOI: 10.5713/ajas.18.0541

4. Li LL, Ma SK, Peng W, Fang YG, Duo HR, Fu HY, Jia GX: Genetic diversity and population structure of Tibetan sheep breeds determined by whole genome resequencing. *Trop Anim Health Pro,* 53, 174-179, 2021. DOI: 10.1007/s11250-021-02605-6

5. Wang HH, Wang X, Li TT, An X, Yin D, Chen N, Ma Y: Regulation of *GDF9* and *CDKN1B* expression in Tibetan sheep testes during different stages of maturity. *Gene Expr Patterns,* 43:119218, 2022. DOI: 10.1016/j.gep.2021.119218

6. Magro-Lopez E, Munoz-Fernandez AM: The role of BMP signaling in female reproductive system development and function. *Int J Mol Sci,* 22 (21): 11927, 2021. DOI: 10.3390/ijms222111927

7. Akhatayeva Z, Bi Y, He Y, Khan R, Li J, Li H, Pan C, Lan X: Survey of the relationship between polymorphisms within the *BMPR1B* gene and sheep reproductive traits. *Anim Biotechnol,* 29: 1-10, 2021. DOI: 10.1080/10495398.2021.1979023

8. Çelikeloğlu K, Tekerli M, Erdoğan M, Koçak S, Hacan Ö, Bozkurt Z: An investigation of the effects of *BMPR1B*, *BMP15*, and *GDF9* genes on litter size in Ramlıç and Dağlıç sheep. *Arch Anim Breed,* 64 (1): 223-230, 2021. DOI: 10.5194/aab-64-223-2021

9. Pourali Dogaheh S, Mirhoseini SZ, Tufarelli V, Hossein-Zadeh NG, Badbarin S, Colonna MA, Seidavi A, Selvaggi M: Investigating the polymorphism of bone morphogenetic protein receptor-1B (*BMPR1B*) gene in Markhoz goat breed. *Animals (Basel),* 10 (9): 1582, 2020. DOI: 10.3390/ani10091582

10. Kumar S, Kumar Rajput P, Bahire SV, Jyotsana B, Kumar V, Kumar D: Differential expression of BMP/SMAD signaling and ovarian-associated genes in the granulosa cells of *FecB* introgressed GMM sheep. *Syst Biol Reprod Med,* 66 (3): 185-201, 2020. DOI: 10.1080/19396368.2019.1695977

11. Zhao YZ: Application of quantitative genetics in sheep breeding. Chinese Sheep Science. 384, China Agriculture Press, Beijing, 2013.

12. Hu KL, Zhao H, Chang HM, Yu Y, Qiao J: Kisspeptin/kisspeptin receptor system in the ovary. *Front Endocrinol,* 8, 1-14, 2018. DOI: 10.3389/fendo.2017.00365

13. Paiva JT, Peixotol MGCD, Bruneli FAT, Alvarenga AB, Oliveira HR, Silva AA, Silva DA, Veroneze R, Silva FF, Lopes PS: Genetic parameters, genome-wide association and gene networks for milk and reproductive traits in Guzerá cattle. *Livest Sci,* 242:104273, 2020. DOI: 10.1016/j.livsci.2020.104273

14. Scaramuzzi RJ, Martin GB: The importance of interactions among nutrition, seasonality and socio-sexual factors in the development of hormone-free methods for controlling fertility. *Reprod Domest Anim,* 2, 129-136, 2008. DOI: 10.1111/j.1439-0531.2008.01152.x

15. Zhang Y, Xue X, Liu Y, Abied A, Ding Y, Zhao S, Wang W, Ma L, Guo J, Guan W, Pu Y, Mwacharo JM, Han J, Ma Y, Zhao Q: Genome-wide comparative analyses reveal selection signatures underlying adaptation and production in Tibetan and Poll Dorset sheep. *Sci Rep,* 11:2466, 2021. DOI: 10.1038/s41598-021-81932-y

16. Liu A, Liu M, Li Y, Chen X, Zhang L, Tian S: Differential expression and prediction of function of lncRNAs in the ovaries of low and high fecundity Hanper sheep. *Reprod Domest Anim,* 56 (4): 604-620, 2021. DOI: 10.1111/rda.13898

17. Sanchez DJD, Vasconcelos FR, Teles-Filho ACA, Viana AGA, Martins AMA, Sousa MV, Castro MS, Ricart CA, Fontes W, Bertolini M, Bustamante-Filho IC, Moura AA: Proteomic profile of pre-implantational ovine embryos produced *in vivo*. *Reprod Domest Anim,* 56 (4): 586-603, 2021. DOI: 10.1111/rda.13897

18. Ren Y, Zhu Y, Baldan D, Fu M, Wang B, Li J, Chen A: Optimizing livestock carrying capacity for wild ungulate-livestock coexistence in a Qinghai-Tibet Plateau grassland. *Sci Rep,* 11:3635, 2021. DOI: 10.1038/s41598-021-83207-y

19. Tsartsianidou V, Sanchez-Molano E, Kapsona VV, Basdagianni Z, Chatziplis D, Arsenos G, Triantafyllidis A, Bnos G: A comprehensive genome-wide scan detects genomic regions related to local adaptation and climate resilience in Mediterranean domestic sheep. *Genet Sel Evol,* 53: 90-99, 2021. DOI: 10.1186/s12711-021-00682-7

20. EEr H, Ma L, Xie X, Ma J, Ma X, Yue C, Ma Q, Liang X, Ding W, Li Y: Genetic polymorphism association analysis of SNPs on the species conservation genes of Tan sheep and Hu sheep. *Trop Anim Health Prod,* 52: 915-926, 2020. DOI: 10.1007/s11250-019-02063-1

21. Fu Z, Akula S, Thorne M, Hellman L: Highly selective cleavage of TH2-Promoting cytokines by the human and the mouse mast cell tryptases, indicating a potent negative feedback loop on TH2 immunity. *Int J Mol Sci,* 20 (20): 5147-5159, 2019. DOI: 10.3390/ijms20205147

22. Zhai M, Xie Y, Liang H, Lei X, Zhao Z: Comparative profiling of differentially expressed microRNAs in estrous ovaries of Kazakh sheep in different seasons. *Gene,* 664 (20): 181-191, 2018. DOI: 10.1016/j.gene.2018.04.025

23. Almeida FRCL, Costermans NGJ, Soede NM, Bunschoten A, Keijer J, Kemp B, Teerds KJ: Presence of anti-Müllerian hormone (AMH) during follicular development in the porcine ovary. *PLoS One,* 13 (7): e0197894, 2018. DOI: 10.1371/journal.pone.0197894

24. Kim S, Shah SB, Graney PL, Singh A: Multiscale engineering of immune cells and lymphoid organs. *Nat Rev Mater,* 4, 355-378, 2019. DOI: 10.1038/s41578-019-0100-9

25. Kranc W, Brazet M, Budna J, Celichowski P, Bryja A, Nawrocki MJ, Ożegowska K, Jankowski M, Chermuła B, Dyszkiewicz-Konwińska M, Jeseta M, Pawelczyk L, Bręborowicz A, Rachoń D, Bruska M, Nowicki M, Zabel M, Kempisty B: Genes responsible for proliferation, differentiation, and junction adhesion are significantly up-regulated in human ovarian granulosa cells during a long-term primary *in vitro* culture. *Histochem Cell Biol,* 151, 125-143, 2019. DOI: 10.1007/s00418-018-1750-1

26. Gentile P, Garcovich S: Advances in regenerative stem cell therapy in androgenic alopecia and hair loss: Wnt pathway, growth-factor, and mesenchymal stem cell signaling impact analysis on cell growth and hair follicle development. *Cells,* 8 (5): 466- 478, 2019. DOI: 10.3390/cells8050466

27. Jia J, Jin J, Chen Q, Yuan Z, Li H, Bian J, Gui L: Eukaryotic expression, CoIP and MS identify BMPR-1B protein–protein interaction network. *Biol Res,* 53 (1): 24-36, 2020. DOI: 10.1186/s40659-020-00290-7

28. Yang J, Li X, Cao YH, Pokharel K, Hu XJ, Chen ZH, Xu SS, Peippo J, Honkatukia M, Kantanen J, Li MH: Comparative mRNA and miRNA expression in European mouflon (*Ovis musimon*) and sheep *(Ovis aries)* provides novel insights into the genetic mechanisms for female reproductive success. *Heredity,* 122, 172-186, 2019. DOI: 10.1038/s41437-018-0090-1

29. Wang F, Chu M, Pan L, Wang X, He X, Zhang R, Tao L, La Y, Ma L, Di R: Polymorphism detection of *GDF9* gene and its association with litter size in Luzhong Mutton sheep (*Ovis aries*). *Animals,* 11 (2): 571-584, 2021. DOI: 10.3390/ani11020571

30. Nair A, Chauhan P, Saha B, Kubatzky KF: Conceptual evolution of cell signaling. *Int J Mol Sci,* 20 (13): 3292-3302, 2019. DOI: 10.3390/ijms20133292

31. Servy EJ, Jacquesson-Fournols L, Cohen M, Menezo YJR: MTHFR isoform carriers. 5-MTHF (5-methyl tetrahydrofolate) vs folic acid: A key to pregnancy outcome: A case series. *J Assist Reprod Genet,* 35, 1431-1435, 2018. DOI: 10.1007/s10815-018-1225-2

32. Sanfins A, Rodrigues P, Albertini DF: *GDF-9* and *BMP-15* direct the follicle symphony. *J Assist Reprod Genet,* 35, 1741-1750, 2018. DOI: 10.1007/s10815-018-1268-4

33. Souza-Fabjan JMG, Batista RITP, Correia LFL, Paramio MT, Fonseca JF, Freitas VJF, Mermillod P: *In vitro* production of small ruminant embryos: Latest improvements and further research. *Reprod Fertil Dev,* 33 (2): 31-54, 2021. DOI: 10.1071/RD20206

34. Xiong X, Lan D, Li J, Lin Y, Zi X: Effects of zinc supplementation during *in vitro* maturation on meiotic maturation of oocytes and developmental capacity in yak. *Biol Trace Elem Res,* 185, 89-97, 2018. DOI: 10.1007/s12011-017-1217-6

35. Almansa-Ordonez A, Bellido R, Vassena R, Barragan M, Zambelli F: Oxidative stress in reproduction: A mitochondrial perspective. *Biology (Basel),* 9 (9): 269-281, 2020. DOI: 10.3390/biology9090269

36. Kowalczyk W, Waliszczak G, Jach R, Dulińska-Litewka J: Steroid receptors in breast cancer: Understanding of molecular function as a basis for effective therapy development. *Cancers,* 13 (19): 4779-1792, 2021. DOI: 10.3390/cancers13194779

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RESEARCH ARTICLE

Prognostic Value of Haptoglobin and Ceruloplasmin Levels Determined in Cows with Adhesive and Non-adhesive Traumatic Reticuloperitonitis

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Abstract: The aim of this study was to compare haptoglobin, ceruloplasmin, some macro mineral levels and hematological parameters in cow with adhesive and non-adhesive traumatic reticuloperitonitis (TRP). For this purpose, 20 cow with adhesive TRP constituted group 1 (G1), 20 cow with non-adhesive TRP constituted group 2 (G2), and 20 healthy cow formed the control group (C). Blood samples were taken from cow with clinical suspicion of TRP, and the inflammation status was examined by performing the glutaraldehyde test. As a result of radiographic inspection, it was decided to perform rumenotomy for the cows determined that the foreign body had penetrated the reticulum. The cows were divided into groups according to the presence or absence of adhesion during the rumenotomy. Total leukocyte count was found to be significantly higher in G1 compared to G2 and C. Calcium, magnesium, phosphorus and albumin were found to be significantly lower in G1 and G2 compared to the C (P<0.05). Haptoglobin and ceruloplasmin were determined to be at the highest level in G1 compared to the other groups (P<0.05). In this study, detection of positive acute phase proteins with higher levels of G1 and lower levels of albumin was found to be important. In conclusion, we think that these parameters will provide important information in determining the presence of adhesion in cows with TRP. In addition, according to the information obtained from the cow owners after rumenotomy, it was learned that the general health status of the cows in G1 was distressed. In the light of these data, the relevant parameters before rumenotomy can give an idea for prognostic evaluation in TRP.

Keywords: *Ceruloplasmin, Cow, Haptoglobin, Prognosis, Traumatic reticuloperitonitis*

Adeziv ve Non-adeziv Travmatik Retikuloperitonitisli İneklerde Haptoglobin ve Seruloplazmin Düzeylerinin Prognostik Değeri

Öz: Bu çalışmanın amacı adeziv ve non-adeziv retikuloperitonitis travmatika'lı (RPT) ineklerde haptoglobin, seruloplazmin, bazı makro mineral düzeylerinin ve hematolojik parametrelerin karşılaştırılması ve ayırıcı tanısal önemi belirlenmeye çalışılmıştır. Bu amaçla çalışmada adeziv RPT'li 20 inek grup 1 (G1), non-adeziv RPT'li 20 inek grup 2 (G2) ve sağlıklı 20 inek kontrol grubu (K) olarak belirlenmiştir. Klinik olarak RPT şüphesi olan sığırlardan kan örnekleri alınarak gluteraldehit testi ile yangı durumu incelendi. Radyografik görüntüleme ile tanı konulduktan sonra sığırlara rumenotomi yapıldı. Rumenotomi sırasında adezyon olup olmamasına göre sığırlar gruplara ayrıldı. Toplam lökosit sayısı G1'de G2 ve kontrole göre anlamlı yüksek bulundu. Kalsiyum, magnezyum, fosfor ve albumin seviyeleri G1 ve G2'de K grubuna göre önemli derecede düşük bulundu (P<0.05). Pozitif akuz faz proteinleri olan haptoglobin ve seruloplazmin diğer gruplara kıyasla G1'de en yüksek seviyede olduğu belirlendi (P<0.05). Bu çalışmada, G1'de pozitif akut faz proteinlerinin yüksek düzeyde ve albuminin ise daha düşük düzeyde saptanması önemli bulunmuştur. Sonuç olarak bu parametrelerin RPT'li ineklerde adezyon varlığının belirlenmesinde önemli bilgiler sağlayacağını düşünüyoruz. Ayrıca rumenotomi sonrası inek sahiplerinden alınan bilgilere göre G1'deki ineklerin genel sağlık durumlarının sıkıntılı olduğu öğrenildi. Bu veriler ışığında rumenotomi öncesi ilgili parametreler RPT'de prognostik değerlendirme için fikir verebilir.

Anahtar sözcükler: *Haptoglobin, İnek, Prognoz, Retikuloperitonitis travmatika, Seruloplazmin*

INTRODUCTION

Traumatic reticuloperitonitis (TRP) is a disease resulting in the development of various degrees of inflammation and complications due to penetration of sharp pointed foreign bodies ingested with feed to the reticulum [1-5]. The reasons for the frequent occurrence of TRP in cow are that they do not use their lips while taking feed in, they

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have no sense of detecting foreign body, they take their food voraciously and swallow it quickly without chewing in large masses, their sensitivity and sense of taste of the tongue is underdeveloped, their papillae on the tongue are turned backward, and their esophagus is wide and produce excessive saliva [4,6]. Many factors cause the formation of the disease including insufficient feeding, pica, penetrating objects randomly thrown into the environment and carelessness of the caregivers in preparing the feed. Foreign bodies coming into the reticulum after ingestion often puncture the reticulum wall and cause local or diffuse peritonitis. Sometimes, it causes more serious complications by sinking into the liver, kidney, spleen, lung and heart, causing various degrees of inflammation in these organs [3,7-10]. As a result of inflammation in the pathophysiology of TRP, the amount of fibrinogen, which is an acute phase protein, increases and will reveal the risk of adhesion formation [3]. As a result of these complications, many hematological and biochemical changes can occur in cow with TRP and serum calcium, magnesium, phosphorus and albumin levels are decreased. As a result of TRP, diffuse inflammation, intestinal malabsorption, liver dysfunction and anorexia can result in a decrease in negative acute phase protein albumin, calcium, magnesium and phosphorus concentrations [2,3,11-14]. Acute phase proteins are classified according to their increase or decrease after infection. Those that increase after infection are called positive acute phase proteins (e.g., haptoglobin and ceruloplasmin), those that decrease are called negative acute phase proteins (e.g., albumin) [15]. Acute phase proteins are proteins produced by the liver as a result of inflammation, tissue injury, infection, neoplastic growth or immunological disorders. The functions of these proteins include protecting the organism from further injury, clearing harmful molecules and residues for the organism, activating the repair process necessary for the organism to return to its normal function and restoring homeostasis. Although there are many acute phase proteins known today, important acute phase proteins for cattle include haptoglobin, serum amyloid A, fibrinogen, albumin, alpha1 acid glycoprotein. There is an increase in positive acute phase proteins and a decrease in negative acute phase proteins in cattle with TRP [3]. Many studies have been reported on the importance of acute phase proteins in different animal diseases. In calves with diarrhea [15], peripartum period sheep [16], Holstein cows [17], in neonatal calves with diarrhea [18], cattle with brucellosis [19], cows with endometritis [20], cattle with foot and mouth disease [21], *Hypoderma* spp. infested cattle [22], infectious respiratory system diseases complex in cattle [23], normal and power-bearing cows [24], sheep infected with *Streptococcus pluranimalium* [25], calves with pneumonia [26] acute phase proteins have been investigated on different animals and diseases.

We planned to conduct this study to determine which operation or platform treatment should be preferred according to the level of acute phase proteins determined in TRP and to determine the importance of measuring the level of acute phase proteins in this decision. In this study, it was aimed to compare the haptoglobin, ceruloplasmin and some macro mineral levels of the cow in the adhesion-formed TRP group compared to the cow in both the control group (C) and the non-adhesion group, and also to evaluate these parameters in terms of diagnosis.

Material and Methods

Ethical Statement

This study was conducted by the approval of the Local Ethics Board for Animal Experiments of Kafkas University (KAU-HADYEK), Research Code: KAU-HADYEK-2021/103), Kars, Türkiye.

Animals

In the study, 20 cows with adhesive TRP, 3-6 years old, Simmental, with clinical complaints of anorexia, pain, depression, hunched posture were diagnosed as TRP following clinical and radiographic examinations. Cases were brought to the clinic between 3-7 d after clinical symptoms appeared. A total of 60 cows have similar clinical symptoms, 20 with adhesive TRP in group 1 (G1), 20 with non-adhesive TRP in group 2 (G2), and 20 healthy cows in the C, were included. In our study, 65% of the cows with TRP gave birth in the last period of pregnancy and 35% of them gave birth recently. C group animals consist of cows of the same age, breed and characteristics, brought from the same barns to our faculty clinic for control purposes. Healthy animals consist of cows of the same age, breed and characteristics, brought to our faculty clinic for control purposes, in the same barn conditions as the sick ones. Since approximately 65% of the cows in the TRP groups were pregnant at last term, 65% of the cows in the C group were also selected from those in the last term. Adhesion was determined during the rumenotomy.

Blood Samples

All cows were blood sampled once by withdrawing 10 mL blood from the jugular vein into serum tubes with gel (BD Vacutainer®, BD, UK) and tubes with K_2EDTA (BD Vacutainer®, BD, UK). Blood samples taken for serum were kept at room temperature for about one hour and centrifuged at 3000 RPM for ten min (Hettich Rotina 380R®, Hettich, Germany). All blood samples were stored at -20°C until analysis.

Biochemical and Hematological Analyses

Blood samples in K_2EDTA were assessed for total leukocyte count (WBC $x10^3/\mu L$) and other hematological parameters

using a complete blood cell count device (VG-MS4e®, Melet Schloesing, France). Complete blood cell count was measured within 15 min immediately after blood collection. Serum calcium, phosphorus, and magnesium were measured with a fully automatic biochemistry device (Mindray BS120®, Mindray Medical Technology, Türkiye). Haptoglobin was determined as reported by Skinner et al.[27], ceruloplasmin was measured by the method of Colombo and Richterich [28], and albumin was measured colorimetrically on device (Epoch, Biotek, USA) using a commercial test kit (Biolabo, France).

Glutaraldehyde Test

The test was performed by mixing 2 mL of blood and 2 mL of 1.4% glutaraldehyde solution. The mixture was put into a 10 mL glass tube. It was turned upside down at 30 sec intervals. Coagulation within 15 min was considered positive. Test result was considered as clotting between 0-5 min severe, 5-10 min moderate and 10-15 min mild inflammation. Coagulations longer than 15 min were considered normal [29].

Radiography

Radiographic images of patients with suspected TRP were taken for definitive diagnosis. Reticulum and diaphragm border were evaluated in radiological examination. Dynamic brand ceiling static x-ray device and FCR prima brand (Fujifilm FCR Prima T2 Veterinary Set®, Medical Technology, Türkiye) imaging unit were used in the radiological evaluation. For this purpose, irradiation doses between 20-40 mA and 80-95 kW were adjusted according to the size of each cow. Radiographic images were taken by irradiation at a distance of 75 cm between the tube and the cassette. Then, the reticulum, diaphragmatic border and chest cavity were examined in detail for the presence of foreign body.

Rumenotomy

Rumenotomy was performed in the patients with foreign body detected as a result of radiological as well as other clinical examinations. The left fossa paralumbalis was prepared for the operation. After shaving and disinfection of the operation area (70% ethyl alcohol + 10% povidone iodine), local infiltrative (reverse L) anesthesia was applied using lidocaine (Vilcain®, Vilsan, Türkiye). Following anesthesia, a 12-15 cm long straight incision was made from the proximal to the distal 7-8 cm caudal to the last ribs, 5-6 cm below the processus transversus of the lumbar vertebrae. After incising the skin, subcutaneous connective tissues, muscles and peritoneum, the rumen were exposed. Before the rumen was opened, the abdominal cavity was examined in detail by inspection and palpation (trans-peritoneal exploration) for adhesions. Then the foreign body was removed from the reticulum by performing routine rumenotomy and all incised layers were closed using appropriate suture materials. Postoperative medications were prescribed and the patient was discharged.

Statistical Analysis

Data were given as mean ± standard error of mean (SEM). The groups were in accordance with the normal distribution according to the histogram, Q-Q graph method and Shapiro-Wilk test. The one-way ANOVA test was used for multiple comparisons of the groups, and the Tukey HSD test was used for post-hoc comparisons. SPSS (SPSS Version 26.0®, Chicago, IL, USA) program was used for all statistical analyses. The differences between the groups in terms of the parameters examined were considered significant at the $P<0.05$ level.

Results

Clinically, anorexia, depression, hunched posture, abdominal pain and groaning were determined in cows with TRP. In addition, 14 cows in G1 were pregnant for 6 months or more, and 6 of them gave birth within 10 d to 3 months. 12 cows in the G2 group were pregnant for 6 months or more, and 8 of them gave birth within 10 days to 3 months. Physical examination findings (rectal temperature, respirations per minute and pulse rate) of all cows in the study were given in the *Table 1*. A partial increase was observed in the mean rectal temperature, respiratory and pulse rates per minute of cows in G1 and G2 compared to the C. While the total leukocyte, neutrophil, and lymphocyte counts were found to be high in TRP, the hemoglobin and red blood cell counts were found to be low *(Table 1)*. Serum calcium, phosphorus, magnesium, albumin, haptoglobin and ceruloplasmin levels of cows in all groups were shown in *Fig. 1*. While calcium, magnesium, phosphorus and albumin levels were found to be lower in the patient groups compared to the C ($P<0.05$, *Fig. 1-A,B,C,D*), positive acute phase proteins haptoglobin and ceruloplasmin were found to be significantly higher ($P<0.001$, *Fig. 1-E,F*).

Glutaraldehyde Test Findings

Glutaraldehyde test revealed very severe inflammation in 16 cows, moderate inflammation in 3 cows, and mild inflammation in 1 cow in G1. Six of the cows had very severe inflammation, 9 had moderate inflammation, and 5 had mild inflammation in G2. C had tested normal with glutaraldehyde.

Radiological Findings

In the radiological examination, it was observed that the foreign body penetrated the reticulum and passed into the abdominal cavity in 23 of the patients. Of these, 15 were in the direction of the base of the reticulum, and 8 of them were in the direction of the diaphragm *(Fig. 2-A)*. In 6 of

Table 1. *Hematology and physical examination findings of cow with traumatic reticuloperitonitis and control group*

Parameters	Groups (Mean ± SEM)			P Value
	G1	G2	C	
Body temperature (°C)	38.67±0.28	38.43±0.26	38.23±0.12	0.364
Breaths/min.	27.80±1.82	29.70±6.11	19.60±0.88	0.140
Heart beats/min.	75.40±4.60	73.80±5.90	65.60±1.78	0.263
Total leukocytes count (x10^3/μL)	23.55±1.90^a	13.00±1.56^b	8.35±0.50^b	<0.001
Lymphocytes (%)	46.31±9.34^{ab}	25.28±2.28^b	48.18±5.32^a	0.030
Monocytes (%)	4.41±0.73^{ab}	2.22±0.30^b	6.89±1.47^a	0.008
Granulocytes (%)	49.28±9.95^{ab}	72.50±2.47^a	44.89±6.47^b	0.021
Lymphocytes count (x10^3/μL)	11.20±2.74^a	2.78±0.39^b	4.03±0.55^b	0.002
Monocytes count (x10^3/μL)	1.11±0.23^a	0.26±0.03^b	0.59±0.15^{ab}	0.004
Granulocytes count (x10^3/μL)	11.24±2.66^a	9.66±1.38^{ab}	3.73±0.57^b	0.013
Red blood cell count (x10^6/μL)	6.16±0.41^b	8.18±0.75^a	7.77±0.48^{ab}	0.042
Mean red cell volume (fL)	45.97±2.31	51.64±1.49	43.50±2.88	0.052
Hematocrit (%)	28.18±2.36^b	42.37±4.45^a	32.20±1.90^{ab}	0.010
Mean erythrocyte hemoglobin (pg)	13.28±0.56^b	15.18±0.49^a	13.85±0.42^{ab}	0.033
Mean erythrocyte hemoglobin volume (g/dL)	29.24±0.93^b	29.57±0.92^b	34.61±1.84^a	0.011
Erythrocyte distribution width (fL)	14.02±0.65	12.36±0.27	13.74±0.47	0.052
Hemoglobin (g/dL)	8.19±0.64^b	12.27±0.98^a	10.30±0.49^{ab}	0.002
Platelet count (x10^3/μL)	568.10±72.20	840.50±172.93	525.40±103.44	0.167
Mean platelet volume (fL)	6.91±0.08	6.72±0.07	6.86±0.09	0.249
Platelets (%)	0.39±0.05	0.57±0.12	0.43±0.07	0.310
Platelet distribution width (fL)	6.81±0.11	6.48±0.22	6.22±0.21	0.100

$^{a, b}$ The mean values with different letters in the same line represent the difference between patient and control groups (P<0.05). The number of cow in all groups is 20; ***SEM:*** *Standard error of mean;* ***G1:*** *Adhesive traumatic reticuloperitonitis group;* ***G2:*** *Non-adhesive traumatic reticuloperitonitis group;* ***C:*** *Control group*

the other 17 cases, foreign bodies were observed within the borders of the reticulum while attached to the magnet; In addition, when the cases were examined in terms of the diaphragm border, it was observed that the diaphragm border was irregular in patients with a foreign body penetrating the reticulum and passing into the abdominal cavity. Abscession was observed at the diaphragm border in 3 of the cases. In non-adhesive cases, it was observed that the foreign body was embedded in the surface of the reticulum or appeared free, and the borders of the diaphragm and reticulum were regular *(Fig. 2-C)*.

Intraoperative Findings

When the abdominal cavity was examined during the operation, it was observed that the adhesions were very severe in cases in which foreign bodies penetrating the reticulum and passed into the abdominal cavity *(Fig. 2-B)*. On the other hand, adhesions were found to be more minimal in cases in which foreign bodies penetrated the reticulum but did not pass into the abdominal cavity, and in cases in which foreign bodies were found to appear free in the reticulum. It was observed that the border of the diaphragm and reticulum was regular in non-adhesive cases where the foreign body was embedded in the surface of the reticulum or the foreign body appeared to be free in the reticulum *(Fig. 2-D)*. It was remarkable that abdominal adhesions were very severe in patients with positive glutaraldehyde test in clinical examinations. In G1, the clinical symptoms were more severe, adhesion was evident on radiographic imaging, severe inflammation was found in the glutaraldehyde test, and the health status of the majority was poor in the information obtained after rumenotomy. In the first week after rumenotomy, cow owners were called and their health status was learned. According to the information given by the cow owners, it was reported that the general health status of 60% (12 to 20) of G1 cows and 15% (3 to 20) of G2 cows were deteriorated. In our study, the majority of foreign bodies causing TRP were wires (72.5%), and nails (17.5%) and other metal objects (10%) were less common.

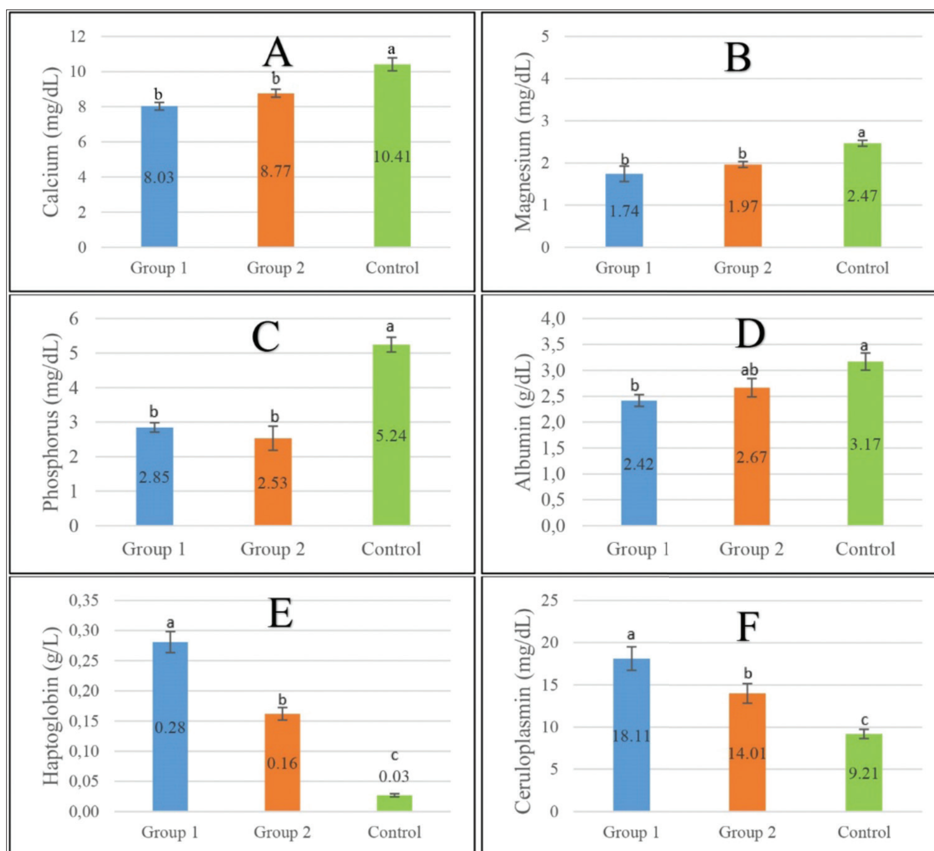

Fig 1. Comparison of mean values of serum calcium, magnesium, phosphorus, albumin, haptoglobin and ceruloplasmin levels of group 1, group 2 and control group cow. **A.** Comparison of mean serum calcium level of cow in group 1, group 2 and control group (P<0.001), **B.** Comparison of mean serum magnesium level of group 1, group 2 and control group cow (P<0.001), **C.** Comparison of mean serum phosphorus level of group 1, group 2 and control group cow (P<0.001), **D.** Comparison of mean serum albumin level of group 1, group 2 and control group cow (P=0.006), **E.** Comparison of mean serum haptoglobin level of group 1, group 2 and control group cow (P<0.001), **F.** Comparison of mean serum ceruloplasmin level of group 1, group 2 and control group cow (P<0.001). Group 1: Adhesive traumatic reticuloperitonitis group. Group 2: Non-adhesive traumatic reticuloperitonitis group

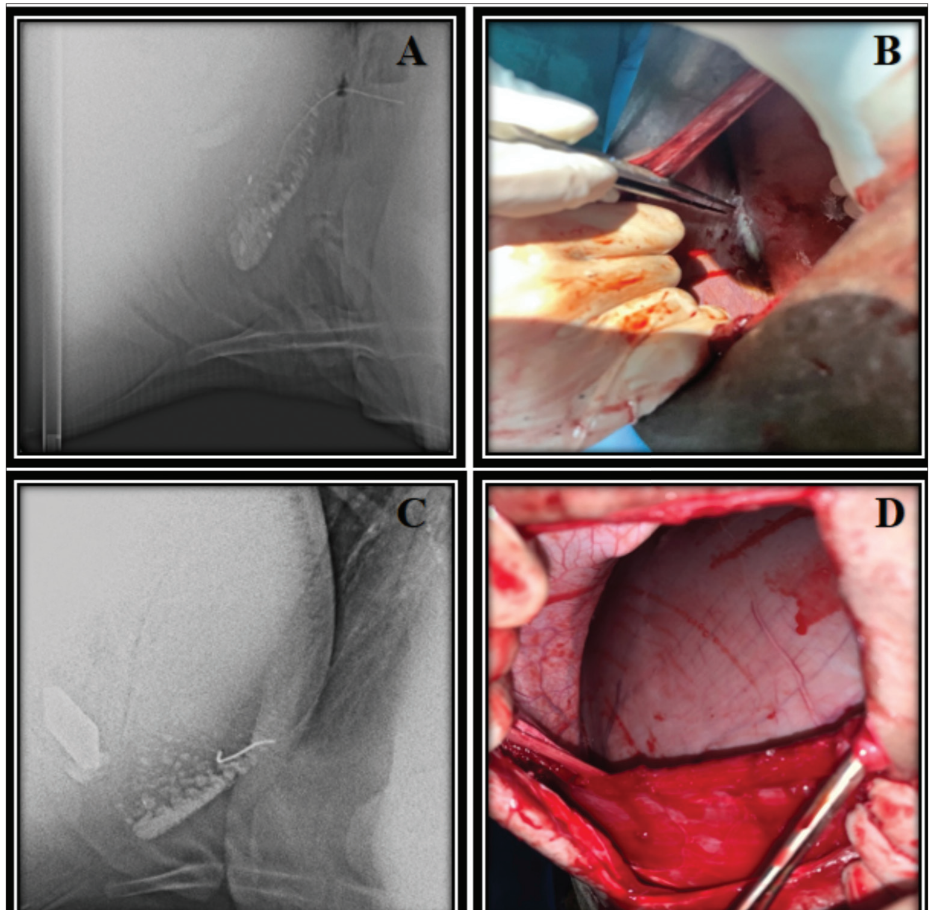

Fig 2. Radiographic and rumenotomy images of cows with adhesive and non-adhesive traumatic reticuloperitonitis. **A.** Radiographic image of a cow with adhesive traumatic reticuloperitonitis. Radiographic image of the foreign body penetrating the reticulum and traveling in the direction of the diaphragm and the image of the abscess at the diaphragm border, **B.** Rumenotomy image of a cow with adhesive traumatic reticuloperitonitis, **C.** Radiographic image of a cow with non-adhesive traumatic reticuloperitonitis. In non-adhesive cases, it was observed that the foreign body was embedded in the surface of the reticulum or appeared free, and the borders of the diaphragm and reticulum were regular, **D.** Rumenotomy image of a cow with non-adhesive traumatic reticuloperitonitis. It was observed that the border of the diaphragm and reticulum was regular in non-adhesive cases where the foreign body was embedded in the surface of the reticulum or the foreign body appeared to be free in the reticulum

Discussion

The fact that 65% of the cows with TRP in our study were at the last period of term and 35% had just given birth supports the information that foreign bodies are taken and penetrate with more feed in this period [3,6]. In the present study, symptoms such as loss of appetite, weight loss, deterioration of general condition, symptoms of pain, signs of indigestion, hunched posture, and reluctance to move were also reported in previous studies in cows with TRP [3,9,30,31]. Studies have reported that metallic objects causing TRP are wires and nails, and often the cranioventral of the reticulum where it sinks [2,3,30]. In animals with TRP, serious changes occur in blood parameters in the acute period. Initially, a severe leukocytosis occurs depending on the severity and the complexity of the infectious agents [3,11]. In our study, leukocytosis in G1 and G2 was also present. In addition, a significant increase was detected in the granulocyte, monocyte and lymphocyte counts in G1 compared to the other groups, while a decrease was observed in the platelet, erythrocyte, hemoglobin and hematocrit percentages. Leukocytosis and anemia might have been the result of TRP related inflammation and bleeding. Studies have reported that the glutaraldehyde test in TRP groups gives positive results from inflammation compared to healthy groups [1,3]. In our study, we think that the reason why the total leukocyte counts were higher in G1 than in G2 was directly proportional to the severity of the inflammation. Depending on the severity of inflammation, the amount of neutrophils in the blood may increase. The fact that the glutaraldehyde test showed more severe inflammation in G1 than in G2 confirms this.

Changes in serum mineral levels were determined in previous studies in cows with TRP [3,14]. TRP causes inappetence food intake and digestive system disorders [32], and thus resulting in decrease of serum calcium and phosphorus levels [3,12,33]. In the presented study, serious decreases in serum calcium, phosphorus and magnesium levels were found in the sick groups as a result of anorexia, dilation of the digestive system due to peritonitis, and ruminal stasis. In addition, the decreased serum calcium level might be associated with hypoalbuminemia [2,3,11]. In addition, since the advanced pregnancy rate in the TRP groups was approximately 65% and the cows in the K group had similar pregnancy status, they were included in the study. In this way, the effect of pregnancy status on mineral levels was minimized.

Acute phase proteins are proteins synthesized by the liver in response to inflammation, tissue damage, infection, and neoplastic growth. The amount of serum albumin may decrease as a result of the destruction caused by infections and inflammation [15,33,34]. In our study, serious decreases were observed in G1 and G2 as the total leukocyte count was higher than the C, positive glutaraldehyde test indicated severe inflammation, and food intake was also stopped, as it could cause disruption in the production of albumin, which is a negative acute phase protein. Haptoglobin levels increase in cases of acute infection, inflammation, and trauma [15], while decreases are observed in recovery or chronic stages [9,35]. It has been reported that increased haptoglobin levels in cow with TRP is due to the continuation of the traumatic situation, stress and surgery [9,35]. Stress can increase even more in the disease [36]. In our study, haptoglobin levels were significantly higher in the sick groups, especially in G1, compared to the C. This increase might have been the result of the traumatic effect of TRP, developing peritonitis, stress and severe inflammation. In addition, the fact that the glutaraldehyde test indicates severe inflammation, the high total leukocyte counts and the observation of serious adhesions during the operation in G1 support this increase in haptoglobin. Ceruloplasmin is used less frequently for diagnostic purposes compared to other acute phase proteins. It protects cells against oxidative damage and has cytoprotective activity [15,37]. It is used in determining the presence of infection and inflammation [19]. In this study, serum ceruloplasmin level may have increased in cows with TRP to protect cells more as a result of peritonitis and severe inflammation. This may also be the case in our study as inflammation was more severe in G1 and the ceruloplasmin levels accordingly increased more in G1.

In conclusion, haptoglobin and ceruloplasmin levels were very high in G1, in whom adhesion was present, and calcium, magnesium, phosphorus and albumin levels were lower than the other groups. The evaluation of these parameters before the operation in cows diagnosed with TRP may provide important information in terms of the presence of adhesion, the severity of the inflammation, the chance of success of the operation and the diagnostic value. We think that these parameters will provide important information in the prognostic evaluation before rumenotomy in TRP.

Availability of Data and Materials

The datasets analyzed during the current study are available from the corresponding author E. Akyüz) on reasonable request.

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Ethical Statement

This study was conducted by the approval of the Local Ethics Board for Animal Experiments of Kafkas University (Research Code: KAU-HADYEK- 2021/103), Kars, Türkiye.

Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

Author Contributions

EA and UA conceived the study. EA and UA collected and analyzed data. EA performed laboratory analyzes and complete blood count procedure. UA performed the radiographic examination and rumenotomy operation. EA and UA have approved and read the final version of the manuscript.

References

1. Gökce G, Özba B, Gökce HI, Erdogan HM, Kırmızıgül AH, Güneş V, Atalan G: Sığırlarda retikuloperitonitis travmatika'nin teşhisinde tripsin inhibitör (Tİ) testinin kullanımı. *Kafkas Univ Vet Fak Derg,* 5 (1): 19-24, 1999.

2. Ward JL, Ducharme NG: Traumatic reticuloperitonitis in dairy cattle. *J Am Vet Med Assoc,* 204 (6): 874-877, 1994.

3. Bozukluhan K, Gökçe Hİ: Retikuloperitonitis travmatika ve retikuloperikarditis travmatikalı sığırlarda bazı akut faz proteinlerin araştırılması. *Erciyes Univ Vet Fak Derg*, 4 (2): 107-113, 2007.

4. Braun U, Gerspach C, Ohlerth S, Warislohner S, Nuss K: Aetiology, diagnosis, treatment and outcome of traumatic reticuloperitonitis in cattle. *Vet J,* 255:105424, 2020. DOI: 10.1016/j.tvjl.2020.105424

5. de Souza VL, Pinto AMSV, Queiroz CRR, de Castro MB, Borges JRJ, Soto-Blanco B, Camara ACL: Traumatic reticuloperitonitis combined with embolic pneumonia and hepatitis as unusual symptoms of foreign body syndrome in a Holstein bull. *Vet Med Sci,* 7, 136-140, 2021. DOI: 10.1002/vms3.341

6. Gül Y: Retikuloperitonitis travmatikanın tanı-ayırıcı tanı ve tedavisindeki son gelişmeler. *FÜ Sağ Bil Vet Derg,* 31 (1): 59-66, 2017.

7. Roth L, King JM: Traumatic reticulitis in cattle: A review of 60 fatal cases. *J Vet Diagn Invest*, 3, 52-54, 1991. DOI: 10.1177/104063879100300111

8. Radostits OM, Blood DC, Gay CC: Traumatic reticuloperitonitis. **In,** Radostits OM, Blood DC, Gay CC (Eds): Veterinary Medicine: A Textbook of the Diseases of Cattle, Sheep, Pigs, Goats and Horses. 8th ed., 278-284, WB Saunders, London, 1994.

9. Kirbas A, Ozkanlar Y, Aktas MS, Ozkanlar S, Ulas N, Erol HS: Acute phase biomarkers for inflammatory response in dairy cows with traumatic reticuloperitonitis. *Isr J Vet Med,* 70 (2): 23-29, 2015.

10. Akyuz E, Aydin U: Clinical biochemistry and hematology in cattle with reticulo-peritonitis traumatic causing complications in the lung-case series-oral presentation. *2nd International Cukurova Agriculture and Veterinary Congress,* 4-5 January, Adana - Türkiye, 2021.

11. Şahal M, Güzel N, Kaya Ü, Bilgili H, Tanyel B: Retikuloperitonitis travmatikalı süt ineklerinde pre- ve post operatif klinik ve biyokimyasal değişiklikler. *Ankara Univ Vet Fak Derg,* 40, 261-80, 1993. DOI: 10.1501/Vetfak_0000001495

12. Roussel AJ, Whitney MS, Cole D: Interpreting a bovine serum chemistry profile: Part: 1. *Vet Med*, 92 (6): 553-558, 1997.

13. Balıkçı E, Günay C: Retikuloperitonitis travmatikalı sığırlarda rumenotomi öncesi ve sonrası bazı klinik, hematolojik, biyokimyasal ve elektrokardiyografik bulguların karşılaştırılması. *FÜ Sağ Bil Derg,* 18 (1): 13-19, 2004.

14. Gökce HI, Gökçe G, Cihan M: Alterations in coagulation profiles and biochemical and haematological parameters in cattle with traumatic reticuloperitonitis. *Vet Res Commun*, 31, 529-537, 2007. DOI: 10.1007/s11259-007-3527-1

15. Erkılıç EE, Merhan O, Kırmızıgül AH, Öğün M, Akyüz E, Çitil M: Salivary and serum levels of serum amyloid a, haptoglobin, ceruloplasmin and albumin in neonatal calves with diarrhoea. *Kafkas Univ Vet Fak Derg*, 25 (4): 583-586, 2019. DOI: 10.9775/kvfd.2018.21424

16. Merhan O, Özcan A: Peripartum dönemdeki koyunlarda seruloplazmin, haptoglobin, fibrinojen, albümin ve transferrin düzeylerinin araştırılması. *Erciyes Univ Vet Fak Derg*, 7 (1): 13-20, 2010.

17. Kuru M, Merhan O, Kaya S, Oral H, Kükürt S: The effect of short term progesterone-releasing intravaginal device treatment on acute inflamation markers for Holstein heifers. *Revue Méd Vét*, 166 (11): 336-340, 2015.

18. Merhan O, Bozukluhan K, Gökçe G, Yılmaz O: İshalli buzağılarda haptoglobin, seruloplazmin ve bazı biyokimyasal parametre düzeylerinin araştırılması. *FÜ Sağ Bil Vet Derg*, 30 (3): 195-198, 2016.

19. Bozukluhan K, Merhan O, Büyük F, Çelebi Ö, Gökçe G: Brusellozisli sığırlarda bazı akut faz proteinlerin düzeyinin saptanması. *Ankara Univ Vet Fak Derg*, 63: 13-16, 2016. DOI: 10.1501/Vetfak_0000002703

20. Kaya S, Merhan O, Kacar C, Colak A, Bozukluhan K: Determination of ceruloplasmin, some other acute phase proteins, and biochemical parameters in cows with endometritis. *Vet World*, 9 (10): 1056-1062, 2016. DOI: 10.14202/vetworld.2016.1056-1062

21. Merhan O, Bozukluhan K, Kiziltepe S, Gokce HI: Investigation of levels of haptoglobin, serum amyloid A, ceruloplasmin and albumin in cattle with foot-and-mouth disease. *Isr J Vet Med*, 72 (4): 14-17, 2017.

22. Merhan O, Bozukluhan K, Gokce HI: Acute phase proteins and biochemical and oxidative stress parameters in *Hypoderma spp.* infested cattle. *J Hell Vet Med Soc*, 68 (4): 535-540, 2017. DOI: 10.12681/jhvms.16049

23. Yilmaz O, Gökçe G: Sığırlarda enfeksiyöz solunum sistemi hastalıkları kompleksinde (BRDC) klinik, hematoloji, biyokimya, oksidatif stres, akut faz proteinler üzerinde araştırmalar. *Atatürk Üniversitesi Vet Bil Derg,* 12 (1): 34-44, 2017. DOI: 10.17094/ataunivbd.309771

24. Bayyit E, Merhan O: Normal ve güç doğum yapan ineklerde bazı akut faz proteinlerinin ve oksidatif stres düzeyinin belirlenmesi. *Atatürk Üniversitesi Vet Bil Derg*, 15 (2): 145-150, 2020. DOI: 10.17094/ataunivbd.688400

25. Kırbaş A, Kaman R, Merhan O, Bozukluhan K, Değirmençay S, Eroğlu MS: Evaluation of acute phase response and oxidative damage in sheep naturally infected with *Streptococcus pluranimalium* using haptoglobin, nitric oxide, and malondialdehyde levels. *FÜ Sağ Bil Vet Derg*, 35 (3): 145-150, 2021.

26. Bozukluhan K, Merhan O, Kiziltepe S, Egritag HE, Akyuz E, Gokce HI: Determination of haptoglobin, some biochemical and oxidative stress parameters in calves with pneumonia. *Fresenius Environ Bull,* 30 (7): 9492-9496, 2021.

27. Skinner JG, Brown RA, Roberts L: Bovine haptoglobin response in clinically defined field conditions. *Vet Rec*, 128, 147-149, 1991. DOI: 10.1136/vr.128.7.147

28. Colombo JP, Richterich R: On the determination of ceruloplasmin in plasma. *Schweiz Med Wochenschr*, 23, 715-720, 1964.

29. Turgut K: Disproteinler ve testleri. **In,** Turgut K (Ed): Veteriner Klinik Laboratuvar Teşhis. 2th ed., 505-506, Bahçıvanlar Yayınevi, Ankara, 2000.

30. Samad A, Awaz KB, Sarkate LB: Diagnosis of bovine traumatic peritonitis I: Strength of clinical signs in predicting correct diagnosis. *J Appl Anim Res*, 6, 13-18, 1994. DOI: 10.1080/09712119.1994.9706019

31. Aiello SE: Traumatic reticuloperitonitis, hemostatic disorders, haematologic reference ranges. **In,** Aiello SE (Ed): The Veterinary Merck Manual. 8th ed., 1-2305, Merck & Co, Pennsylvania, 1998.

32. Gökçe G, Şendil Ç, Sural E: 1996 yılında Kafkas Üniversitesi Veteriner Fakültesi Kliniklerine getirilen hayvanların iç hastalıklarının istatistiksel değerlendirmesi. *Kafkas Univ Vet Fak Derg*, 3, 181-186, 1996.

33. Akyüz E, Gökce G: Neopterin, procalcitonin, clinical biochemistry, and hematology in calves with neonatal sepsis. *Trop Anim Health Prod,* 53:354, 2021. DOI: 10.1007/s11250-021-02779-z

34. Talkhan OFA, Salama SEM, Kholy MME, Mosallam SA, Atwa EI: Bacterial agent of respiratory manifestation in cattle and associated biochemical alterations in Menoufea Governorate. *J Nat Sci*, 7 (9): 26-30, 2009.

35. Petersen HH, Nielsen JP, Heegard PMH: Application of acute phase protein measurements in veterinary clinical chemistry. *Vet Res,* 35, 163-187, 2004. DOI: 10.1051/vetres:2004002

36. Akyüz E, Makav M, Kuru M, Bayram P: The effect of *Tarantula cubensis* extract on gentamicin-induced acute kidney injury in ovariectomized rats. *Kafkas Univ Vet Fak Derg*, 27 (5): 559-566, 2021. DOI: 10.9775/kvfd.2021.25390

37. Coşkun A, Şen İ: Sığırlarda akut faz proteinleri ve klinik kullanım alanları. *Sağlık Bil Derg*, 20 (3): 240-246, 2011.

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Research Article

Association Between Virulence Genes and Serovars, Sequence Types of *Glaesserella (Haemophilus) parasuis* Isolates from the Nasal Cavity of Live Piglets

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Abstract: This study analyzed the 19 virulence genes (VGs) of 117 *Glaesserella (Haemophilus) parasuis (G. parasuis)* isolates from the nasal cavities of live piglets from the south of China and assessed the associations between VGs and serovars, sequence types (STs) of these isolates. The detection rate of 19 VGs ranged from 1.7% to 95.2%, with *vacJ* and *clpP* (95.7%) as the most prevalent. Of the 117 *G. parasuis* isolates, 105 were assigned to ten distinct serovars (1, 2, 4-10 and 15), and twelve of the isolates tested were non-typable (NT). The serovar 10 (17.9%) was the most prevalent. The *G. parasuis* isolates belonging to the same ST and serovar harbored different VGs, and all isolates exhibited considerable genetic heterogeneity. Significant correlations were found between VGs and serovars, different pathogenic serovar groups, and members of clade 2 (based on ST). The results complement epidemiological data of *G. parasuis* and will help the scientific community understand the extreme genetic diversity and pathogenesis of *G. parasuis,* which will aid in the development of *G. parasuis* vaccines.

Keywords: *Glaesserella (Haemophilus) parasuis, Virulence gene, Serovar,* Sequence type*, Live piglet*

Canlı Domuz Yavrularının Burun Boşluğundan İzole Edilen *Glaesserella (Haemophilus) parasuis*'in Virülans Genleri İle Serovar ve Sekans Tipleri Arasındaki İlişki

Öz: Bu çalışmada, Çin'in güneyinde canlı domuz yavrularının burun boşluklarından elde edilen 117 *Glaesserella (Haemophilus) parasuis (G. parasuis)* izolatının 19 virülans geni (VG'ler) analiz edildi ve VG'ler ile serovarlar ve sekans tipleri (ST'ler) arasındaki ilişki değerlendirdi. 19 VG'nin pozitiflik oranı %1.7 ile %95.2 arasında değişmekte olup, en yaygın (%95.7) *vacJ* ve *clpP* genleri saptandı. 117 *G. parasuis* izolatının 105'i on farklı serovar (1, 2, 4-10 ve 15) içerisinde yer alırken, test edilen izolatlardan 12'si serotiplendirilemedi (NT). Serovar 10 (%17.9) en yaygın olanıydı. Aynı sekans tipi ve serovara ait olan *G. parasuis* izolatları farklı VG'ler barındırır iken, tüm izolatlar önemli ölçüde genetik heterojenite sergiledi. VG'ler ile serovarlar, farklı patojenik serovar grupları ve ST tabanlı monofiletik grup 2 (klad 2) üyeleri arasında önemli korelasyonlar saptandı. Bulgular, *G. parasuis*'in epidemiyolojik özelliklerini tamamlamakta olup, bilim camiasına, *G. parasuis* etkenine karşı aşı geliştirilmesine katkı sağlayacak geniş genetik çeşitliliğinin ve patogenezinin aydınlatılması yönünde yardımcı olacaktır.

Anahtar sözcükler: *Glaesserella (Haemophilus) parasuis, Virülans gen, Serovar, Sekans tipi, Canlı domuz yavrusu*

Introduction

Glaesserella (Haemophilus) parasuis (G. parasuis), the pathogen that causes Glässer's disease, has brought huge economic losses to the global swine industry [1,2]. *G. parasuis* is a commensal bacterium in the swine upper respiratory tract that contains strains ranging from non-virulent to highly virulent. Virulent strains can invade and cause systemic disease under certain conditions [3-5].

To date, 15 serovars have been identified, in addition to some non-typable (NT) strains [6,7]. Serovar identification of the isolates is the basis for designing vaccination programs [8]. Some earlier studies suggested that *G. parasuis* serovars were virulence markers and could be divided into three pathogenic groups [2]. However, later studies found that isolates allocated into non-pathogenic serovars can also cause disease, and virulence of the isolates allocated to the same serovar can vary greatly [9-11]. Thus, it remains

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unclear whether serovar can be used as a marker of virulence in *G. parasuis*.

It is generally believed that a single virulence gene (VG) may not be a decisive factor in triggering the pathogenesis of multifactorial diseases such as Glässer's disease, and the pathogenesis of bacteria often depends on the interaction and expression regulation of many VGs. Thus, a comprehensive analysis of VGs in clinical isolates may be helpful to predict the pathogenicity of novel *G. parasuis* isolates as they are identified. Although the characteristics of *G. parasuis* isolates from clinical cases have been extensively studied, an in-depth analysis of *G. parasuis* isolates from the swine upper respiratory tract has not been performed. In this study, we analyzed the characteristics, including serovars and VGs, of *G. parasuis* isolates from the nasal cavities of live piglets in the south of China. Our results provide more information on the epidemiology and pathogenesis of *G. parasuis*.

Material and Methods

Identification and Serotyping

Nasal swabs were collected from the nasal cavities of live piglets without obvious clinical symptoms of Glässer's disease between 2007 and 2016 in three provinces (Guangdong, Jiangxi, and Shanghai) in the south of China. Nasal swabs were inoculated on blood agar medium with 0.0025% of NAD immediately after sampling. Suspect *G. parasuis* colonies were identified by NAD-dependency and 16S rRNA PCR [12]. The isolates underwent molecular serotyping via a multiplex PCR assay described in Howell et al.[13].

Table 1. *Primers used to amplify VGs*

VGs	Primers	Sequence (5'→3')	Product Size
hhdA	*hhdAF*	GGTTCTAGTTCACAAACAGCCAATAC	964
	hhdAR	GATATTTACCCCTGCCTTCATTGTATC	
hhdB	*hhdBF*	ATCTTGCCCTGATTAGAGAGTAGGAGT	557
	hhdBR	GTGAATATAGCCCTTATCCAAATAGGC	
fhuA	*fhuAF*	ATGGTTTGGTTGTAATGGAGTATC	563
	fhuAR	AACAACGCCAGCTAGGCTTGTACT	
vta1	*vta1F*	TTTAGGTAAAGATAAGCAAGGAAATCC	406
	vta1R	CCACACAAAACCTACCCCTCCTCC	
wbgY	*wbgYF*	TTAGGGCTTGTCGCCCTATTTC	380
	wbgYR	GAAGCACTATCTGTAATACCAGGC	
fimB	*fimBF*	CTAAGAGAGAGCAGGGCGATAGAA	386
	fimBR	TGTCACCACAATGGCTCAGGTTGA	
hsdR	*hsdRF*	GCAAGCTTACTCTCGTACTAACCG	410
	hsdRR	AGGCTCCACTAGGTTCTTCTACTC	
nhaC	*nhaCF*	CATATTGTGGTACAAGGTGGCGAG	415
	nhaCR	CTAATACGGAAGTCACTGTACCGC	
H0254	*H0254F*	CAGTGAAAGTCGTGATGTGGAACC	397
	H0254R	GGACGTTCGTTCACATCTTGTTCG	
capD	*capDF*	CGAAGGGAGTGTTTCTATCA	958
	capDR	GAGTTTCTCACCAGGTCTAA	
rfaE	*rfaEF*	GCAGGGCGAGCGTTGGATAA	524
	rfaER	TGGGTCGGTAAATGGAATGG	
lsgB	*lsgBF*	ATGAATTTGATTATTTGTATGACTCCATTT	969
	lsgBR	CTATTGGCATGTGTAGTCAATTACTTC	
HPM1370	*HPM1370F*	ATGCTAAAAAGAGTGTTTGATATTTTC	540
	HPM1370R	TATATTATGATTAACATAATC	
HPM1371	*HPM1371F*	ATGAACTTTCTACCATTCGCCCTTCCCG	520
	HPM1371R	ATTATATTTGAATCCAGGTTCAATG	
HPM1372	*HPM1372F*	ATGAAATTGTCTGTCTTAATGGCTGT	720
	HPM1372R	TCCGCCAAATGTACATCATCAC	
HPM1373	*HPM1373F*	ATGAAATTGTCTGTCTTAATGGCTGT	462
	HPM1373R	CTCTCATACCATACCCCAACTCAGG	
clpP	*clpPF*	AGAGTGAGGGCGTTGAGT	331
	clpPR	TTCTTGTTTCGGGTGTTT	
cheY	*cheYF*	CCTTATGATGCCGTAGTTCTCG	443
	cheYR	TCAAGAGCGTTGCTACTGACCT	
vacJ	*vacJF*	ACCGTGCCATGTGGAAAGTC	377
	vacJR	TAAATCTTGACGAGGCGTTGC	

VG Analysis

Nineteen VGs were analyzed using PCR as previously described [14-23]. Details of all primers used are listed in *Table 1*.

Sequence Types (STs) Analysis

A STs analysis was carried out using the Multi-locus Sequence Typing (MLST) method as previously described [24,25]. A neighbor-joining tree was built using the MEGA version 5.0 software based on the MLST target sequences.

Statistical Analyses

Chi-square and Fisher's exact tests were used to assess the associations between serovars, ST, and VGs using SPSS version 18.0, and p values lower than 0.05 were considered statistically significant associations.

Results

Identification and Serotyping

A total of 117 *G. parasuis* isolates were obtained from 710 nasal swab samples. Of the 117 *G. parasuis* isolates, 105 were assigned to ten distinct serovars, and twelve of the isolates tested were NT. Serovar 10 (17.9%) was the most prevalent, followed by serovars 15 (14.5%), 6 (12.0%), 8 (11.1%), 4 (8.5%), 9 (7.7%), 1 (7.7%), 7 (6.0%), 5/12 (4.3%), and 2 (0.9%) *(Fig. 1-A)*. Serovars 3, 11, 13, and 14 were not identified. Serovars 4, 6, 15, and NT were observed in all three provinces. However, serovar 2 was observed only in Shanghai and serovar 7 was observed only in Jiangxi *(Fig. 1-B)*.

VG Analysis

The VGs *vacJ* and *clpP* (95.7%) were the most prevalent, followed by *cheY* (93.2%), *rfaE* (92.3%), *hsdR* (91.5%), *capD* (88.9%), *fhuA* (40.2%), *vta1* (35.9%), *hhdA* (33.3%), *hhdB* (26.5%), *HPM1372* (22.2%), *nhaC* (21.4%), *lsgB* (19.7%), *H0254* (10.3%), *fimB* (10.3%), *wbgY* (7.7%), *HPM1373* (6.8%), *HPM1371* (5.3%), *HPM1370* (1.7%) *(Fig. 2)*. All *G. parasuis* isolates were clustered according to the presence of VGs. Four clusters were obtained (clusters A, B, C, and D) *(Fig. 3)*. Cluster A includes serovars 1, 2, 4, 6, 7, 8, 9, 10, 15, and NT isolates, harboring 4 to 11 VGs; Cluster B includes serovars 4, 5/12, 6, and NT isolates, harboring 9 to 17 VGs; Cluster C includes serovars 1, 7, and 10, harboring 5 to 8 VGs; and Cluster D includes only NT isolates, harboring 0 to 4 VGs. Interestingly, some serovars were distributed in 2 or 3 clusters. For example, serovars 4 and 6 were found in clusters A and B, serovars 1, 7, and 10 were found in clusters A and C, and NT isolates were found in clusters A, B, and D *(Fig. 3)*.

Association Between Serovars and VGs

The distribution of VGs in the isolates allocated to different serovars varied greatly, and a significant correlation was found between serovars and some VGs. A significant

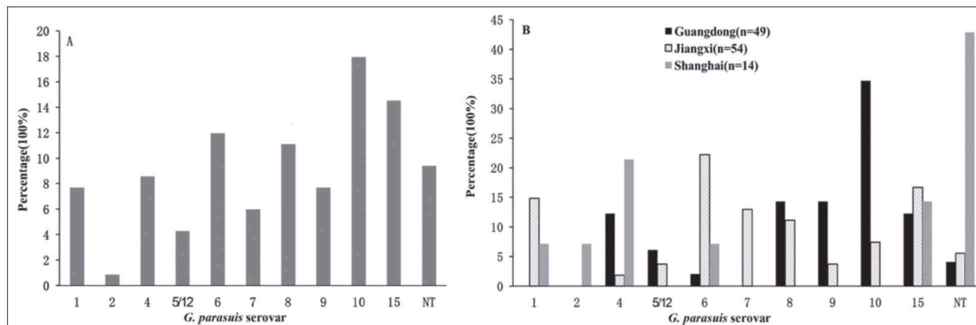

Fig 1. The distribution of serovar in all 117 isolates(A) and in difference provinces(B)

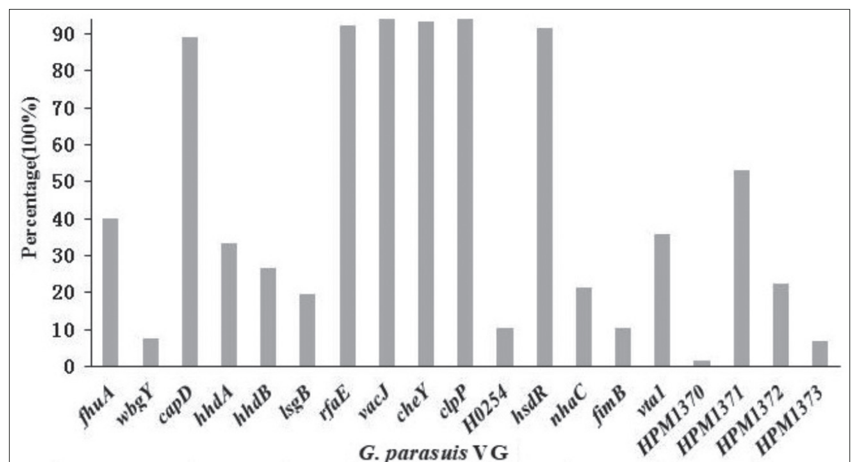

Fig 2. The distribution of 19 VGs in all 117 *G. parasuis* isolates

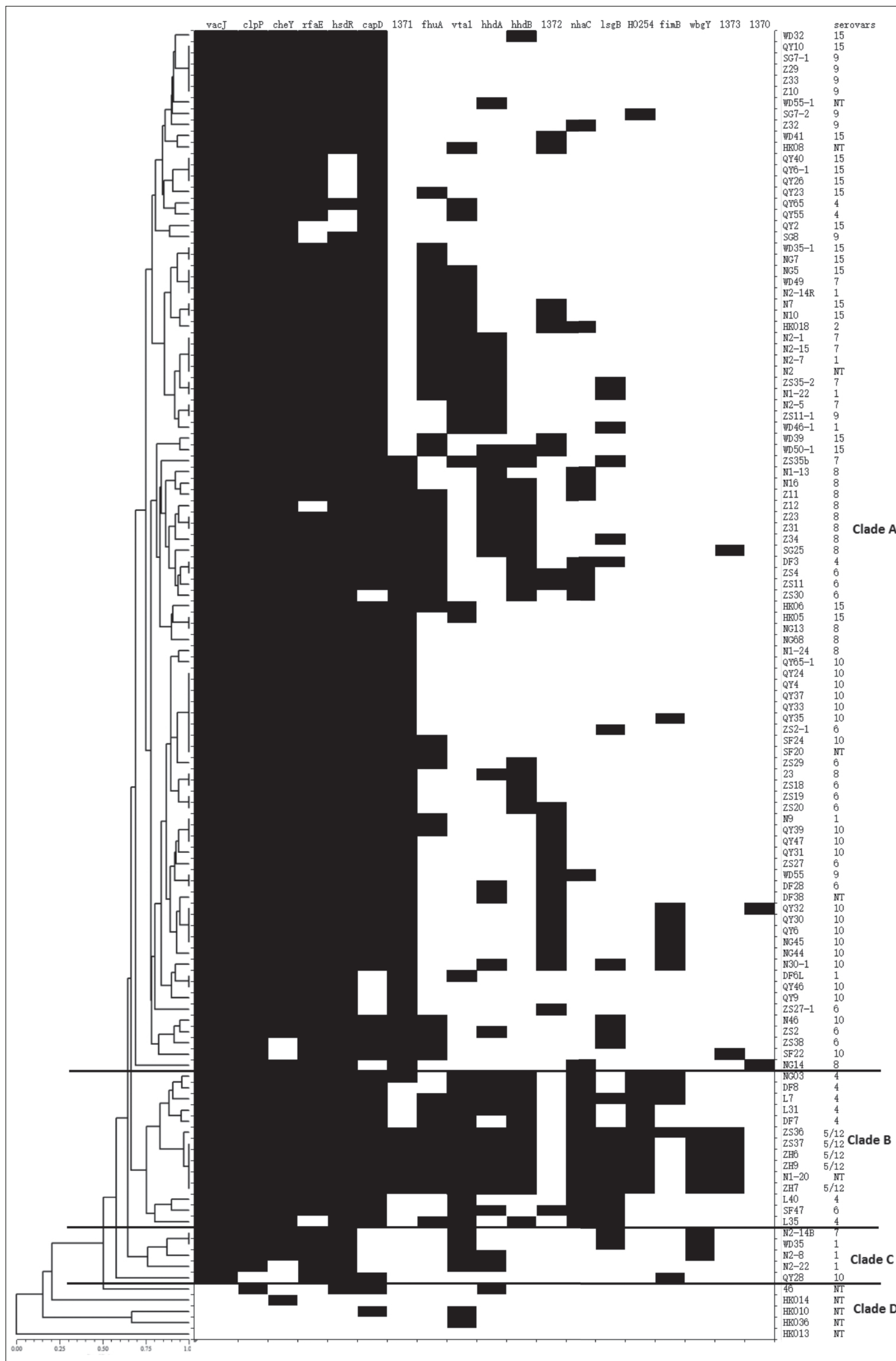

Fig 3. Clustering of *G. parasuis* isolates based on VGs

positive correlation was found between the following: serovar 1 and *vta1*; serovar 4 and *hhdB, H0254, nhaC,* and *vta1*; serovar 5/12 and *fhuA, wbgY, hhdA, hhdB, lsgB, H0254, nhaC, vta1,* and *HPM1373*; serovar 6 and both *HPM1371,* and *HPM1372*; serovar 7 and both *hhdA* and *vta1*; serovar 8 and *hhdA, hhdB,* and *HPM1371*; serovar 10 and *fimB, HPM1371,* and *HPM1372*; serovar 15 and *hsdR*. However, a significant negative correlation was found between serovar 1 and *capD*, serovar 4 and *HPM1371,* serovar 6 and *vta1*, serovar 8 and *vta1*, serovar 9 and both *fhuA* and *HPM1371*, and the following: serovar 10 and *fhuA*, *hhdA*, *hhdB*, *nhaC*, and *vta1*, serovar 15 and *hhdA*, *lsgB*, *nhaC*, and *HPM1371*, and NT and r*faE*, *vacJ*, *cheY*, *clpP*, and *hsdR* (P<0.05, Table 2).

***Table 2.** Association between serovars and VGs of G. parasuis isolates*

Serovar	VGs	VG +	VG−	−VG +	−VG−	OR	95% CI	P
1	*capD*	5	4	99	9	0.11	0.03-0.48	0.009
	vta1	8	1	34	74	17.41	2.09-144.78	0.001
5/12	*fhuA*	5	0	42	70	∞	/	0.009
	wbgY	5	0	4	108	∞	/	0.000
	hhdA	5	0	34	78	∞	/	0.003
	hhdB	5	0	26	86	∞	/	0.001
	lsgB	5	0	18	94	∞	/	0.000201
	H0254	5	0	7	105	∞	/	0.000005
	nhaC	5	0	20	92	∞	/	0.000317
	vta1	5	0	37	75	∞	/	0.005
	HPM 1373	5	0	3	109	∞	/	0.000
10	*fhuA*	4	17	43	53	0.29	0.09-0.93	0.047
	hhdA	1	20	38	58	0.08	0.01-0.62	0.002
	hhdB	0	21	31	65	0	/	0.001
	nhaC	0	21	25	71	0	/	0.006
	fimB	8	13	4	92	14.15	3.73-53.68	0.000094
	vta1	0	21	42	54	0	/	0.000031
	HPM1371	20	1	42	54	25.71	3.31-199.41	0.000007
	HPM 1372	9	12	17	79	3.49	1.27-9.59	0.019
4	*hhdB*	7	3	24	83	8.07	1.94-33.61	0.003
	H0254	5	5	7	100	14.29	3.33-61.37	0.001
	nhaC	8	2	17	90	21.18	4.13-108.52	0.000053
	vta1	9	1	33	74	20.18	2.46-165.85	0.000391
	HPM 1371	2	8	60	47	0.2	0.04-0.99	0.044
15	*hhdA*	1	16	38	62	0.1	0.01-0.78	0.011
	lsgB	0	17	23	77	0	/	0.023
	hsdR	12	5	95	5	0.13	0.03-0.52	0.006
	nhaC	0	17	25	75	0	/	0.022
	HPM 1371	2	15	60	40	0.09	0.02-0.42	0.000336
8	*hhdA*	9	4	30	74	5.55	1.59-19.41	0.009
	hhdB	8	5	23	81	5.63	1.68-18.87	0.005
	vta1	0	13	42	62	0	/	0.004
	HPM 1371	13	0	49	55	∞	/	0.000158
6	*vta1*	1	13	41	62	0.12	0.02-0.95	0.017
	HPM 1371	13	1	49	54	14.33	1.81-113.61	0.001
	HPM 1372	7	7	19	84	4.42	1.39-14.10	0.014
7	*hhdA*	5	2	34	76	5.59	1.03-30.26	0.040
	vta1	7	0	35	75	∞	/	0.001
9	*fhuA*	0	9	47	61	0	/	0.011
	HPM 1371	1	8	61	47	0.1	0.01-0.83	0.012
NT	*rfaE*	6	5	102	4	0.05	0.01-0.24	0.000289
	vacJ	6	5	106	0	0	/	0.000003
	cheY	7	4	102	4	0.07	0.01-0.34	0.003
	clpP	7	4	105	1	0.02	0-0.20	0.000212
	hsdR	7	4	100	6	0.11	0/03-0.48	0.007

***VG +:** Number of isolates in the corresponding serovar but carrying the VG; **VG−:** Number of isolates in the corresponding serovar but no carrying the VG **−VG +:** Number of isolates no in the corresponding serovar but carrying VG; **−VG −:** Number of isolates no in the corresponding serovar but no carrying VG*

Table 3. Association between pathogenic serovar group and VGs of G. parasuis isolates								
Pathogenic Serovar Group	**VGs**	**VG +**	**VG−**	**−VG +**	**−VG−**	**OR**	**95% CI**	**P**
Highly pathogenic group	*wbgY*	7	28	1	70	17.5	2.06-148.84	0.002
	hhdB	5	30	25	46	0.31	0.11-0.90	0.038
	fimB	9	26	3	68	7.85	1.97-31.28	0.002
	HPM 1371	27	8	32	39	4.11	1.64-10.28	0.002
	HPM 1373	6	29	1	70	14.48	1.67-125.66	0.005
Moderately pathogenic group	*hsdR*	22	6	78	0	0	/	0.0002
	vta1	15	13	22	56	2.94	1.21-7.17	0.021
	HPM 1371	4	24	55	23	0.07	0.02-0.22	0.000
Non-pathogenic group	*H0254*	1	42	10	53	0.13	0.02-1.06	0.026
	fimB	0	43	12	51	0	/	0.001
	vta1	9	34	28	35	0.33	0.14-0.80	0.014

VG +: *Number of isolates in the corresponding serovar but carrying the VG;* ***VG−:*** *Number of isolates in the corresponding serovar but no carrying the VG*
−VG +: *Number of isolates no in the corresponding serovar but carrying VG;* ***−VG −:*** *Number of isolates no in the corresponding serovar but no carrying VG*

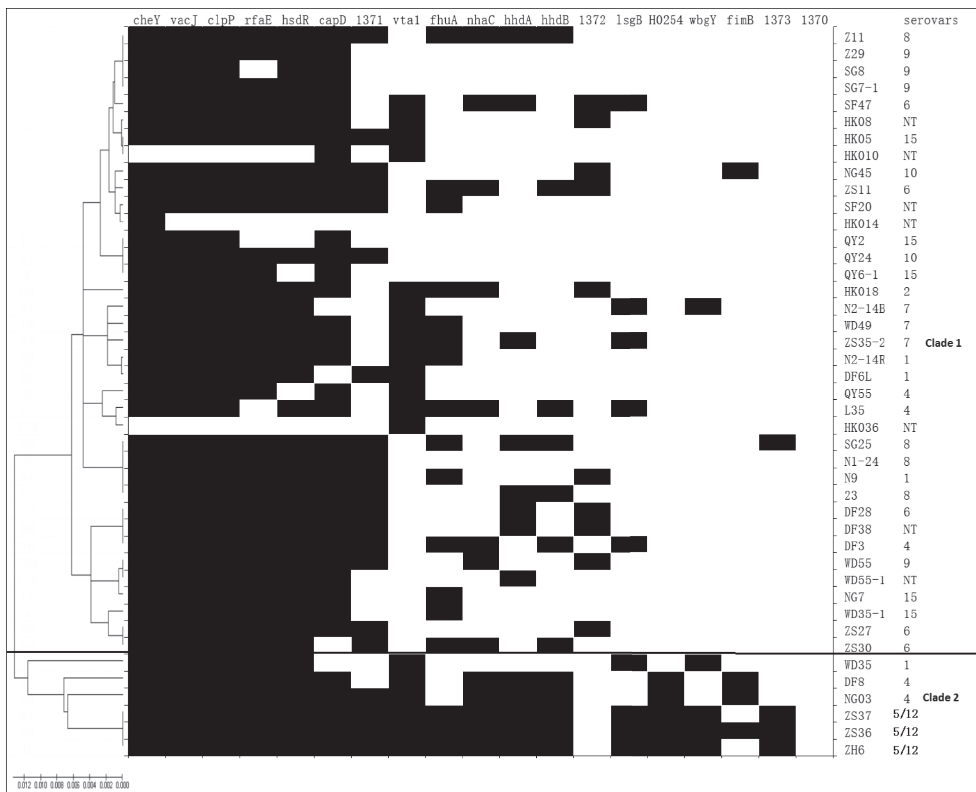

Fig 4. Neighbour-joining tree based on the MLST target sequences of 43 *G. parasuis* isolates

Oliveira and Pijoan [2] reported that *G. parasuis* was divided into three groups based on different serovars: highly pathogenic serovars (1, 5, 10, 12, 13, and 14), moderately pathogenic serovars (2, 4, and 15), and non-pathogenic serovars (3, 6, 7, 8, 9, and 11). The current study identified a significant correlation between different pathogenic serovar groups and several VGs. The highly pathogenic serovars had a significant positive association with *wbgY*, *fimB*, *1371*, and *1373*, and a significant negative association with *hhdB*. The moderately pathogenic serovars had a significant positive association with *hsdR* and *vta1,* and a significant negative association with *HPM1371*. The non-pathogenic serovars had a significant negative association with *H0254*, *fimB*, and *vta1* (P<0.05, *Table 3*).

Association Between ST and VGs

The ST analysis revealed two major clades (clade 1 and clade 2) based on the MLST target sequences of 43 *G. parasuis* isolates. Clade 1 includes 37 isolates of serovars 1, 2, 4, 6, 7, 8, 9, 10, 15, and NT, harboring 1 to 11 VGs each.

Table 4. Association between MLST clade and VGs of G. parasuis isolates

VG	Clade1+	Clade1−	Clade2+	Clade2−	OR	95% CI	P
vta1	13	24	6	0	0	/	0.004
nhaC	8	29	5	1	0.06	0.01-0.59	0.007
hhdA	8	29	5	1	0.06	0.01-0.59	0.007
hhdB	7	30	5	1	0.05	0.01-0.50	0.004
lsgB	5	32	4	2	0.08	0.01-0.56	0.01
H0254	0	37	5	1	0	/	0.000006
wbgY	1	36	4	2	0.01	0-0.14	0.001
fimB	1	36	3	3	0.03	0-0.38	0.006
HPM 1373	1	36	3	3	0.03	0-0.38	0.006

+: Number of isolates in the corresponding clade but carrying the VG; −: Number of isolates in the corresponding clade but no carrying the VG

Clade 2 includes 6 isolates of serovars 1, 4 and 5, harboring 8 to 16 VGs each *(Fig. 4)*. Interestingly, isolates in the second clade had a significantly increased probability of containing the VGs *vta1*, *nhaC*, *hhdA*, *hhdB*, *lsgB*, *H0254*, *wbgY*, *fimB*, and *1373* (P<0.05, Table 4).

Discussion

In the study, a total of 117 *G. parasuis* isolates were obtained from 710 nasal swab samples from three provinces (Guangdong, Jiangxi, and Shanghai) in the south of China, the isolation rate was 16.5%, slightly higher than previous studies (14.6%) [26]. Ten distinct serovars were identified, serovars 10, 15, 6, and 8 were the dominant serovars identified in this study, with the detection frequency exceeding 10%. This differs from a previous report that the dominant serovars of strains in diseased pigs are 5 and 4 [27-31]. This difference may be uniquely associated with isolates from the nasal cavity of live piglets. In another study of *G. parasuis* isolates from the piglet nasal cavity by Zhang et al.[26], the dominant serovars in 6 provinces of China (Beijing, Shandong, Henan, Shanghai, Sichuan, and Chongqing) were 7, 3, 2, and 11 (over 10%). Those authors did not identify any isolates representing serovars 14 and 15. In the current study, we did not isolate any *G. parasuis* strains from serovars 3 and 11, and we only isolated a single strain from serovar 2. This suggests that serovars of *G. parasuis* from the swine nasal cavity exhibit a complex regional distribution across provinces in China. In both the current study and the study conducted by Zhang et al.[26], the detection frequency of serovars 4 and 5 was relatively low. Strains in serovars 4 and 5 are widely regarded as pathogenic strains, and they are most often identified from pigs with Glässer's disease. Although the detection frequency of serovars 4 and 5 was not high in live piglets, these isolates may nonetheless cause disease when an animal is under stress. Of note, the dominant serovars identified in this study, serovar 10 and serovar 15, were previously considered to be highly and moderately pathogenic, respectively. These two serovars have rarely been isolated in diseased pigs in China. Further attention and research are required to determine whether the presence of strains from serovars 10 and 15 in the respiratory tract of live piglets would cause localized disease, or even a potential disease epidemic.

In this study, all *G. parasuis* isolates were divided into four clusters according to the presence of VGs. Though serovars 2, 5, 8, 9, 10, and 15 were only distributed in one cluster, isolates belonging to the same serovar harbored different VGs. These differences were also present among strains that belonged to the same ST and serovar. For example, strains SG25 and N1-24, isolated from different farms, were both allocated to ST185 and serovar 8, and possessed seven identical VGs. However, strain SG25 had five more VGs than N1-24. Similarly, strains OY2 and QY6-1, isolated from the same farm, were allocated to ST255 and serovar 15, but strain QY6-1 has one more VG *(rfaE)* than OY2. Interestingly, strain QY6, isolated from the nasal cavity of the same piglet as strain QY6-1, also harbored *rfaE*. These results suggest that *G. parasuis* isolates may undergo multiple gene exchanges while coexisting in the respiratory tract. The VGs of isolates allocated to the same ST and serovar varied greatly, which may lead to differences in the pathogenicity and immunogenicity of strains belonging to the same ST and serovar. Once these strains invade the host tissues and organs, they may cause localized disease and eventually become epidemics. At that point, even if the serovars of commercially available vaccines and pathogenic strains were the same, the differences in VGs may lead to immune failures. That scenario would pose a substantial challenge to the development of a new vaccine.

Van et al.[31] reported that the detection frequency of the VGs *vta1*, *HPM-1371*, *capD*, *HPM-1372*, *lsgB*, *HPM-1373*, and *HPM-1370* was 62.5%, 35.7%, 30.3%, 12.5%, 8.9%, 8.9%, and 0%, respectively. Boerlin et al.[17] reported that the detection frequency of *vta1*, *hsdR*, *fimB*, *nhaC*, *fhuA*, *capD*, *wbgY*, and *H0254* was 92.5%, 47.9%, 37.2%, 38.3%,

38.3%, 23.4%, 22.3%, and 17%, respectively; Turni et al.[32] reported that the detection frequency of *hhdA* and *hhdB* was 36% and 13.3%, respectively, which differs from our results for most of the above VGs. Although previous studies [31] have shown that the VGs *lsgB*, *fhuA*, *capD*, *HPM-1372*, and *HPM-1373* were not observed in any isolates from non-pathogenic serovar group, our results showed that 8 of 43 isolates from the non-pathogenic serovar group were positive for *lsgB*, 16 were positive for *fhuA*, 39 were positive for *capD*, 8 were positive for *HPM-1372*, and 1 was positive for *HPM-1373*. Our results indicate that the distribution of VGs in *G. parasuis* is diverse and complex.

Olvera et al.[16] reported that isolates without *vtaA1* are generally avirulent. In this study, the presence of *vta1* was associated with a significantly decreased probability of membership in the non-pathogenic serovar group. This indicates that isolates allocated to the non-pathogenic serovar group may be avirulent based on this *vta1* analysis. Similarly, a significantly increased probability of harboring *vta1* was observed in the highly pathogenic serovars 1 and 5. Based on only the above analysis, the virulences predicted by the serovar and *vtaA1* analyses were consistent. However, all 21 serovar 10 isolates were *vtaA1* negative in the study, which indicates that serovar 10 isolates may be avirulent, but serovar 10 belonged to highly pathogenic serovars according to the previous research [2], so, the results of virulence prediction by the serovar and *vtaA1* analyses were in opposition. The correlation between serovars and VGs varied greatly among different serovars, even if the isolates belonged to the same pathogenic serovar group. For example, serovar 1 was only positively associated with *vta1*, while serovar 5 was positively associated with 9 VGs. Although the average number of VGs in the three pathogenic serovar groups was similar, the highly pathogenic serovars had a significant positive association with 4 VGs, the moderately pathogenic serovars had a significant positive association with 2 VGs, and no VGs had a positive association with non-pathogenic serovars. A previous study showed that *G. parasuis* MLST STs can be classified into two clades, with clade one almost completely containing avirulent or attenuated STs, and clade two mainly containing virulent STs [25,33]. In the current study, the detection frequency of VGs in clade two was much higher than that in clade one. While all isolates of clade two were *vtaA1* positive, only 30% of clade one isolates were *vtaA1* positive. We found a significant positive correlation between clade two and 9 VGs. Based on the VG analyses, it appears that isolates belonging to clade two are more virulent than isolates belonging to clade one. Overall, our results show that VG analyses may be a supplementary method for accurately allocating serovars or genotypes of *G. parasuis* into different pathogenic groups.

Availability of Data and Materials

The authors declare that data supporting the findings of this study are available upon request.

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Conflict of Interest

The authors declare no conflict of interest.

Authors' Contributions

LP and XYX conceived the experiments and wrote the paper. All authors performed the experiments. All authors have interpreted the data, revised the manuscript, and approved the final version.

References

1. Pilchová V, Seinige D, Hennig-Pauka I, Büttner K, Abdulmawjood A, Kehrenberg C: Development and validation of a loop-mediated isothermal amplification (LAMP) assay for rapid detection of *Glaesserella (Haemophilus) parasuis*. *Microorganisms*, 9 (1): 41, 2020. DOI: 10.3390/microorganisms9010041

2. Oliveira S, Pijoan C: *Haemophilus parasuis*: New trends on diagnosis, epidemiology and control. *Vet Microbiol*, 99 (1): 1-12, 2004. DOI: 10.1016/j.vetmic.2003.12.001

3. Zhang Y, Dong Y, Xu Y, Wang Z, Yu N, Liu H, Wang L: The development of a SYBR green I multiple real-time fluorescence PCR assay for detection of *Actinobacillus pleuropneumoniae, Haemophilus parasuis* and *Pasteurella multocida*. *Kafkas Univ Vet Fak Derg*, 28 (1): 11-17, 2022. DOI: 10.9775/kvfd.2021.26202

4. Mahmmod YS, Correa-Fiz F, Aragon V: Variations in association of nasal microbiota with virulent and non-virulent strains of *Glaesserella (Haemophilus) parasuis* in weaning piglets. *Vet Res*, 51 (1): 7, 2020. DOI: 10.1186/s13567-020-0738-8

5. Blanco-Fuertes M, Correa-Fiz F, López-Serrano S, Sibila M, Aragon V: Sow vaccination against virulent *Glaesserella parasuis* shapes the nasal microbiota of their offspring. *Sci Rep*, 12 (1): 3357, 2022. DOI: 10.21203/rs.3.rs-1192355/v1

6. Kielstein P, Rapp-Gabrielson VJ: Designation of 15 serovars of *Haemophilus parasuis* on the basis of immunodiffusion using heat-stable antigen extracts. *J Clin Microbiol*, 30 (4): 862-865, 1992. DOI: 10.1128/jcm.30.4.862-865.1992

7. Peng L, Yuan X, Fang R, Liu-Fu W, Wen Q, Yang X: Serovars, antimicrobial susceptibility and molecular characteristics of *Haemophilus parasuis* isolates in Southern China. *Kafkas Univ Vet Fak Derg*, 26 (4): 491-497, 2020. DOI: 10.9775/kvfd.2019.23587

8. Hau SJ, Eberle KC, Brockmeier SL: Importance of strain selection in the generation of heterologous immunity to *Glaesserella (Haemophilus) parasuis*. *Vet Immunol Immunopathol*, 234:110205, 2021. DOI: 10.1016/j.vetimm.2021.110205

9. Dazzi CC, Guizzo JA, Prigol SR, Kreutz LC, Driemeier D, Chaudhuri S, Schryvers AB, Frandoloso R: New pathological lesions developed in pigs by a "non-virulent" strain of *Glaesserella parasuis*. *Front Vet Sci*, 7:98, 2020. DOI: 10.3389/fvets.2020.00098

10. Wan X, Li X, Osmundso T, Li C, Yan H: Whole-genome sequence analyses of *Glaesserella parasuis* isolates reveals extensive genomic variation and diverse antibiotic resistance determinants. *Peer J*, 8 (6):e9293, 2020. DOI: 10.7717/peerj.9293

11. Álvarez-Estrada A, Gutiérrez-Martín CB, Rodríguez-Ferri EF, Martínez-Martínez S: Transcriptomics of *Haemophilus (Glasserella) parasuis* serovar 5 subjected to culture conditions partially mimetic to natural infection for the search of new vaccine antigens. *BMC Vet Res*, 14 (1): 326, 2018. DOI: 10.1186/s12917-018-1647-1

12. Angen O, Oliveira S, Ahrens P, Svensmark B, Leser TD: Development of an improved species specific PCR test for detection of *Haemophilus parasuis*. *Vet Microbiol*, 119 (2-4): 266-276, 2007. DOI: 10.1016/j.vetmic.2006.10.008

13. Howell KJ, Peters SE, Wang J, Hernandez-Garcia J, Weinert LA, Luan SL, Chaudhuri RR, Angen Ø, Aragon V, Williamson SM, Parkhill J, Langford PR, Rycroft AN, Wren BW, Maskell DJ, Tucker AW, BRaDP1T Consortium: Development of a multiplex PCR assay for rapid molecular serotyping of *Haemophilus parasuis*. *J Clin Microbiol*, 53 (12): 3812-3821, 2015. DOI: 10.1128/JCM.01991-15

14. Sack M, Baltes N: Identification of novel potential virulence-associated factors in *Haemophilus parasuis*. *Vet Microbiol*, 136 (3-4): 382-386, 2009. DOI: 10.1016/j.vetmic.2008.11.008

15. Zhou H, Yang B, Xu F, Chen X, Wang J, Blackall PJ, Zhang P, Xia Y, Zhang J, Ma R: Identification of putative virulence-associated genes of *Haemophilus parasuis* through suppression subtractive hybridization. *Vet Microbiol*, 144 (3-4): 377-383, 2010. DOI: 10.1016/j.vetmic.2010.01.023

16. Olvera A, Pina S, Macedo N, Oliveira S, Aragon V, Bensaid A: Identification of potentially virulent strains of *Haemophilus parasuis* using a multiplex PCR for virulence-associated autotransporters *(vtaA)*. *Vet J*, 191 (2): 213-218, 2012. DOI: 10.1016/j.tvjl.2010.12.014

17. Boerlin P, Poljak Z, Gallant J, Chalmers G, Nicholson V, Soltes GA, MacInnes JI: Genetic diversity of *Haemophilus parasuis* from sick and healthy pigs. *Vet Microbiol*, 167 (3-4): 459-467, 2013. DOI: 10.1016/j.vetmic.2013.07.028

18. Wang X, Xu X, Wu Y, Li L, Cao R, Cai X, Chen H: Polysaccharide biosynthesis protein capD is a novel pathogenicity-associated determinant of *Haemophilus parasuis* involved in serum-resistance ability. *Vet Microbiol*, 164 (1-2): 184-189, 2013. DOI: 10.1016/j.vetmic.2013.01.037

19. Zhang B, Yu Y, Zeng Z, Ren Y, Yue H: Deletion of the *rfaE* gene in *Haemophilus parasuis* sc096 strain attenuates serum resistance, adhesion and invasion. *Microb Pathog*, 74, 33-37, 2014. DOI: 10.1016/j.micpath.2014.07.006

20. Lawrence P, Bey R: Map-based comparative genomic analysis of virulent *Haemophilus parasuis* serovars 4 and 5. *J Genomics*, 3, 59-71, 2015. DOI: 10.7150/jgen.10924

21. He L, Wen X, Yan X, Ding L, Cao S, Huang X, Wu R, Wen Y: Effect of *cheY* deletion on growth and colonization in a *Haemophilus parasuis* serovar 13 clinical strain EP3. *Gene,* 577 (1): 96-100, 2016. DOI: 10.1016/j.gene.2015.11.046

22. Huang J, Wang X, Cao Q, Feng F, Xu X, Cai X: *ClpP* participates in stress tolerance and negatively regulates biofilm formation in *Haemophilus parasuis*. *Vet Microbiol*, 182, 141-149, 2016. DOI: 10.1016/j.vetmic.2015.11.020

23. Zhao L, Gao X, Liu C, Lv X, Jiang N, Zheng S: Deletion of the *vacJ* gene affects the biology and virulence in *Haemophilus parasuis* serovar 5. *Gene*, 603, :42-53, 2017. DOI: 10.1016/j.gene.2016.12.009

24. Olvera A, Cerda-Cuellar M, Aragon V: Study of the population structure of *Haemophilus parasuis* by multilocus sequence typing. *Microbiolgy*, 152 (12): 3683-3690, 2006. DOI: 10.1099/mic.0.29254-0

25. Mullins MA, Register KB, Brunelle BW, Aragon V, Galofré-Mila N, Bayles DO, Jolley KA: A curated public database for multilocus sequence typing (MLST) and analysis of *Haemophilus parasuis* based on an optimized typing scheme. *Vet Microbiol*, 162 (2-4): 899-906, 2013. DOI: 10.1016/j.vetmic.2012.11.019

26. Zhang P, Zhang C, Aragon V, Zhou X, Zou M, Wu C, Shen Z: Investigation of *Haemophilus parasuis* from healthy pigs in China. *Vet Microbiol*, 231, 40-44, 2019. DOI: 10.1016/j.vetmic.2019.02.034

27. Qi B, Li F, Chen K, Ding W, Xue Y, Wang Y, Wang H, Ding K, Zhao Z: Comparison of the *Glaesserella parasuis* virulence in mice and piglets. *Front Vet Sci*, 8:659244, 2021. DOI: 10.3389/fvets.2021.659244

28. Macedo N, Gottschalk M, Strutzberg-Minder K, Van CN, Zhang L, Zou G, Zhou R, Marostica T, Clavijo MJ, Tucker A, Aragon V: Molecular characterization of *Glaesserella parasuis* strains isolated from North America, Europe and Asia by serotyping PCR and LS-PCR. *Vet Res,* 52 (1): 68, 2021. DOI: 10.1186/s13567-021-00935-9

29. Wang Z, Zhao Q, Wei H, Wen X, Cao S, Huang X, Wu R, Yan Q, Huang Y, Wen Y: Prevalence and seroepidemiology of *Haemophilus parasuis* in Sichuan province, China. *Peer J*, 5:e3379, 2017. DOI: 10.7717/peerj.3379

30. Ma L, Wang L, Chu Y, Li X, Cui Y, Chen S, Zhou J, Li C, Lu Z, Liu J, Liu Y: Characterization of Chinese *Haemophilus parasuis* isolates by traditional serotyping and molecular serotyping methods. *Plos One*, 11 (12):e0168903, 2016. DOI: 10.1371/journal.pone.0168903

31. Van CN, Thanh TVT, Zou G, Jia M, Wang Q, Zhang L, Ding W, Huang Q, Zhou R: Characterization of serotypes and virulence genes of *Haemophilus parasuis* isolates from Central Vietnam. *Vet Microbiol*, 230 (1): 117-122, 2019. DOI: 10.1016/j.vetmic.2019.02.008

32. Turni C, Singh R, Blackall PJ: Virulence-associated gene profiling, DNA fingerprinting and multilocus sequence typing of *Haemophilus parasuis* isolates in Australia. *Aust Vet J*, 96 (6): 196-202, 2018. DOI: 10.1111/avj.12705

33. Wang L, Ma L, Liu Y, Gao P, Li Y, Li X, Liu Y: Multilocus sequence typing and virulence analysis of *Haemophilus parasuis* strains isolated in five provinces of China. *Infect Genet Evol*, 44, 228-233, 2016. DOI: 10.1016/j.meegid.2016.07.015

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Research Article

The Effect of Rat Adipose-derived Stem Cells in Bone Tissue Regeneration

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Abstract: Stem cell approaches has been increasing in the conventional medicine. This study was planned to determinate the efficacy of adipose-derived stem cells (ADSCs) on the bone fracture's regeneration by histologically, morphologically and radiologically. Primarily isolated cells were cultivated and identified for the obtaining of the third passage mesenchymal stem cells in the cell culture laboratory. The right tibias (applicated ADSCs) were planned as experimental group and the left tibias were studied as control on the clinically healthy 10 rats. The middle diaphysis of the right and left tibias was transversally cut by a thin saw and then fixed by intramedullary pin. Before surgical closure of the incised area, 10^6 ADSCs were injected on an absorbent material, which covered the cut site of the right tibia, in experimental group. At postoperative 45th day, cranio-caudal and lateral radiographs of the tibias pointed out that there was callus formation in the caudal and lateral parts of the right tibia. After sacrification of the animals, preparations were made for histological examinations. The results indicate that there were clear differences in the mitotic activity; and also, the experimental tibias were found to be rich in blood vessel network. As a conclusion, it was found that ADSCs can have a positive potential effect on osteogenesis in bone tissue.

Keywords: *ADSCs, Bone Tissue Regeneration, Histologically, Radiologically, Rat*

Sıçan Yağ Dokusu Kökenli Kök Hücrelerin Kemik Dokusu Rejenerasyonuna Etkisi

Öz: Kök hücre teknolojisi, geleneksel tıbbi yaklaşımları geliştirmektedir. Çalışmada, yağ doku kökenli kök hücrelerin (ADSC) kemik kırığının rejenerasyon süreci üzerindeki etkinliği histolojik, morfolojik ve radyolojik olarak değerlendirilmesi amaçlandı. Öncelikli olarak; izole edilmiş hücreler, hücre kültürü laboratuvarında üçüncü pasaj mezenkimal kök hücrelerin elde edilmesi için kültürlendi ve tanımlandı. Klinik olarak sağlıklı 10 rat üzerinde; ADSC uygullanan sağ tibialar deney grubu olarak ve sol tibialar kontrol olarak planlandı. Sağ ve sol tibianın orta diyafizi ince bir testere ile enine kesildi. Sağ kaval kemiği üzerinde, deney grubuna emici bir malzeme üzerine 10^6 ADSC enjekte edildi. Deney ve kontrol gruplarında tibia üzerinde kemik kırığı oluşturulduktan sonra intramedüller tespit yapıldı. Operasyondan 45 gün sonra tibianın kranio-kaudal ve lateral grafileri çekilerek; sağ tibianın kaudal ve lateralinde kallus oluşumu dikkati çekti. Sakrifikasyon sonrası histolojik incelemeler için hazırlıklar yapıldı ve mitotik aktivitede belirgin farklılıklar olduğu saptandı. Aynı zamanda deneysel tibiaların kan damarı ağı açısından zengin olduğu görüldü. Sonuç olarak, ADSC 'lerin kemik dokusunda osteogenez için olumlu bir potansiyel etki oluşturacabileceği saptandı.

Anahtar sözcükler: *ADSCs, Kemik Doku Rejenerasyonu, Histoloji, Radyoloji, Sıçan*

Introduction

Mesenchymal stem cells (MSCs) are attractive therapeutic models for regenerative medicine due to their pluripotent features. These are also undifferentiated cells that have capable of self-renewal with symmetric and asymmetric divisibility for tissue regeneration. In recent years, applications of MSCs have received increasing attention. Easily extracted from bone marrow and fat, MSCs differentiate into different cell lines according to the needs of certain biomedical applications [1,2]. MSCs are suitable cell sources for tissue regeneration. Thus, *in vitro* studies have been

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focused on usage of MSCs in bone defect and degenerative bone-cartilage disease known as progenitor stem cells [1,3].

MSCs from different tissues exhibit various *in vitro* characteristics including their proliferation capacity and differentiation potential, which influence their applicability [4]. Lendeckel et al.[5] stated that ADSCs have more proliferative capacity than bone marrow derived mesenchymal stem cells (BMSCs). When compared to other sources, adipose tissue is popular stem cell source because it is easily accessible, abundant, and has less painful collection procedure. Adipose tissue derived mesenchymal stem cells (ADSCs) have a capacity of self-renewable, multipotency and transdifferentiate to several different specific cell phenotypes such as cartilage, bone, fat, tendon and muscle tissue [6,7].

Mohamed-Ahmed et al.[8] shown that equal amounts of bioactive factors are given both of stem cells, ADSCs have inferior capacity and immuno-modulatory effects than BMSCs. ADSCs stimulate macrophage cells however inhibit T cells and dendritic cells, inducing angiogenesis, a decrease in apoptosis and fibrosis leads to an increase in anti-inflammation process [(IL-6, IL-10, IL-13), (TNF-α), (TGF-β), (TLR 2, TLR4), (VEGF), (b-FGF)] [9]. In tissue engineering, MSCs can be considered as an alternative cell source to ADSCs [10]. BMSCs have also some difficulties, such as low MSC concentrations and difficulty in collecting [11,12]. Moreover, the differentiation of ADSCs into osteoblasts makes them preferable cells for bone tissue engineering [13].

The aim of this study was to evaluate the efficacy of ADSCs on the bone fracture's regeneration process by histologically and radiologically.

Material and Methods

Ethical Statement

This study was approved by the Bursa Uludag University Animal Experiments Local Ethics Committee (Approval no: 2018-02/03).

Animals

Totally 13 male Sprague Dawley rats (clinically healthy, about 300 g, 3 months-old) were included in the study. Among them, 10 rats were used for establishment of bone regeneration, and 3 rats were used for harvesting adipose tissue.

Isolation of ADSCs

Approximately 1-1.5 g adipose tissue per rat was obtained under sterile conditions. The non-enzymatic stem cell method was performed for the isolation of ADSCs as reported previously [14-17]. After adipose tissue was rinsed with sterile D-PBS (Cat No: BSS-1006), adipose tissue fragments were chopped into 2-3 mm thickness and transferred into rat-MSCs specific growth medium (Cat No: RAXMD-03011-440). Growth medium contained 100 units/mL penicillin-100 µg/mL streptomycin (Cat No: TMS-AB2-C), 2 mM L-glutamine (Cat No: G7513), and 10% heat-inactivated Fetal Bovine Serum (Cat No: TMS-013-B). Tissue fragments were incubated in 25 cm^2 tissue culture flask under standard culture conditions. The culture medium was replaced with fresh medium once every three days on passage 1, passage 2 and passage 3.

Third-passaged (P3) cells were subcultured in order to collect the cells for stem cell transplantation. For this purpose, the cultured P3 cells were centrifugated at 1000 rpm, 25°C for 4 min. After the supernatant was discarded, the pellet was filtered with 70-micron filter to use MSCs. The 10^6 ADSCs were prepared by insulin syringes to apply the fractured area of the tibias.

Adipogenic, Osteogenic, Chondrogenic Differentiation

After isolating and expanding ADSCs, P3 cells (7×10^4 cells per well) were seeded within 24 well plates. The standard medium was replaced with the MSCgo™ Adipogenic Differentiation Basal Medium (Cat No: 05-330-1B, 05-331-1-01 and 05-332-1-15); when cells reached 80% confluency. After two weeks, the cells were stained with Oil Red O [18].

ADSCs (1.5×10^6 P3 cells per well) were seeded within 24 well culture plates, and then MSCgo™ Osteogenic Differentiation Medium (Cat No: 05-440-1B) was added, when cells reached 70% confluency. After 21 days, cells were stained with Alizarin Red [18].

ADSCs (1.5×10^6 P3 cells per well) were seeded within 96 well culture plates. The MSCgo™ chondrogenic differentiation basal medium (Cat No: 05-220-1B and 05-221-1D) was added when cells reached 80 confluency. The cells were incubated within the chondrogenic medium for 21 days and stained with Alcian Blue [18].

Characterization of ADSCs by Immunohistochemistry

Forty thousand ADSCs were seeded on eight-well plastic culture slide for immunostaining. P3 cells were fixed with 4% paraformaldehyde (Merck, Germany, Cat No: 1.04005.1000) for 1 h at room temperature. The cells were incubated with CD 90 (1:900 dilution, Cat number: ab225), CD 105 (1:150 dilution, Cat No: ab156756), CD 45 (1:200 dilution, Cat No: ab8879) and CD 11b (1:200 dilution, Cat No: ab10558) stem cell surface primary for 18 h at +4°C in humidity chamber. The cells were then incubated with secondary antibody and streptavidin-peroxidase (Cat No: TP-125-HL) for 10 min, respectively. Finally, the bound antibody complexes were stained with chromogen solutions under light microscopic determination and then counter stained with hematoxylin [19].

Surgical Experimental Protocol

In 10 rats, the right tibias were evaluated as experimental group (application to ADSCs) and the left tibias were planned as control. Before surgery, clinical examinations, mediolateral and craniocaudal radiographies of the bilateral tibias were taken in all rats to evaluate cortical and medullar radio-opacity and to calculate the medullar and cortical space thickness of the tibias.

Premedication and induction were performed using xylazine HCl (5 mg/kg, im.) and ketamine HCl (50 mg/kg, im.), respectively. Isoflurane was inhaled to all rats for general anesthesia and maintenance. The animals were restrained in dorsal recumbence and bilateral tibial regions were prepared aseptically for surgery. The medial longitudinal incisions were made, and medial surfaces of the tibias were exposed following to dissections of the soft tissues. Hemorrhages were controlled with electro-coagulation. The middle diaphysis of the right and left tibias was transversally cut by a thin saw, and then a suitable diameter (0.5-1 mm) Kirschner pin was intramedullary inserted for fixation to the tibias. The surgical incisions of the left tibial regions were closed routinely. Moreover, on the right tibia, 10^6 the ADSCs was injected on an absorbent material and this material was covered circumferentially on the cutting surfaces of tibia, were applied in groups *(Fig. 1)*. All soft tissues and skin incisions were sutured routinely.

Postoperatively, carprofen (5 mg/kg, sc, qd) and cefazolin Na (20 mg/kg, im, bid) were administered as analgesic and antibiotic for 5 days, respectively. The skin sutures were taken at postoperative 10th day.

Clinical examinations (general condition, inspection and palpation of the surgical area) of the rats were routinely performed at each week in order to evaluate the health status of the rats and to assess the local surgical area regarding tissue healing. At the end of the planned study time (2 months), tibial regions of all rats were clinically examined again, and then all rats were decapitated to obtain the tibias of the rats for histological examinations, as follows.

Histological Examination

After decapitation, the tibias were quickly harvested and the bone tissue cut into small pieces. Specimens were fixed in 10% neutral buffered formalin for 3 days. After the fixation of specimens, they were decalcified in ethylenediaminetetraacetic acid (EDTA) solution. Until the specimens were soft enough to allow cutting, solution was changed day by day. Depending on the size of the samples, this process took two weeks or more [20,21]. When decalcification was complete, specimens were briefly rinsed in water. After dehydration in 70% ethanol, 80% ethanol, 96% ethanol, and absolute ethanol, specimens were embedded in paraffin wax and then 6-7 µm thickness tissue sections were cut and stained with Crossman's triple staining method [22]. Histology sections were examined micro-scopically (Nikon® Eclipse 80i Microscope, Netherlands). Photographs were taken with attached camera (Nikon®, Digital Sight DS-L1).

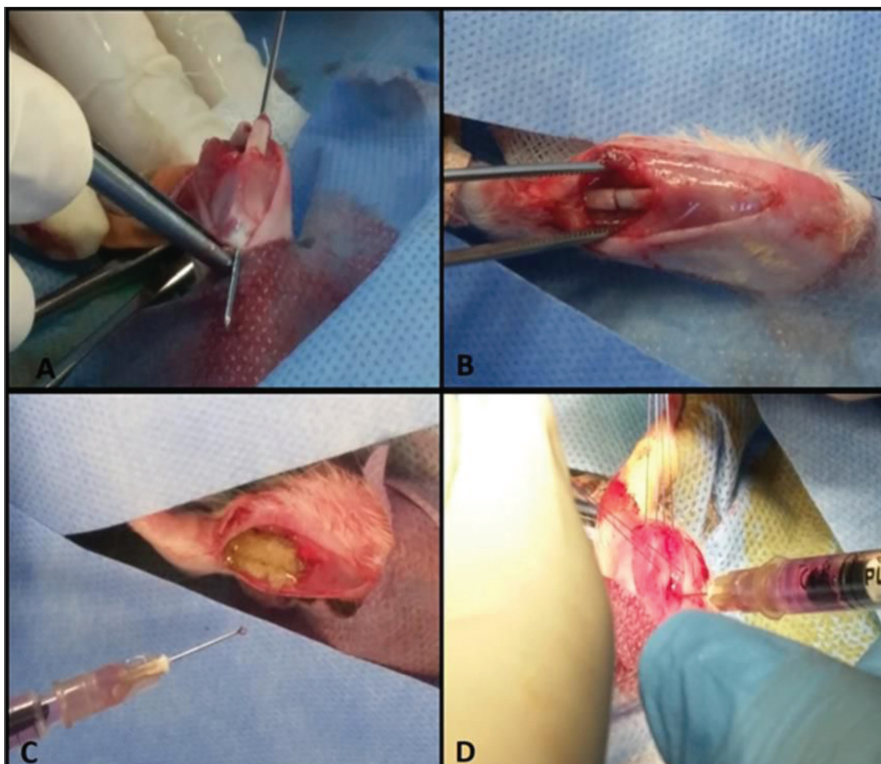

Fig 1. The middle diaphysis of the tibia was transversally cut by a thin saw, and then a 0.5-1 mm Kirschner pin was intramedullary inserted for fixation (A, B). On the right tibia, the applications in groups were injected the ADSCs on an absorbent material which was covered circumferentially on the cutting surfaces of the tibia (C, D)

Results

Characterization and Differentiation of the ADSCs

Lipogenic, osteogenic, chondrogenic differentiation capability, positive and negative characterization were used to identify and confirm the ADSCs. On day 3; ADSCs were observed to adhere to culture flasks *(Fig. 2-A)* and contained both fibroblast-like cells with spindle shape and migrating cells with round shape by inverted microscopy *(Fig. 2-B)*. The first week isolation of ADSCs, the mononuclear cells are adhered to the flask. ADSCs reached 80% confluency on day 7-8. The second and third passage of ADSCs reached 80% confluency on day 13-18 *(Fig. 2-C)* respectively. P3 cells were determined by lipogenic, osteogenic and chondrogenic differentiation capability *in vitro* that detected using Oil red O, Alizarin Red and Alcian Blue staining, respectively by inverted microscopy *(Fig. 3-A,C)*. ADSCs are characterized by the presence or absence of certain surface markers by the expression of CD 90, CD 105 *(Fig. 4-A,B)* and CD 11b, CD 45 *(Fig. 4-C,D)* respectively. ADSCs were showed positive reaction for CD 90, CD 105 and negative reaction for CD 45, CD 11b, as assessed by immuno-histochemistry.

Histological Results of the Bone Histogenesis

In postoperative 2nd months, new bone formation areas were examined histologically in the fractured parts of tibias, and they were determined normal in both tibias with triple stain. The morphology of the cells in the new bone formation areas commonly showed mitotic activity of the osteoblast cells around the fractured part of the tibias. In addition, the proliferation of the osteoblast cells increased in the callus zone especially experimental tibias than control tibias *(Fig. 5)*, and also bone matrix was strongly eosinophilic in experimental tibias *(Fig. 6)*. The eosinophilic reactions were weaker in the controls than the experimental tibias, two months after operation *(Fig. 6)*. The morphology of the cells pointed out common mitotic activity and there were much more blood vessels in the new bone formation areas in experimental tibias *(Fig. 5)*.

Clinical and Radiological Results

Clinically, there were no clinical abnormalities such as local tissue damage, ecchymosis and secondary surgical wound complications in the rats of groups at the postoperative early stage.

Radiographs taken on postoperative 21st day revealed radiopaque appearance (callus formation) around to fractured parts of tibias. In the postoperative 45th days' radiographs, there was callus formation around to fractured line, and the fractured line was clearly determined in these radiographs, as well *(Fig. 7)*. At 45th day, craniocaudal and lateral radiographs of the tibias in a rat, it might be clearly seen that there was callus formation in the caudal and lateral parts of the right tibia (arrows) and bilateral visible fractured lines.

Discussion

The stem cell transplantation system for osteogenesis

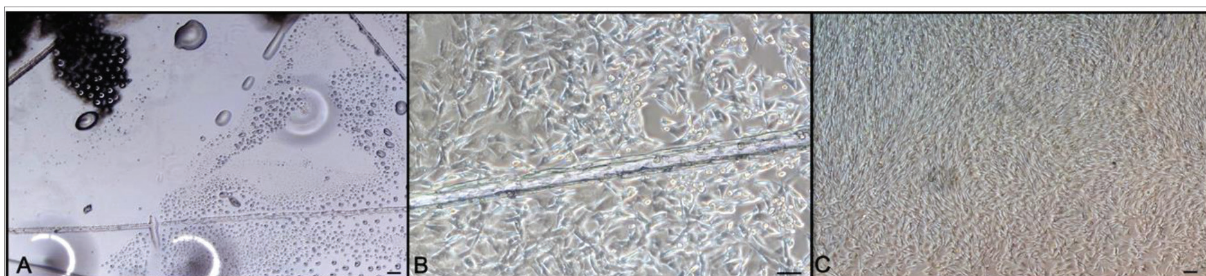

Fig 2. Microscopic evaluation of ADSCs: On day 3, isolation of ADSCs (A); ADSCs were detected with fibroblast-like cells morphology and migrating cells (B); On day13- 18, ADSCs reached 80% confluence (C) (Bar: 100 µm)

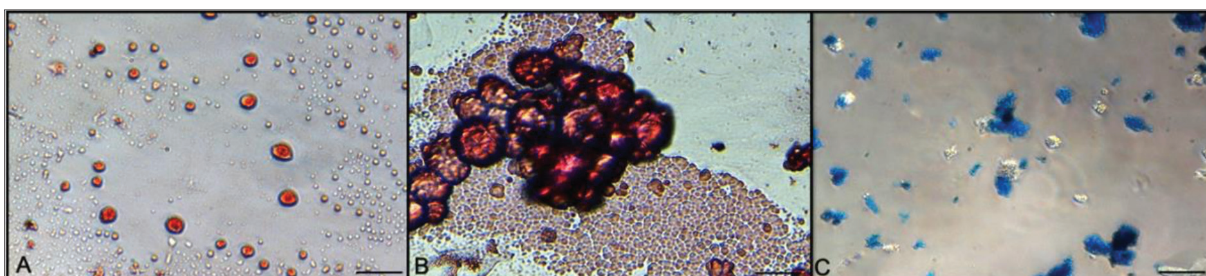

Fig 3. Adipogenic, osteogenic, chondrogenic differentiation of ADSCs were shown by Oil Red O (A), Alizarin red (B) and Alcian Blue (C) staining (Bar 25 µm)

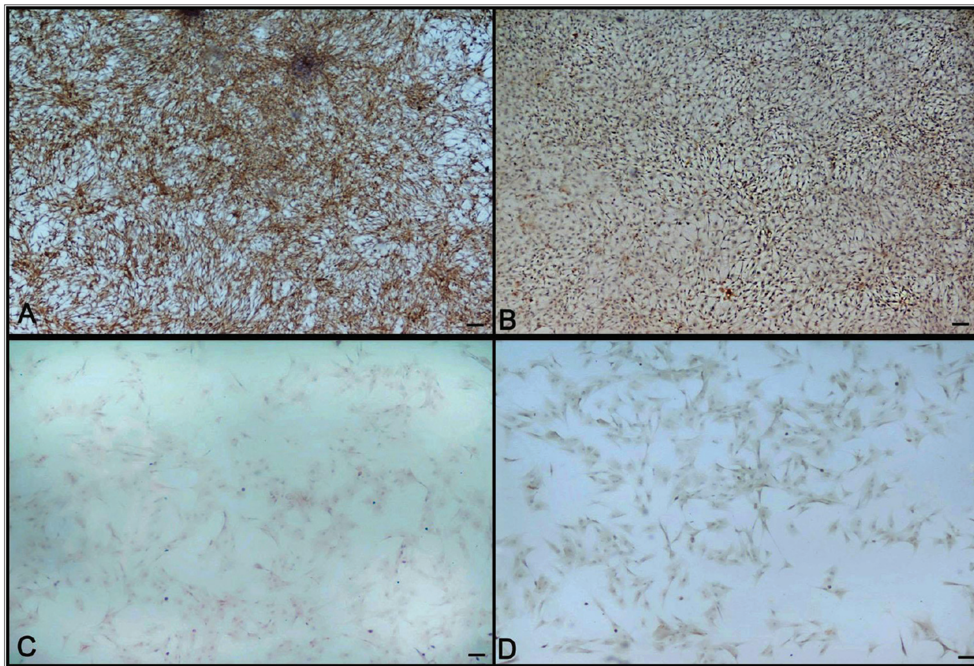

Fig 4. In P3 ADSCs: CD90 (A) and CD105 (B) were expressed positive reaction; CD 11b (C) and CD 45 (D) expressed negative reaction by immunohistochemistry (Bar 100 μm)

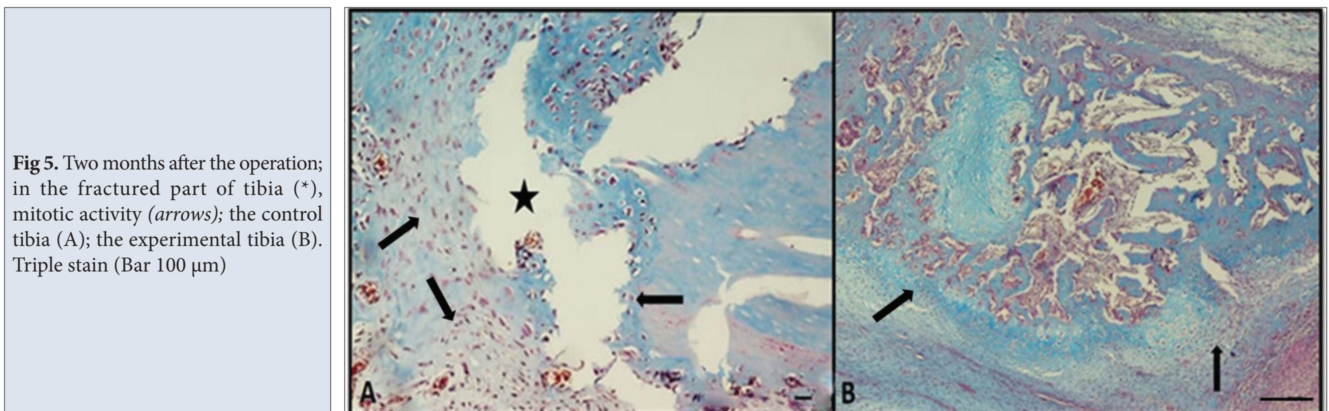

Fig 5. Two months after the operation; in the fractured part of tibia (*), mitotic activity *(arrows);* the control tibia (A); the experimental tibia (B). Triple stain (Bar 100 μm)

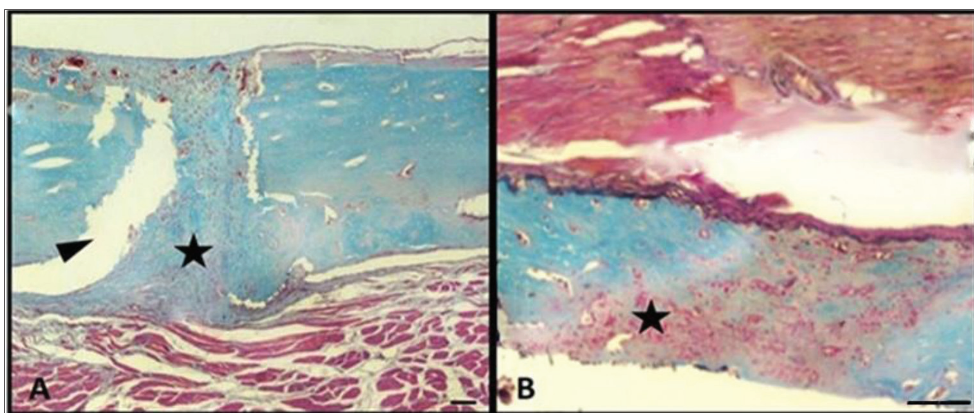

Fig 6. At 60th day after the operation; the control tibia (A); the experimental tibia (B). Fractured part of tibia *(arrow head)* the matrix of decalcified bone of the experimental tibias (B) were showed strongly eosinophilic reaction (*) than the control tibias. Triple stain (Bar 100 μm)

has been investigated histologically, clinically and radiologically in this study. ADSCs were preferred, because adipose tissue was a rich source of stem cells; thus, this study aimed to evaluate the osteogenic ability of grafted ADSCs on an absorbent material in rat tibias.

The distinct mechanisms of tissue regeneration mediated by stem cells are elucidated for many tissues [23,24]. Osteogenic regeneration depends on several factors including the biomaterial, bone quality, and surgical technique [25,26]. ADSCs and their extracellular secretions are major factors in osteogenesis; in this way, ADSCs represent an alternative

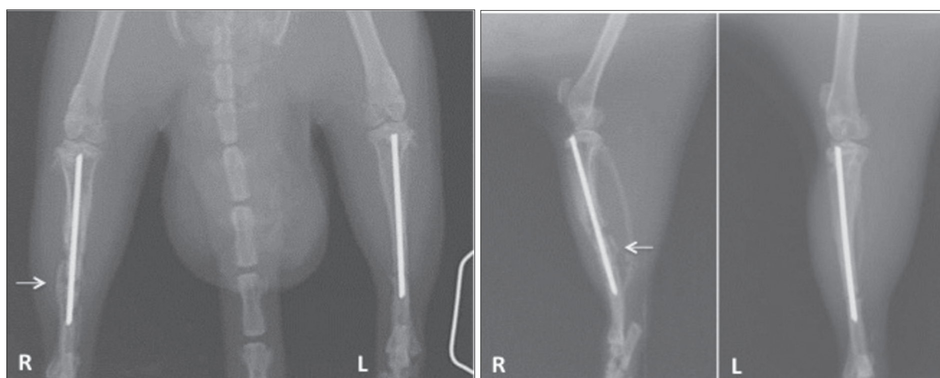

Fig 7. At 45th day after the operation, cranio-caudal and lateral radiographs of the tibias in a rat, it may be clearly seen that there was callus formation in the caudal and lateral parts of the experimental (right) tibia *(arrows)* and bilateral visible fractured lines

source of osteogenic cell potential [27,28]. In the presented study, it has been showed that expression pattern of cell surface markers on P3 cells are associated with ADSCs. These markers (CD90, CD105, CD 11b, CD 45) have also been used to determine multi-lineage differentiation capabilities of ADSCs and examined P3 cells are determined by lipogenic, osteogenic and chondrogenic differentiation capability *in vitro* that detected using Oil Red O, Alizarin red and Alcian Blue staining, respectively. As a result of these findings, passage 3 cells ADSCs were safely used in the injected with an absorbent material. The osteo differentiation of P3 cells increases with induction by osteogenic culture medium. The capability of these cells to secrete mineralized extracellular matrix (ECM) was confirmed by the gradual increase in alizarin red staining (illustration of mineralized nodules secreted by differentiated cells).

Because of the role of MSCs in tissue engineering, ADSCs can be considered as a preferable cell source, as periosteal stem cells have a higher proliferation rate and osteogenic potential [29]. This also indicates that both BMSSCs and ADSCs are unable to differentiate into osteoblasts/odontoblasts *in vivo* early in the transplantation process, but can also induce host cells to participate in tissue regeneration with the formation of a hematopoietic marrow and a pulp. Connective tissue aids osteogenesis by stimulating blood vessel growth and hematopoietic marrow formation in BMSSC transplants [23]. In this presented study, new bone formations (callus) were investigated radiologically and histologically, and then the experimental group results evaluated significant differences regarding new bone formation as compared with the control group. At two months after postoperatively, there was no necrosis in both groups and there was granulation tissue. Moreover, osteoblastic cell proliferation and new bone formation (callus) were higher in the experimental group than in the control group. According to these results, it could be implied that adipose tissue-derived stem cells had potentiate osteogenesis.

Important role of the periosteum and endosteum is nutrition of bone cells and provision of osteoblasts for bone histogenesis and repairmen [30]. These cells synthesize and secrete the osteoid matrix that is composed of glycoproteins and collagen. The secreted osteoid matrix has a high affinity for calcium salts that are brought into the area of bone formation by the circulatory system [31]. In this study, at two months after surgery the right (experimental) tibias had more blood vessels than the left (control) tibias. It was observed that the developmental difference in the ADSCs-treated groups sections were demonstrated with respect to the proliferation activity. The mitotic activity is prominent on the callus area of the right (experimental) tibias. It has been shown that ADSCs also regulate osteogenic activity and bone regeneration. In this way, in the experimental tibias, regeneration improved earlier compared with the control tibias, and the bone regeneration by osteoblasts is observed at fractured part of the right tibias. Furthermore, a strong eosinophilic reaction in the bone matrix of the right tibias than left tibia was also detected. The presence of the eosinophilic mineralized bone matrix area was suggested the presence of bone histogenesis [32]. These results provide evidence that ADSCs was stimulated osteoblast activity and increases mineralized bone volume. Development of the new growth factors in ADSCs cultures would be induce superior osteogenesis [33]. ADSCs has also been found to act on chondrocytes and osteoblast stimulating their proliferation. The histologically and radiologically results of this study strongly support the mesenchymal stem cells obtained from adipose tissue of rats has been found to act on osteoblast activity and increases mineralized bone volume. In addition, allogeneic ADSCs to be an effective agent that can be used to increase bone regeneration and osteogenesis.

As a conclusion, it can be emphasized that ADSCs has superiority for osteogenesis and easily accessible therapeutic agent, and veterinary practitioners can be use ADSCs as a ready-to-use product to increase the bone healing follow up surgery.

Availability of Data and Materials

The datasets during and/or analyzed during the current

study available from the corresponding author (T. İlhan) on reasonable request.

Funding Support

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Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Statement

This study was approved by the Bursa Uludag University Animal Experiments Local Ethics Committee (Approval no: 2018-02/03).

Author Contributions

Tİ, HS and HE planned, designed the experiment, analyzed all data and drafted manuscript. CÖA and Eİ collected samples, participated in cell culture analysis and collect data. Tİ, CÖA and Eİ responsible of histological examination and immunohistochemistry protocol. MÇ, UC and HS participated in surgical experimental protocol. All authors have read and agreed to the published version of the manuscript.

References

1. Han Y, Li X, Zhang Y, Han Y, Chang F, Ding J: Mesenchymal stem cells for regenerative medicine. *Cells*, 8 (8):886, 2019. DOI: 10.3390/cells8080886

2. Yoshimura H, Muneta T, Nimura A, Yokoyama A, Koga H, Sekiya I: Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle. *Cell Tissue Res,* 327, 449-462, 2007. DOI: 10.1007/s00441-006-0308-z

3. Jimenez-Puerta GJ, Marchal JA, López-Ruiz E, Galvez-Martin P: Role of mesenchymal stromal cells as therapeutic agents: Potential mechanisms of action and implications in their clinical use. *J Clin Med*, 9, 445, 2020. DOI: 10.3390/jcm9020445

4. Heo JS, Choi Y, Kim HS, Kim HO: Comparison of molecular profiles of human mesenchymal stem cells derived from bone marrow, umbilical cord blood, placenta and adipose tissue. *Int J Mol Med,* 37, 115-125, 2016. DOI: 10.3892/ijmm.2015.2413

5. Lendeckel S, Jödicke A, Christophis P, Heidinger K, Wolff J, Fraser JK, Hedrick MH, Berthold L, Howaldt HP: Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: Case report. *J Cranio-Maxillofacial Surg,* 32, 370-373, 2004. DOI: 10.1016/j.jcms.2004.06.002

6. Mazini L, Rochette L, Amine M, Malka G: Regenerative capacity of adipose derived stem cells (ADSCs), comparison with mesenchymal stem cells (MSCs). *Int J Mol Sci,* 20 (10): 2523, 2019. DOI: 10.3390/ijms20102523

7. Mildmay-White A, Khan W: Cell surface markers on adipose-derived stem cells: A systematic review. *Curr Stem Cell Res Ther,* 12 (6): 484-492, 2017. DOI: 10.2174/1574888X11666160429122133

8. Mohamed-Ahmed S, Yassin MA, Rashad A, Espedal H, Idris SB, Finne-Wistrand A, Mustafa K, Vindenes H, Fristad I: Comparison of bone regenerative capacity of donor-matched human adipose-derived and bone marrow mesenchymal stem cells. *Cell Tissue Res,* 383 (3): 1061-1075, 2021. DOI: 10.1007/s00441-020-03315-5

9. Jin HJ, Bae YK, Kim M, Kwon SJ, Jeon HB, Choi SJ, Kim SW, Yang YS, Oh W, Chang JW: Comparative analysis of human mesenchymal stem cells from bone marrow, adipose tissue, and umbilical cord blood as sources of cell therapy. *Int J Mol Sci,* 14 (9): 17986-8001, 2013. DOI: 10.3390/ijms140917986

10. Caplan AI: Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J Cell Physiol,* 213 (2): 341-347, 2007. DOI: 10.1002/jcp.21200

11. De Ugarte DA, Morizono K, Elbarbary A, Alfonso Z, Zuk PA, Zhu M, Dragoo JL, Ashjian P, Thomas B, Benhaim P, Chen I, Fraser J, Hedrick MH: Comparison of multi-lineage cells from human adipose tissue and bone marrow. *Cells Tissues Organs,* 174 (3): 101-109, 2003. DOI: 10.1159/000071150

12. Mafi R, Hindocha S, Mafi P, Griffin M, Khan WS: Sources of adult mesenchymal stem cells applicable for musculoskeletal applications - A systematic review of the literature. *Open Orthop J,* 5, 242-248, 2011. DOI: 10.2174/1874325001105010242

13. Gastaldi G, Asti A, Scaffino MF, Visai L, Saino E, Cometa AM, Benazzo F: Human adipose-derived stem cells (hASCs) proliferate and differentiate in osteoblast-like cells on trabecular titanium scaffolds. *J Biomed Mater Res*, 94 (3): 790-799, 2010. DOI: 10.1002/jbm.a.32721

14. Çerçi E, Erdost H: Rapid, practical and safe isolation of adipose derived stem cells. *Biotech Histochem*, 96 (2): 138-145, 2021. DOI: 10.1080/10520295.2020.1776895

15. Çerçi E, Erdost H: Non enzymatic isolation of adipose tissue and stromal vascular fraction derived cells. *Ankara Univ Vet Fak Derg,* 67, 295-301, 2020. DOI: 10.33988/auvfd.660139

16. Ghorbani A, Jalali SA, Varedi M: Isolation of adipose tissue mesenchymal stem cells without tissue destruction: A non-enzymatic method. *Tissue Cell,* 46 (1): 54-58, 2014. DOI: 10.1016/j.tice.2013.11.002

17. Özen A, Gül Sancak İ, Ceylan A, Özgenç Ö: Isolation of adipose tissue-derived stem cells. *Turk J Vet Anim Sci,* 40 (2): 137-141, 2016. DOI: 10.3906/vet-1505-54

18. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH: Multilineage cells from human adipose tissue: Implications for cell-based therapies. *Advances in Tissue Eng,* 7 (2): 211-228, 2001. DOI: 10.1089/107632701300062859

19. Elie N, Plancoulaine B, Signolle JP, Herlin P: A Simple way of quantifying immunostained cell nuclei on the whole histologic section. *Cytometry A,* 56 (1): 37-45, 2003. DOI: 10.1002/cyto.a.10075

20. Luna LG: Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology, 9-10. McGraw-Hill Book Company, New York, USA, 1968.

21. Bancroft JD, Gamble M: Theory and Practice of Histological Techniques. 274-280. Elsevier, Churchill Livingstone, 2002.

22. Crossmann G: A modification of Mallory's connective tissue stain with a discussion of the principles involved. *Anat Rec,* 69, 33-38, 1937. DOI: 10.1002/ar.1090690105

23. Koc ON, Gerson SL, Cooper BW, Dyhouse SM, Haynesworth SE, Caplan AI, Lazarus HM: Rapid hematopoietic recovery after coinfusion of autologous blood stem cells and culture expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high dose chemotherapy. *J Clin Oncol,* 18 (2): 307-316, 2000. DOI: 10.1200/JCO.2000.18.2.307

24. Budgude P, Kale V, Vaidya A: Mesenchymal stromal cell-derived extracellular vesicles as cell-free biologics for the *ex vivo* expansion of hematopoietic stem cells. *Cell Biol Int*, 44 (5): 1078-1102, 2020. DOI: 10.1002/cbin.11313

25. Sakka S, Coulthard P: Bone quality: A reality for the process of osseointegration. *Implant Dent,* 18 (6): 480-485, 2009. DOI: 10.1097/ID.0b013e3181bb840d

26. Yoon HG, Heo SJ, Koak JY, Kim SK, Lee SY: Effect of bone quality and implant surgical technique on implant stability quotient (ISQ) value. *J Adv Prosthodont,* 3 (1): 10-15, 2011. DOI: 10.4047/jap.2011.3.1.10

27. Stramandinoli-Zanicotti RT, Sassi LM, Rebelatto CLK, Boldrine-Leite LM, Brofman PRS, Calvalho AL: The effect of bone marrow-derived stem cells associated with platelet-rich plasma on the osseointegration of immediately placed implants. *J Clin Exp Dent*, 13 (1):e8-e13, 2021. DOI: 10.4317/jced.56743

28. Yu M, Zhou W, Song Y, Yu F, Li D, Na S, Zou G, Zhai M, Xie C: Development of mesenchymal stem cell-implant complexes by cultured cells sheet enhances osseointegration in type 2 diabetic rat model. *Bone,* 49 (3): 387-394, 2011. DOI: 10.1016/j.bone.2011.05.025

29. Ferreti C, Mattioli-Belmonte M: Periosteum derived stem cells for regenerative medicine proposals: Boosting current knowledge. *World J Stem Cells,* 6 (3): 266-277, 2014. DOI: 10.4252/wjsc.v6.i3.266

30. Chen X, Wang Z, Duan N, Zhu G, Schwarz EM, Xie C: Osteoblast–osteoclast interactions. *Connect Tissue Res,* 59 (2): 99-107, 2018. DOI: 10.1080/03008207.2017.1290085

31. Florencio-Silva R, Rodrigues da Silva Sasso G, Sasso-Cerri E, Simoes MJ, Cerri PS: Biology of bone tissue: Structure, function, and factors that influence bone cells. *Biomed Res Int,* 2015 (Article ID: 421746): 1-37, 2015. DOI: 10.1155/2015/421746

32. Stewart S, Bryant SJ, Ahn J, Hankenson KD: Bone Regeneration Translational Regenerative Medicine. 313-333. Academic Press, USA, 2015.

33. Chen Y, Lin J, Yu Y: Mesenchymal Stem Cells in Human Health and Diseases. **In,** Ahmed HK El-Hashas (Editor). 127-143. Academic Press, USA, 2020.

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Research Article

Effects of Prestorage Application of Gum Arabic Coating on the Quality of Table Eggs During Storage

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Abstract: The aim of this study was to investigate the effect of gum arabic coating on the quality characteristics of table eggs during 28 days of storage at 4°C and 25°C. Treatments were compared in a 2 x 4 factorial design with two different storage temperatures (4°C and 25°C) and five prestorage coating applications (control, 1% gum arabic (G1), 5% gum arabic (G5), and 10% gum arabic (G10) solution). Egg quality characteristics that were measured included weight loss, specific gravity, shell strength, Haugh unit, yolk index, and albumen pH. The eggs coated with 10% gum arabic solution had the lowest egg weight loss (2.71%), and albumen pH (9.18), and the highest egg specific gravity (1.057 g/cm3) at the end of storage (P<0.05). During the storage period, there were no significant differences between gum arabic coated and control eggs for shell strength, Haugh unit or yolk index. Eggs stored at 4°C for 28 days had a lower weight loss (1.86 %) and albumen pH (9.15), and higher egg specific gravity (1.068 g/cm^3), Haugh unit (69.96) and yolk index (0.41) than eggs stored at 25°C. The results suggest that applying a 10% gum arabic coating to eggs before they are stored might be a simple and effective way to keep them fresh.

Keywords: *Coating, Egg quality, Gum arabic, Storage, Table eggs*

Sofralık Yumurtalarda Depolama Öncesi Gam Arabik Kaplama Uygulamasının Yumurta Kalitesine Etkileri

Öz: Bu çalışmanın amacı gam arabik kaplaması uygulanmış sofralık yumurtların 4°C ve 25°C'de 28 günlük depolama süresince yumurta kalite özelliklerine etkisini araştırmaktır. Muameleler, iki farklı depolama sıcaklığı (4°C ve 25°C) ve beş adet depolama öncesi uygulama (kontrol, %1 gam arabik (G1), %5 gam arabik (G5) ve %10 gam arabik (G10)) ile 2 x 4 faktöriyel deneme deseninde karşılaştırılmıştır. Yumurta kalite özellikleri olarak yumurta ağırlık kaybı, özgül ağırlık, kabuk mukavemeti, Haugh birimi, yumurta sarısı indeksi ve ak pH'sı incelenmiştir. Depolama sonunda en düşük yumurta ağırlığı kaybı (%2.71) ve ak pH'sı (9.18) ve en yüksek yumurta özgül ağırlığı (1.057 g/cm3) değerleri %10 gam arabik solusyonu ile kaplanmış yumurtalarda tespit edilmiştir (P<0.05). Depolama süresince gam arabik kaplı yumurtalar ve kontrol yumurtaları arasında yumurta kabuğu mukavemeti, Haugh birimi ve yumurta sarısı indeksi bakımından önemli bir fark bulunmamıştır. 4°C'de 28 gün süre ile depolanan yumurtaların yumurta ağırlık kaybı (%1.86) ve ak pH (9.15)'ı 25°C'de depolanan yumurtalara göre daha düşük, yumurta özgül ağırlığı (1.068 g/cm^3), Haugh birimi (69.96) ve yumurta sarısı indeksi (0.41) değerlerinin ise daha yüksek olduğu tespit edilmiştir (P<0.05). Sonuç olarak, %10 gam arabik kaplama uygulamasının, depolama süresince yumurta kalitesini korumanın basit ve etkili bir yolu olabileceği düşünülmektedir.

Anahtar sözcükler: *Kaplama materyali, Yumurta kalitesi, Gam arabik, Depolama, Sofralık yumurta*

Introduction

Eggs are an important source of animal protein and also contain various nutritional compounds such as unsaturated fatty acids, vitamins, and minerals [1]. The quality of an egg is the highest when it is laid, and deterioration occurs depending on both environmental conditions and the duration of storage [2]. Depending on the storage conditions, a loss in egg weight [3], decreases in albumen height and Haugh unit [4-6], egg yolk index [6,7] and an increase in albumen pH [4-6,8] may be observed. After eggs are collected from the egg production system, they must

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be stored under appropriate conditions. On the other hand, eggs can be exposed to unexpected and undesired conditions at all the stages through farm to retail. Nowadays, both researchers and producers have focused on new technologies such as pulsed light, sonication, high hydrostatic pressure, ozonation, ultraviolet light, and coating to extend the shelf life of eggs and minimize the negative effects of environmental conditions to which they are exposed [9,10].

Various coating materials, such as propolis [7,11], chitosan [12-14], proteins [15], oils [16] and starch [17] have been used to eliminate or reduce the aforementioned problems during storage. Some coating materials extend the shelf-life of eggs. These materials prevent the penetration of microorganisms through the shell and the evaporation of albumen water from the shell, thereby reducing economic losses [17,18]. Gums are widely used as thickening, gelling and stabilizing agents [19,20] in many different areas including foods, creams and lotions, adhesives, inks, paper coating and ceramics. Due to the film forming feature, gum arabic has been used as an edible coating directly or in combination with different materials in recent years to extend the shelf life of fruits and vegetables as well as meat products such as meatballs [21-23]. Such long-term preservation is possible as the gum arabic film minimizes contact between the external environment and the coated material. Gum arabic films have been widely used in recent years as a coating material because they are natural, water soluble, can be easily removed and do not have negative effects on human health when consumed [24,25]. Upadhyaya et al.[26] investigated the efficacy of gum arabic-based coating materials for reducing *Salmonella enteritidis* on egg shell, and found that gum arabic-based coating significantly reduced the *Salmonella enteritidis* in the egg shell. Zhang et al.[27] evaluated the effects of pullulan with added glycerin, gum arabic, lysozyme, and chitinase on egg shell microbial activity and egg quality during storage, and reported that the composite pullulan coating could effectively inhibit the growth of *Escherichia coli, Staphylococcus aureus, Salmonella* sp., *Mucor* sp., and *Aspergillus* sp. and therefore have a good preservative effect.

According to the literature survey, there is a lack of knowledge about the usability of gum arabic as an egg coating material. So this study aimed to determine the changes in some quality parameters in table eggs stored at 4 and 25°C for 28 days after coated with different concentrations of gum arabic solution.

Material and Methods

Materials

A total of four hundred table eggs were obtained from H&N Nick-Chick laying hens (40 weeks old) reared on a commercial poultry farm (Konya, Turkey). The hens were fed a layer diet containing 2800 kcal of ME/kg and 17% crude protein.

Preparation of Solutions

Three different gum arabic solutions at concentrations of 1, 5, and 10% were used in this study. Briefly, 10, 50, and 100 g of gum arabic were weighed into separate flasks, and each flask was filled to 1000 ml with ultra pure water. To obtain homogeneous solutions, each gum arabic: water mixture was stirred using a magnetic stirrer (MSH-20D, Wisestir, Daihan) at 500 rpm and room temperature for 10 hours, and then kept in a fridge at +4°C without stirring overnight. The gum arabic solutions were prepared the day before use.

Application of Gum Arabic Solutions

Four hundred eggs were randomly divided into four equal groups. The eggs in the first group (one hundred) weren't treated with any gum arabic solutions and were used as a control group (C). The eggs in the second, third, and fourth groups were treated with gum arabic solution containing 1%, 5%, and 10%, respectively, and these experimental groups were named as G1, G5, and G10, in the same order. The solutions (1000 mL of each solution) were sprayed onto the eggs, using a hand sprayer (Mertcan, Turkey) to cover the whole surface. After application, 20 eggs were taken away from each group to make experiments at the beginning of the storage, and the rest of the eggs in the groups were also splitted into eight groups. The subdivided groups were stored at 4°C or 25°C for 7, 14, 21, or 28 days. Ten eggs were analyzed from each group (control, G1, G5, and G10) at every period and storage temperature. Eggs were collected daily and stored in ambient conditions for one day before the application of gum arabic. The air flow of the storage cabinets (Qualitec, Gc-1000, Turkey) was measured at 0.15 m/sn.

Egg Quality Analysis

After the eggs were coated, they were numbered and weighed, so that the egg weight loss during storage could be calculated. The egg weight loss, specific gravity, eggshell breaking strength, Haugh unit, yolk index, and albumen pH of ten eggs from each group were measured at the end of storage periods of 7, 14, 21, and 28 days, respectively. Egg weight was measured using a balance and recorded to the nearest 0.01 g. Specific gravity was estimated by the Archimedes' method, using the following formula: Specific gravity = Egg weight (g)/(Egg weight – Egg weight in water) [28]. Eggshell strength (kg) was measured with a device for measuring eggshell resistance (Egg Force Reader, 06-UM-001, Version B, Orka Food Tech. Ltd., Hong Kong, China). The height of the albumen was measured using a digital height gauge (Egg Analyser, 05-UM-001, Version B, Orka Food Tech. Ltd., Hong

Kong, China). The Haugh unit was calculated using the following formula: Haugh unit = 100 log $(H + 7.57 - 1.7W^{0.37})$, where H is albumen height in millimeters and W is egg weight in grams [29]. After the eggs had been broken, the yolk was separated from the albumen and placed on a flat glass surface. The height of the yolk was measured using a digimatic height gauge (Tresna, IP54, USA), and the width of the yolk was measured using a micrometer caliper (Mitutoyo, Japan). The yolk index was calculated using the following formula: Yolk index = Yolk height/ Yolk diameter [30]. Albumen pH was measured using a pH meter (Mettler Toledo, Switzerland).

Statistical Analysis

The study was organized as a randomized plot of trials, with three covering materials (1%, 5%, and 10% gum arabic), and a control group. At each measurement period, egg quality analyses were carried out in 10 replicates for each group. Variance homogeneity and Gaussian distribution, which are parametric test assumptions, were checked by Levene's test and the Shapiro Wilks test, respectively. The parametric test conditions were satisfied by various transformations of non-normally distributed data. The data on egg weight, egg weight loss, specific gravity, shell strength, Haugh unit, yolk index, and albumen pH were analyzed using one way analysis of variance (ANOVA) followed by a post hoc Tukey's multiple comparison test for comparison between different treatment groups. A value of $P<0.05$ was considered statistically significant (Minitab 16).

Results

Egg Weight Loss (%)

Table 1 shows the effects of storage temperature, gum coating, and storage x gum coating interaction on egg weight loss (%). Storage x gum coating interaction had a significant effect on egg weight loss only at 21 and 28 days of storage ($P<0.05$). The lowest egg weight loss was recorded in G10 after 21 and 28 days of storage at 25°C. However, there was no significant difference in egg weight loss among treatment groups at 4°C after 28 days of storage. Egg weight loss was 3.23% higher in the eggs stored at 25°C than in the eggs stored at 4°C after 28 days of storage.

Egg Specific Gravity (g/cm³)

Egg specific gravity values are given in *Table 2*. The effect of storage temperature x gum coating interaction on the specific gravity of eggs was significant only on day 28 of the storage period ($P<0.05$). No significant differences in egg specific gravity were found among treatment groups at 4°C after 28 days of storage, while the highest egg specific gravity was found in G10 after 28 days of storage at 25°C. Egg specific weight was affected by storage temperature in all storage periods ($P<0.05$). The specific gravity of eggs stored at 4°C was higher than those stored at 25°C in all periods.

Egg Shell Strength (kg)

Table 3 presents eggshell strength values and the effects

Table 1. *The effect of storage temperature, gum coating and their interaction on egg weight loss*

Treatment		n	Fresh Egg Weight (g)	Egg Weight Loss (%)			
				7 days	14 days	21 days	28 days
Storage Temperature (°C)	25	40	53.10	1.38	2.56	3.83	5.09
	4	40	54.68	0.65	0.82	1.30	1.86
	SEM		0.578	0.107	0.046	0.066	0.104
	P-value		>0.05	<0.05	<0.05	<0.05	<0.05
Gum Arabic [1]	C	20	54.48	0.82	1.73	2.48^{b}	3.82^{a}
	G1	20	54.90	1.28	1.80	2.92^{a}	3.56^{b}
	G5	20	52.88	1.03	1.70	2.64^{ab}	3.80^{ab}
	G10	20	53.32	0.92	1.54	2.21^{c}	2.71^{c}
	SEM		0.817	0.151	0.065	0.093	0.147
	P-value		>0.05	>0.05	>0.05	<0.05	<0.05
Storage Temperature (°C) x Gum Arabic	25 x C	10	52.92	1.23	2.60	3.82^{b}	5.52^{a}
	25 x G1	10	53.63	1.41	2.70	4.37^{a}	5.36^{a}
	25 x G5	10	52.21	1.37	2.57	3.75^{b}	5.60^{a}
	25 x G10	10	53.64	1.50	2.40	3.37^{c}	3.90^{b}
	4 x C	10	56.03	0.41	0.85	1.14^{ef}	2.12^{c}
	4 x G1	10	56.16	1.16	0.91	1.48^{de}	1.77^{c}
	4 x G5	10	53.54	0.69	0.83	1.53^{d}	2.01^{c}
	4 x G10	10	53.00	0.34	0.67	1.05^{f}	1.52^{c}
	SEM		1.156	0.213	0.092	0.131	0.207
	P-value		>0.05	>0.05	>0.05	<0.05	<0.05

*[a-f] Means within a column with different superscripts differ significantly (P<0.05); [1] **C:** Control (with no application); **G1:** 1% gum arabic coating; **G5:** 5% gum arabic coating; **G10:** 10% gum arabic coating; **SEM:** Standard Error Mean*

Table 2. *The effect of storage temperature, gum coating and their interaction on egg specific gravity*

Treatment		n	Fresh Egg Specific Gravity (g/cm^3)	Egg Specific Gravity (g/cm^3)			
				7 days	14 days	21 days	28 days
Storage Temperature (°C)	25	40	1.089	1.071	1.059	1.046	1.031
	4	40	1.089	1.081	1.077	1.073	1.068
	SEM		0.001	0.001	0.001	0.001	0.001
	P-value		>0.05	<0.05	<0.05	<0.05	<0.05
Gum Arabic[1]	C	20	1.088	1.077	1.069	1.062^{ab}	1.047^{b}
	G1	20	1.089	1.075	1.066	1.055^{c}	1.048^{b}
	G5	20	1.089	1.075	1.068	1.058^{bc}	1.046^{b}
	G10	20	1.088	1.079	1.069	1.064^{a}	1.057^{a}
	SEM		0.001	0.001	0.001	0.001	0.002
	P-value		>0.05	>0.05	>0.05	<0.05	<0.05
Storage Temperature x Gum Arabic	25 x C	10	1.086	1.072	1.058	1.048	1.027^{c}
	25 x G1	10	1.089	1.071	1.058	1.041	1.027^{c}
	25 x G5	10	1.091	1.068	1.058	1.045	1.026^{c}
	25 x G10	10	1.089	1.074	1.061	1.051	1.045^{b}
	4 x C	10	1.090	1.082	1.079	1.075	1.066^{a}
	4 x G1	10	1.089	1.079	1.074	1.069	1.069^{a}
	4 x G5	10	1.088	1.081	1.077	1.070	1.066^{a}
	4 x G10	10	1.088	1.083	1.077	1.077	1.070^{a}
	SEM		0.002	0.002	0.002	0.002	0.002
	P-value		>0.05	>0.05	>0.05	>0.05	<0.05

*[a-c] Means within a column with different superscripts differ significantly (P<0.05); [1] **C:** Control (with no application); **G1:** 1% gum arabic coating, **G5:** 5% gum arabic coating; **G10:** 10% gum arabic coating; **SEM:** Standard Error Mean*

of storage temperature, gum coating, and storage x gum coating interaction. The effect of storage temperature on eggshell strength was only significant on day 14 of storage (P<0.05). The egg shell strength of eggs stored at 25°C was higher than those stored at 4°C on the 14th day of storage.

Haugh Unit

In *Table 4*, the effects of storage temperature, gum coating, and their interaction on the Haugh unit are presented. Storage temperature had a significant effect on the Haugh unit at 14, 21, and 28 days of storage (P<0.05). The Haugh unit of eggs stored at 4°C was higher than that of eggs stored at 25°C on the 14th, 21st, and 28th days of storage. There were no effects of gum treatments and their interaction on the Haugh unit in any of the storage periods.

Yolk Index

The effects of storage temperature, gum coating, and their interaction on the yolk index are shown in *Table 5*. Storage temperature x gum coating interaction had a statistically significant effect on the yolk index only on day 21 of storage (P<0.05). The lowest yolk index was recorded in G1 after 21 days of storage at 25°C, while the highest yolk index was obtained for C after 21 days of storage at 4°C.

Albumen pH

In *Table 6*, the effects of storage temperature, gum coating, and their interaction on albumen pH are presented. Storage temperature x gum coating interaction effects on the albumen pH of eggs were significant only on the 7th, 14th, and 21st days of storage (P<0.05). The lowest albumen pH was obtained in G10 after 14 and 21 days of storage at 4°C.

The effect of storage temperature on albumen pH was significant at 14, 21, and 28 days of storage (P<0.05). The albumen pH of eggs stored at 4°C was lower than the albumen pH of eggs stored at 25°C on the 14th, 14th, and 21st days of storage.

Discussion

As can be seen from *Table 1*, the effect of gum arabic coating on egg weight loss (%) was significant only on the 21st and 28th days (P<0.05). The lowest egg weight loss was found in G10 after 21 and 28 days of storage (P<0.05). No significant differences in egg weight loss were found between the C and G5 after 21 and 28 days of storage. Similar results were observed in eggs coated with oils [31], chitosan [32] and proteins [33]. Ideally, egg weight loss should be low during storage. Egg weight loss during the storage period occurs as water in the albumen evaporates through the eggshell pores [34-37]. This study found that when gum is applied to eggs, especially 10% gum, it makes a protective layer that stops water from evaporating through the egg shell pores, which reduces egg weight loss while they are in storage.

The effect of gum arabic coating on egg specific gravity was significant on the 21st and 28th days of storage (P<0.05). The highest egg specific gravity was detected in G10 after 28 days of storage. Egg specific gravity is considered an

Table 3. The effect of storage temperature, gum coating and their interaction on egg shell strength

Treatment		n	Fresh Egg Shell Strength (kg)	Egg Shell Strength (kg)			
				7 days	14 days	21 days	28 days
Storage Temperature (°C)	25	40	3.916	3.678	4.322	4.620	4.681
	4	40	4.219	3.811	4.665	4.529	4.611
	SEM		0.163	0.122	0.111	0.081	0.099
	P-value		>0.05	>0.05	<0.05	>0.05	>0.05
Gum Arabic[1]	C	20	4.213	3.845	4.348	4.637	4.778
	G1	20	4.126	3.650	4.608	4.376	4.617
	G5	20	3.955	3.568	4.681	4.511	4.531
	G10	20	3.977	3.914	4.336	4.772	4.659
	SEM		0.213	0.172	0.156	0.114	0.139
	P-value		>0.05	>0.05	>0.05	>0.05	>0.05
Storage Temperature (°C) x Gum Arabic	25 x C	10	4.147	3.868	3.901	4.738	4.899
	25 x G1	10	3.841	3.767	4.536	4.409	4.573
	25 x G5	10	3.963	3.224	4.572	4.543	4.344
	25 x G10	10	3.715	3.851	4.278	4.788	4.910
	4 x C	10	4.278	3.822	4.795	4.535	4.657
	4 x G1	10	4.411	3.534	4.680	4.344	4.661
	4 x G5	10	3.947	3.912	4.789	4.479	4.718
	4 x G10	10	4.239	3.976	4.394	4.756	4.409
	SEM		0.302	0.243	0.221	0.161	0.197
	P-value		>0.05	>0.05	>0.05	>0.05	>0.05

[1] ***C:*** *Control (with no application);* ***G1:*** *1% gum arabic coating;* ***G5:*** *5% gum arabic coating;* ***G10:*** *10% gum arabic coating;* ***SEM:*** *Standard Error Mean*

Table 4. The effect of storage temperature, gum coating and their interaction on Haugh unit

Treatment		n	Fresh Egg Haugh Unit	Haugh Unit			
				7 days	14 days	21 days	28 days
Storage Temperature (°C)	25	40	82.03	73.79	61.70	64.11	62.99
	4	40	77.56	76.22	75.72	78.29	69.96
	SEM		1.937	1.461	1.752	0.869	1.410
	P-value		>0.05	>0.05	<0.05	<0.05	<0.05
Gum Arabic[1]	C	20	82.84	71.91	69.45	69.25	67.31
	G1	20	80.38	77.29	66.35	71.59	64.93
	G5	20	80.14	73.73	67.50	70.77	67.19
	G10	20	75.82	77.09	71.52	73.19	66.48
	SEM		2.737	2.066	2.422	1.229	1.994
	P-value		>0.05	>0.05	>0.05	>0.05	>0.05
Storage Temperature (°C) x Gum Arabic	25 x C	10	85.10	74.81	59.88	63.70	61.87
	25 x G1	10	80.17	73.19	58.12	63.48	63.25
	25 x G5	10	83.71	70.81	62.70	63.11	64.72
	25 x G10	10	79.13	76.35	66.10	66.16	62.13
	4 x C	10	80.59	69.01	79.01	74.80	72.74
	4 x G1	10	80.58	81.38	74.59	79.70	66.61
	4 x G5	10	76.56	76.65	72.32	78.43	69.66
	4 x G10	10	72.51	77.83	76.95	80.22	70.83
	SEM		3.868	2.921	3.351	1.737	2.816
	P-value		>0.05	>0.05	>0.05	>0.05	>0.05

[1] ***C:*** *Control (with no application);* ***G1:*** *1% gum arabic coating;* ***G5:*** *5% gum arabic coating;* ***G10:*** *10% gum arabic coating;* ***SEM:*** *Standard Error Mean*

important indicator of egg shell quality [38] Eggs with a higher specific gravity have a stronger eggshell, which is good for the egg industry [39].

The effects of gum coating and storage temperature x gum coating interaction on egg shell strength were not significant in any of the storage periods *(Table 3)*. Yuceer and Caner [40] stated that there were no significant differences in shell strength between coated and uncoated eggs during storage. On the other hand, the shell of uncoated (control) eggs exhibited significantly lower puncture strength at both the top and bottom than chitosan-coated eggs [41]. Similarly, Yüceer and Caner [42] reported that coating with

***Table 5.** The effect of storage temperature, gum coating and their interaction on egg yolk index*

Treatment		**n**	**Fresh Egg Yolk Index**	**Yolk Index**			
				7 days	**14 days**	**21 days**	**28 days**
Storage Temperature (°C)	25	40	0.43	0.38	0.37	0.38	0.27
	4	40	0.44	0.43	0.45	0.53	0.41
	SEM		0.004	0.003	0.005	0.005	0.006
	P-value		>0.05	<0.05	<0.05	<0.05	<0.05
Gum Arabic[1]	C	20	0.43	0.40	0.41^{a}	0.47	0.33^{ab}
	G1	20	0.43	0.41	0.41^{a}	0.45	0.32^{b}
	G5	20	0.43	0.40	0.41^{a}	0.46	0.35^{ab}
	G10	20	0.43	0.41	0.40^{b}	0.46	0.36^{a}
	SEM		0.006	0.005	0.007	0.007	0.009
	P-value		>0.05	>0.05	<0.05	>0.05	<0.05
Storage Temperature (°C) x Gum Arabic	25 x C	10	0.42	0.37	0.38	0.38^{dc}	0.28
	25 x G1	10	0.43	0.38	0.37	0.36^{d}	0.24
	25 x G5	10	0.44	0.38	0.36	0.39^{dc}	0.28
	25 x G10	10	0.43	0.39	0.35	0.39^{c}	0.29
	4 x K	10	0.45	0.43	0.45	0.55^{a}	0.38
	4 x G1	10	0.44	0.43	0.45	0.54^{ab}	0.41
	4 x G5	10	0.43	0.42	0.46	0.53^{ab}	0.42
	4 x G10	10	0.43	0.42	0.43	0.52^{b}	0.42
	SEM		0.009	0.007	0.010	0.010	0.012
	P-value		>0.05	>0.05	>0.05	<0.05	>0.05

*[a-d] Means within a column with different superscripts differ significantly (P<0.05); [1] **C:** Control (with no application); **G1:** 1% gum arabic coating; **G5:** 5% gum arabic coating; **G10:** 10% gum arabic coating; **SEM:** Standard Error Mean*

***Table 6.** The effect of storage temperature, gum coating and their interaction on albumen pH*

Treatment		**n**	**Fresh Albumen pH**	**Albumen pH**			
				7 days	**14 days**	**21 days**	**28 days**
Storage Temperature (°C)	25	40	8.80	9.19	9.41	9.34	9.37
	4	40	8.79	9.19	9.12	9.03	9.15
	SEM		0.024	0.039	0.011	0.011	0.012
	P-value		>0.05	>0.05	<0.05	<0.05	<0.05
Gum Arabic[1]	C	20	8.79	9.21	9.27^{b}	9.25^{a}	9.28^{a}
	G1	20	8.78	9.19	9.33^{a}	9.24^{a}	9.30^{a}
	G5	20	8.79	9.13	9.31^{a}	9.20^{a}	9.29^{a}
	G10	20	8.81	9.25	9.16^{c}	9.07^{b}	9.18^{b}
	SEM		0.033	0.055	0.015	0.016	0.017
	P-value		>0.05	>0.05	<0.05	<0.05	<0.05
Storage Temperature (°C) x Gum Arabic	25 x C	10	8.82	9.11^{ab}	9.47^{a}	9.40^{a}	9.40
	25 x G1	10	8.76	9.29^{a}	9.49^{a}	9.40^{a}	9.40
	25 x G5	10	8.81	9.22^{ab}	9.44^{a}	9.31^{b}	9.39
	25 x G10	10	8.80	9.16^{ab}	9.26^{b}	9.26^{b}	9.30
	4 x C	10	8.76	9.30^{a}	9.08^{d}	9.10^{c}	9.16
	4 x G1	10	8.80	9.10^{ab}	9.16^{c}	9.07^{c}	9.19
	4 x G5	10	8.78	9.03^{b}	9.18^{c}	9.08^{c}	9.19
	4 x G10	10	8.81	9.33^{a}	9.07^{d}	8.89^{d}	9.07
	SEM		0.047	0.077	0.022	0.022	0.024
	P-value		>0.05	<0.05	<0.05	<0.05	>0.05

*[a-d] Means within a column with different superscripts differ significantly (P<0.05); [1] **C:** Control (with no application); **G1:** 1% gum arabic coating; **G5:** 5% gum arabic coating; **G10:** 10% gum arabic coating; **SEM:** Standard Error Mean*

shellac and lysozyme-chitosan significantly increased egg shell strength. The shell strength of a table egg is an important economic issue for egg producers. The higher the breaking strength, the fewer losses due to breakage during the collection, transportation, and storage of the egg, which will provide an economic gain.

The fact that coating eggs with gum arabic did not affect the Haugh unit in any storage period *(Table 4)* was found to be consistent with the results reported by Xu et al.[32] who found no significant difference in the Haugh unit between chitosan coated and control eggs after 31 and 36 days of storage at 25°C. Similarly, there were no significant differences in the Haugh unit between control and eggs coated with whey protein concentrate and rice bran oil for 28 days at room temperature [43]. On the other hand, the Haugh unit of coated eggs was significantly higher than that of control eggs during storage [13]. The Haugh unit is the primary indicator of quality in the egg industry [33] and the higher the Haugh unit, the better the albumen quality of the egg. The Haugh unit decreases with the decrease of the albumen height during storage as a result of the increase in clusterin and ovoinhibitory concentrations in albumen and the disordering of the ovalbumin structure [44].

The effect of storage temperature on the yolk index was significant in all storage periods (P<0.05). The yolk index of eggs stored at 4°C was higher than the yolk index of eggs stored at 25°C on the 7th, 14th, 21st, and 28th days of storage. This result seems to be in agreement with studies showing that the yolk index decreases significantly with increasing storage temperature [45-48]. The effect of the gum treatment on yolk index was significant at 14 and 28 days of storage (P<0.05). The lowest yolk index was in G10 on the 14th day of storage, but the yolk index of G10 did not significantly differ from control on the 28th day of storage. These results conflict with the findings of Caner and Cansiz [13], Xu et al.[32], Safavi and Javanmard [43] who found that coated eggs exhibited a significantly higher yolk index than un-coated eggs during storage. The egg yolk index is a measure of the strength of the yolk vitelline membrane and can be used to indicate freshness. The higher the egg yolk index, the better the yolk quality [49]. As a result of the weakening of the egg yolk vitelline membrane, the water in the egg white diffuses into the egg yolk and the egg yolk index decreases [45,48].

The effect of gum treatment on albumen pH was significant at 14, 21, and 28 days of storage (P<0.05). After 14, 21, and 28 days, G10 had a lower albumen pH than the other treatment groups (P<0.05). These results are consistent with the studies of Biladeau and Keener [33], and Caner and Cansiz [41] in which eggs were coated using chitosan and whey protein, respectively. As the storage period increases, albumen pH increases due to the removal of CO_2 through pores in the eggshell [36,48,50]. According to our results, the gum arabic coating acts as a barrier against CO_2 loss from the albumen through the eggshell pores.

As a result, coating the eggs with gum arabic had no effect on eggshell strength, Haugh unit, or yolk index. In addition, it was determined that the eggs coated with 10% gum arabic had better results in terms of egg weight, specific gravity, and white pH compared to the eggs in the other experimental groups. It is thought that 10% gum arabic coating on table eggs will be effective in maintaining freshness. Similarly, as the storage temperature increased, most of the egg quality characteristics decreased. This study may contribute to the preservation of egg quality characteristics for a longer period of time during storage in the egg industry. Future research should focus on combining gum arabic with different coating materials to enhance its physical barrier properties.

Availability of Data and Materials

The datasets used and/or analyzed during the present study are available from the corresponding author (a. Aygün) on reasonable request.

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Competing Interests

The authors have no competing interests to declare that are relevant to the content of this article.

Authors' Contributions

VS: Conceptualization, Investigation; AA: Conceptualization, Validation, Supervision, Writing - review & editing; HC: Methodology; DN: Methodology; MA: Methodology

References

1. Chambers JR, Zaheer K, Akhtar H, Abdel-Aal E-SM: Chicken eggs. **In,** Hater P (Ed): Egg Innovations and Strategies for Improvementsed. 1-9, Elsevier, 2017.

2. Usaga J, Acosta O, Sullivan EK, Padilla-Zakour OI: Pickling eggs. **In,** Hater P (Ed): Egg Innovations and Strategies for Improvementsed., 405-413, Elsevier, 2017.

3. Akyurek H, Okur AA: Effect of storage time, temperature and hen age on egg quality in free-range layer hens. *J Anim Vet Adv,* 8 (10): 1953-1958, 2009.

4. Scott T, Silversides FG: The effect of storage and strain of hen on egg quality. *Poult Sci,* 79 (12): 1725-1729, 2000. DOI: 10.1093/ps/79.12.1725

5. Aktan S: Effects of age and storage duration on relationships among albumen quality traits and egg weight in Japanese quails. *J Anim Vet Adv,* 10 (Suppl.): 3340-3344, 2011. DOI: 10.3923/javaa.2011.3340.3344

6. Baylan M, Canogullari S, Ayasan T, Copur G: Effects of dietary selenium source, storage time, and temperature on the quality of quail eggs. *Biol Trace Elem Res,* 143 (2): 957-964, 2011. DOI: 10.1007/s12011-010-8912-x

7. Copur G, Camci O, Sahinler N, Gul A: The effect of propolis egg shell coatings on interior egg quality. *Arch Geflugelkd,* 72 (1): 35-40, 2008.

8. Silversides FG, Budgell K: The relationships among measures of egg albumen height, pH, and whipping volume. *Poult Sci,* 83 (10): 1619-1623, 2004. DOI: 10.1093/ps/83.10.1619

9. Agregán R, Munekata PES, Putnik P, Pateiro M, Bursać Kovačević D, Zavadlav S, Lorenzo JM: The use of novel technologies in egg processing. *Food Rev Int,* 1-21, 2021. DOI: 10.1080/87559129.2021.1980887

10. Yüceer M, Caner C: The impact of coatings and novel processing techniques on the functionality of table eggs during extended storage period at ambient temperature. *J Food Process Preserv,* 45 (3):e15261, 2021. DOI:

10.1111/JFPP.15261

11. Sahinler N, Gul A, Copur G: Chemical composition and preservative effect of Turkish propolis on egg quality durig storage. *Asian J Chem,* 21 (3): 1877-1886, 2009.

12. Bhale S, No HK, Prinyawiwatkul W, Farr AJ, Nadarajah K, Meyers SP: Chitosan coating improves shelf life of eggs. *J Food Sci,* 68 (7): 2378-2383, 2003. DOI: 10.1111/j.1365-2621.2003.tb05776.x

13. Caner C, Cansiz O: Effectiveness of chitosan-based coating in improving shelf-life of eggs. *J Sci Food Agric,* 87 (2): 227-232, 2007. DOI: 10.1002/jsfa.2698

14. Yi SZ, Liu Q, Xu D, Li DM: Preservation effects of chitosan/nano-montmorillonite coating on clean eggs. *Packag Eng,* 7:12, 2017.

15. Caner C, Yuceer M: Maintaining functional properties of shell eggs by ultrasound treatment. *J Sci Food Agric,* 95 (14): 2880-2891, 2015. DOI: 10.1002/jsfa.7029

16. Jones DR, Ward GE, Regmi P, Karcher DM: Impact of egg handling and conditions during extended storage on egg quality. *Poult Sci,* 97 (2): 716-723, 2018. DOI: 10.3382/ps/pex351

17. Eddin AS, Ibrahim SA, Tahergorabi R: Egg quality and safety with an overview of edible coating application for egg preservation. *Food Chem,* 296, 29-39, 2019. DOI: 10.1016/j.foodchem.2019.05.182

18. Aygun A: The eggshell microbial activity. **In,** Hester P (Ed): Egg Innovation and Strategies for Improvemented. 135-144, Academic Press, Elsevier, Oxford, United Kingdom, 2017.

19. Saha D, Bhattacharya S: Hydrocolloids as thickening and gelling agents in food: A critical review. *J Food Sci Tchnol,* 47 (6): 587-597, 2010. DOI: 10.1007/s13197-010-0162-6

20. Cong L, Zou B, Palacios A, Navarro ME, Qiao G, Ding Y: Thickening and gelling agents for formulation of thermal energy storage materials - A critical review. *Renew Sust Energ Rev*, 155:111906, 2022. DOI: 10.1016/j.rser.2021.111906

21. Ali A, Maqbool M, Ramachandran S, Alderson PG: Gum arabic as a novel edible coating for enhancing shelf-life and improving postharvest quality of tomato *(Solanum lycopersicum* L.) fruit. *Postharvest Biol Technol,* 58 (1): 42-47, 2010. DOI: 10.1016/j.postharvbio.2010.05.005

22. Salehi F: Edible coating of fruits and vegetables using natural gums: A review. *Int J Fruit Sci,* 20 (Supppl. 2): S570-S589, 2020. DOI: 10.1080/15538362.2020.1746730

23. Zhou X, Zong X, Zhang M, Ge Q, Qi J, Liang J, Xu X, Xiong G: Effect of konjac glucomannan/carrageenan-based edible emulsion coatings with camellia oil on quality and shelf-life of chicken meat. *Int J Biol Macromol,* 183, 331-339, 2021. DOI: 10.1016/j.ijbiomac.2021.04.165

24. Ali BH, Ziada A, Blunden G: Biological effects of gum arabic: A review of some recent research. *Food Chem Toxicol,* 47 (1): 1-8, 2009. DOI: 10.1016/j.fct.2008.07.001

25. Xu T, Gao C, Feng X, Yang Y, Shen X, Tang X: Structure, physical and antioxidant properties of chitosan-gum arabic edible films incorporated with cinnamon essential oil. *Int J Biol Macromol,* 134, 230-236, 2019. DOI: 10.1016/j.ijbiomac.2019.04.189

26. Upadhyaya I, Yin HB, Nair MS, Chen CH, Lang R, Darre MJ, Venkitanarayanan K: Inactivation of *Salmonella enteritidis* on shell eggs by coating with phytochemicals. *Poult Sci,* 95 (9): 2106-2111, 2016. DOI: 10.3382/ps/pew152

27. Zhang Y, Guo S, Zhang B, Ma M, Cai Z, Huang X, Fu X: Preparation and application of composite antibacterial pullulan coating in the preservation of eggs. *Food Sci,* 40 (11): 213-219, 2019. DOI: 10.7506/spkx1002-6630-20180315-188

28. Wells R: A study of the hen's egg. *British Egg Marketing Board Symposium,* Edinburgh. 207-249, 1968.

29. Haugh RR: The Haugh unit for measuring egg quality. *US Egg Poult Mag,* 43, 522-573, 1937.

30. Funk E: The relation of the yolk index determined in natural position to the yolk index as determined after separating the yolk from the albumen. *Poult Sci,* 27 (3): 367-367, 1948.

31. Homsaard N, Kodsangma A, Jantrawut P, Rachtanapun P, Leksawasdi N, Phimolsiripol Y, Seesuriyachan P, Chaiyaso T, Sommano SR, Rohindra D, Jantanasakulwong K: Efficacy of cassava starch blending with gelling agents and palm oil coating in improving egg shelf life. *Int J Food Sci Technol*, 56, 3655-3661, 2020. DOI: 10.1111/ijfs.14675

32. Xu D, Wang J, Ren D, Wu X: Effects of chitosan coating structure and changes during storage on their egg preservation performance. *Coatings,* 8 (9): 317, 2018. DOI: 10.3390/coatings8090317

33. Biladeau A, Keener KM: The effects of edible coatings on chicken egg quality under refrigerated storage. *Poult Sci,* 88 (6): 1266-1274, 2009. DOI: 10.3382/ps.2008-00295

34. Vlčková J, Tůmová E, Míková K, Englmaierová M, Okrouhlá M, Chodová D: Changes in the quality of eggs during storage depending on the housing system and the age of hens. *Poult Sci*, 98, 6187-6193, 2019. DOI: 10.3382/ps/pez401

35. Pires PGS, Leuven AFR, Franceschi CH, Machado GS, Pires PDS, Moraes PO, Kindlein L, Andretta I: Effects of rice protein coating enriched with essential oils on internal quality and shelf life of eggs during room temperature storage. *Poult Sci,* 99 (1): 604-611, 2020. DOI: 10.3382/ps/pez546

36. Eroglu M, Baykalir Y, Erisir Z: The effect of different storage period on some egg quality characteristics and ovalbumin levels in goose eggs. *Emir J Food Agr,* 33 (8): 699-703, 2021. DOI: 10.9755/ejfa.2021.v33.i8.2741

37. Drabik K, Próchniak T, Kasperek K, Batkowska J: The use of the dynamics of changes in table eggs during storage to predict the age of eggs based on selected quality traits. *Animals,* 11 (11): 3192, 2021. DOI: 10.3390/ani11113192

38. Ingram DR, Hatten III LF, Homan KD: A study on the relationship between eggshell color and eggshell quality in commercial broiler breeders. *Int J Poult Sci,* 7, 700-703, 2008. DOI: 10.3923/IJPS.2008.700.703

39. De Ketelaere B, Govaerts T, Coucke P, Dewil E, Visscher J, Decuypere E, De Baerdemaeker J: Measuring the eggshell strength of 6 different genetic strains of laying hens: Techniques and comparisons. *Br Poult Sci,* 43 (2): 238-244, 2002. DOI: 10.1080/00071660120121454

40. Yuceer M, Caner C: Antimicrobial lysozyme–chitosan coatings affect functional properties and shelf life of chicken eggs during storage. *J Sci Food Agric,* 94 (1): 153-162, 2014. DOI: 10.1002/jsfa.6322

41. Caner C, Cansız Ö: Chitosan coating minimises eggshell breakage and improves egg quality. *J Sci Food Agric,* 88 (1): 56-61, 2008. DOI: 10.1002/jsfa.2962

42. Yüceer M, Caner C: The effects of ozone, ultrasound and coating with shellac and lysozyme-chitosan on fresh egg during storage at ambient temperature. Part II: Microbial quality, eggshell breaking strength and FT-NIR spectral analysis. *Int J Food Sci Technol,* 55 (4): 1629-1636, 2020. DOI: 10.1111/ijfs.14422

43. Safavi M, Javanmard M: Effect of whey protein-rice bran oil incorporated *Zataria multiflora* extract edible coating on chemical, physical and microbial quality of chicken egg. *Iran Food Sci Tech Res J,* 11, 738-746, 2016. DOI: 10.22067/IFSTRJ.V1394I6.50003

44. Sheng L, Huang M, Wang J, Xu Q, Hammad HHM, Ma M: A study of storage impact on ovalbumin structure of chicken egg. *J Food Eng,* 219, 1-7, 2018. DOI: 10.1016/j.jfoodeng.2017.08.028

45. Samli HE, Agma A, Senkoylu N: Effects of storage time and temperature on egg quality in old laying hens. *J Appl Poult Res,* 14 (3): 548-553, 2005. DOI: 10.1093/japr/14.3.548

46. Akarca G, Istek Ö, Tomar O: The effect of resin coating on the quality characteristics of chicken eggs during storage. *J Food Sci,* 86 (4): 1243-1257, 2021. DOI: 10.1111/1750-3841.15686

47. Güler G, Şen A, Korkmaz Turgud F, Tahtabiçen E, Ağma Okur A, Samli HE: Serbest gezen tavuklardan elde edilen yumurtaların propolis ekstraktı ile kaplanmasının raf ömrü ve kalite parametrelerine etkileri. *J Tekirdag Agric Fac,* 19 (1): 89-100, 2022. DOI: 10.33462/jotaf.893121

48. Didar Z: Effects of coatings with pectin and *Cinnamomum verum* hydrosol ıncluded pectin on physical characteristics and shelf life of chicken eggs stored at 30°C. *Nutr Food Sci Res,* 6 (4): 39-45, 2019. DOI: 10.29252/nfsr.6.4.39

49. Stadelman W: The preservation of quality in shell eggs. **In,** Stadelman W, Cotterill OJ (Eds): Egg Science and Technologyed. 67-79, Food Products Press, New York, London, 1995.

50. Mathew AO, Olufemi AM, Foluke A, Oguntunji A: Relationship of temperature and length of storage on pH of internal contents of chicken table egg in humid tropics. *Biotechnol Anim Husb,* 32 (3): 285-296, 2016. DOI: 10.2298/BAH1603285M

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Research Article

Effects of Oxidative Stress-Related Major Molecular Parameters on Milk Composition in Weaning Period of Damascus Goats [1]

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Abstract: The weaning process in goat breeding is applied in various methods depending on the breeding administration. In this study, blood and milk samples have been collected from Damascus goats at the weaning day (postpartum 105th day-weaned day) and a week later (post-weaned day). In addition to determining cortisol and MDA in plasma, COX-2 and NFE2L2 activities have been investigated both mRNA and protein levels from leucocytes and plasma. Compositional parameters of milk have also been analyzed and the possible relations between studied parameters have been investigated. While expression levels of COX-2 and SCC of milk decreased, pH of milk was increased in post-weaned day samples. The MDA, FFDM, protein, lactose and freezing point were decreased in the post-weaned day. Milk fat was negatively correlated with NFE2L2, and milk protein had positively correlated with SCC and FFDM. On the other hand, lactose was positively correlated with FFDM and protein. In addition, most of the compositional parameters positively correlated with a freezing point; they were negatively correlated with electrical conductivity. According to the results obtained from the study, it is thought that the decrease in milk fat in goats may be an indicator of increased oxidative stress in lactating goats due to the increase in NFE2L2 protein, which has a central role in the antioxidant response.

Keywords: *COX-2, Damascus goat, Gene expression, NFE2L2, Weaning in goats*

Sütten Kesim Dönemindeki Damascus Keçilerinde Oksidatif Stresle İlişkili Majör Moleküler Parametrelerin Süt Bileşimi Üzerine Etkileri

Öz: Keçi yetiştiriciliğinde sütten kesme işlemi, yetiştirme yönetimine bağlı olarak çeşitli yöntemlerle uygulanmaktadır. Bu çalışmada Damascus keçilerinden sütten kesim günü (postpartum 105. gün-Sütten kesim günü) ve bir hafta sonra (sütten kesim sonrası) kan ve süt örnekleri alınmıştır. Plazmada kortizol ve MDA'nın belirlenmesine ek olarak, COX-2 ve NFE2L2 aktiviteleri lökosit ve plazmada hem mRNA hem de protein düzeyinde araştırılmıştır. Süt kompozisyon parametreleri de analiz edilmiş ve çalışılan parametreler arasındaki olası ilişkiler araştırılmıştır. Sütten kesim sonrası alınan örneklerde COX-2 ekspresyon seviyesi ve SCC azalırken, süt pH'sı artmıştır. Sütten kesim sonrası MDA, FFDM, protein, laktoz ve donma noktası seviyeleri düşmüştür. Süt yağı, NFE2L2 ile negatif korelasyon gösterirken süt proteini, SCC ve FFDM ile pozitif korelasyona sahip olmuştur. Bununla birlikte laktoz, FFDM ve protein ile pozitif korelasyon göstermiştir. Ek olarak, kompozisyon parametrelerinin çoğu donma noktası ile pozitif, elektriksel iletkenlik ile negatif korelasyon göstermiştir. Çalışmadan elde edilen sonuçlara göre, keçilerde süt yağındaki azalmanın, antioksidan yanıtta merkezi rolü olan NFE2L2 proteinindeki artışa bağlı olarak laktasyondaki keçilerde artan oksidatif stresin bir göstergesi olabileceği düşünülmektedir.

Anahtar sözcükler: *COX-2, Damascus keçisi, Gen ekspresyonu, Keçilerde sütten kesim, NFE2L2*

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Introduction

Goat is one of the precious farm animals in the supply of quality milk and dairy products [1]. It is reported that there are more than 1000 goat breeds and more than 1 billion goats worldwide [2,3]. Breeding of goats is mainly preferred for milk, and dairy products. Goat milk is an exceedingly valuable animal product, especially for children, the elderly and those with food allergies. As in other mammals, milk is an essential requirement for the nutritional necessities and maintenance of health in goat kids [4,5]. Although there are various changes between species, the milk sucking period, which begins with colostrum in ruminants after birth, ends with the transition to roughage and concentrated feeds. Weaning is an important stress factor affecting the health and growth performance of kids [5,6]. However, physiological changes related to stress and oxidative stress that may occur due to weaning in goats are known to a limited extent [7]. On the other hand, the possible weaning-related changes in milk composition are directly related to milk quality are potentially crucial for goat breeding.

The weaning process in goat breeding is applied in various methods and periods depending on the breeding administration [8,9]. Milk production is also maintained in varying periods after weaning. In this process, oxidative stress may occur in mammals. It is known that oxidative status crucially effects the physiology of the organism and composition of milk [10]. Although there are studies on physiological changes in kids after weaning, there are few studies investigating the remarkable changes in goats [5,6]. The study aimed to determine plasma cortisol and MDA (Malondialdehyde) levels in the weaning process in Damascus goats. COX-2 ((Cyclooxygenase 2) and NFE2L2 (Nuclear Factor Erythroid 2-Related Factor 2) activities were also investigated both mRNA and protein levels in leucocytes and plasma. The milk composition was analyzed in this process, and the possible relations between studied parameters were investigated.

Material and Methods

Ethical Statement

The study was approved by Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee (Approval no: 2021/02-08).

Animals and Sample Collection

The study was conducted on a private farm in Hatay Province in Türkiye in May 2021 (36°18’65.6”N and 36°18’63.9”E). Healthy and lactating Damascus goats were used in the study (n=24). The health status of goats was evaluated by veterinarians. Animals were selected by random sampling method from a herd of approximately 100 goats on similar ages. The animals were at the 3th-4th lactation period and gave singleton birth (44-50 months old). Approximately 2 m^2/goat area was arranged for each animal. Animals were fed with 1 kg/goat concentrate feed (88.91% dry matter, 16.51% crude protein, and 2650 kcal/kg total Metabolic Energy) and 1 kg/goat dry alfalfa.

Animals were weaned at postpartum 105th day (105±5). Blood and milk samples were collected from goats at the weaning day (Weaned) and a week later (post-weaned) at the morning milking time. While blood samples were collected from the left jugular vein into EDTA-containing tubes with a volume of 10 mL, approximately 150 mL milk samples from each animal were collected into falcon tubes with 50 mL (three tubes were used). Sterile conditions were complied with during sampling stages and nuclease-free falcons were used. Before collecting milk samples, goat’s udders were washed with alcohol-based disinfectant and cleaned with water and sterile cotton gauze swabs. The samples were transferred to the laboratory of Genetics in a cold chain within about 30 min.

Determination of Composition, pH, and Somatic Cell Count of Milk

Milk samples were manually homogenized before analysis and 10 mL of each sample was stored at -80°C for MDA analysis. While compositional parameters were measured with a milk analyzer (Milkotester Master Classic LM2-P1, Bulgaria), the pH of samples was determined with a pH meter (Hanna pH meter, HI83141, USA). At the same time, somatic cell counts of samples were determined via somatic cell counter (Lactoscan SCC 6010, Bulgaria). Validated devices were used to measure the composition, pH, and somatic cell count of the samples.

Plasma and Leucocyte Collection from Blood Samples

Blood samples were centrifuged at 3000 xg for 10 min at +4°C for the plasma and leucocyte collection. While obtained plasma was portioned to 1.5 mL tubes, the leucocyte layer of samples was transferred to new tubes and treated with Red Blood Cell Lysis Buffer [11]. Then the samples were centrifuged at 3000 xg for 10 min at +4°C and aqua phases were discarded. Approximately 1 mL of TRIzol Reagent (Thermo Fisher Scientific, USA, Cat No: 15596018) was added to leucocytes pellets and samples were homogenized by pipetting and stored at –80°C until molecular analysis.

RNA Isolation and cDNA Synthesis

According to the modified Trizol method, total RNA isolation was performed from leucocytes kept in Trizol Reagent [12]. Following the chloroform-isopropyl alcohol-ethyl alcohol steps, the total RNA pellets were obtained. According to the pellet sizes, samples were diluted with 30-50 µL nuclease-free water. Purity (A260/280) and RNA concentration were measured by the nucleic acid meter

(SMA-1000 Spectrophotometer, Merinton, China). In addition, the integrity of RNA was evaluated with 1% agarose gel electrophoresis.

Following the elimination of possible genomic DNA contamination via DNA digestion kit (DNase I, RNase free, Thermo Fisher Scientific, USA, Cat no: EN0521), cDNA was synthesized using RevertAid First Strand cDNA synthesis kit (Thermo Fischer Scientific, USA, Cat no: K1621). Thermal cycler (BioRad T100, USA) protocol was as follows: 10 min at 25°C, 120 min at 37°C and 5 min at 85°C, respectively. Then, all samples were completed to 200 µL with nuclease-free water and kept at –20°C until the gene expression analysis.

Real-Time PCR Application

Amplifications of *COX-2*, *NFE2L2,* and *ACTB* genes were performed in Real-Time PCR (Rotor-gene Q MDx 5plex HRM, Qiagen, USA). SYBR Green dye-containing kit (Power SYBR Green PCR Master Mix, Thermo Fisher Scientific, USA, Cat no: 4367659) was used for the amplification. *ACTB* was selected as the housekeeping gene. Each sample was studied in duplicate and a 10 µL volume sample was used for the reaction. The Real-Time PCR protocol was as follows: 10 min at 95°C, followed by 15 s at 95°C, 60 s at 60°C, and 40 cycles. Forward and Reverse sequences of primers were presented in *Table 1*.

Determination of MDA Levels and ELISA Application

The levels of MDA from plasma and milk samples were measured spectrophotometrically according to the method reported by Esterbauer and Cheeseman [15]. Cortisol, COX-2, and NFE2L2 levels of plasma samples were determined by goat-specific ELISA kits (E0021Go, E1375Go, E1376Go, respectively, Bioassay Technology Laboratory, USA) at 450 nm with ELISA reader (AMR-100, Allsheng, China).

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics Software Version 23.0. The sample size was calculated with G*Power software (Version 3.1.9.2). Result of the sample size calculation showed that the minimum number of Damascus goats was 24, considering an effect size of 0.30, an alpha value of 0.05, and a power of 0.80. The variables were examined as parametric test assumptions. Spearman correlation coefficient was performed to assess the correlation between SCC, MDA, compositional parameters of milk, plasma concentration levels of cortisol, MDA, COX-2, NFE2L2. A mixed model for repeated measures was used to test the differences in each variable between sampling periods (Weaned, Post-weaned). Animals were included as a random factor in all models, while sampling time was included as a fixed factor. Pairwise comparisons were made using a Bonferroni adjustment. Gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. The results were determined by comparing to "Weaned day" and presented as fold changes [16]. Differences with P<0.05 were considered statistically significant. Results were calculated as "Mean ± Standard Error of Mean" and presented as a figure.

Results

The SCC of the Post-Weaned day samples were found to be significantly lower (P<0.001). On the other hand, pH values of milk increased on post-weaned days (P<0.05). While the fat percentage and electrical conductivity were found to be similar between sampled days, MDA levels, FFDM (Fat-Free Dry Matter), protein, lactose, and freezing point increased in the post-weaned day *(Fig. 1)*.

The purity (A260/280=1.85±0.01) and concentration (384.51±23.80 ng/µL) values of samples were appropriate for conversion to cDNA and gene expression analysis. Compared to Weaned day samples, the *COX-2* gene was downregulated almost 2-fold in samples of post-weaned day (P<0.05). As with *NFE2L2* gene expression results, COX-2 and NFE2L2 protein levels were found to be similar in plasma on both days *(Fig. 2)*.

According to the correlation analysis, variable and significant correlations were found between studied parameters *(Table 2)*. Milk fat levels were negatively correlated with NFE2L2 protein levels; milk protein was positively correlated with SCC and FFDM (P<0.05). On the other hand, lactose was positively correlated with FFDM and milk protein. In addition, while most of the composition parameters were positively correlated with the freezing point, a negative correlation was found between electrical conductivity and these parameters at varying significance levels (P<0.05) *(Table 2)*.

Table 1. *Forward and reverse sequences of primers used in the study*

Genes	Forward and Reverse Sequences	Product Length	Reference
COX-2	F: 5'-GTAGGCCAGGAGGTCTTTGG-3' R: 5'-GCCTGCTTGTCTGGAACAAC-3'	142 bp	[10]
NFE2L2	F: 5'-CCAACTACTCCCAGGTAGCCC-3' R: 5'-AGCAGTGGCAACCTGAACG-3'	227 bp	[13]
ACTB	F: 5'-CTTCCAGCCGTCCTTCCT-3' R: 5'-TGTTGGCATACAGGTCCTTTC-3'	105 bp	[14]

Bp: *Base-pair*

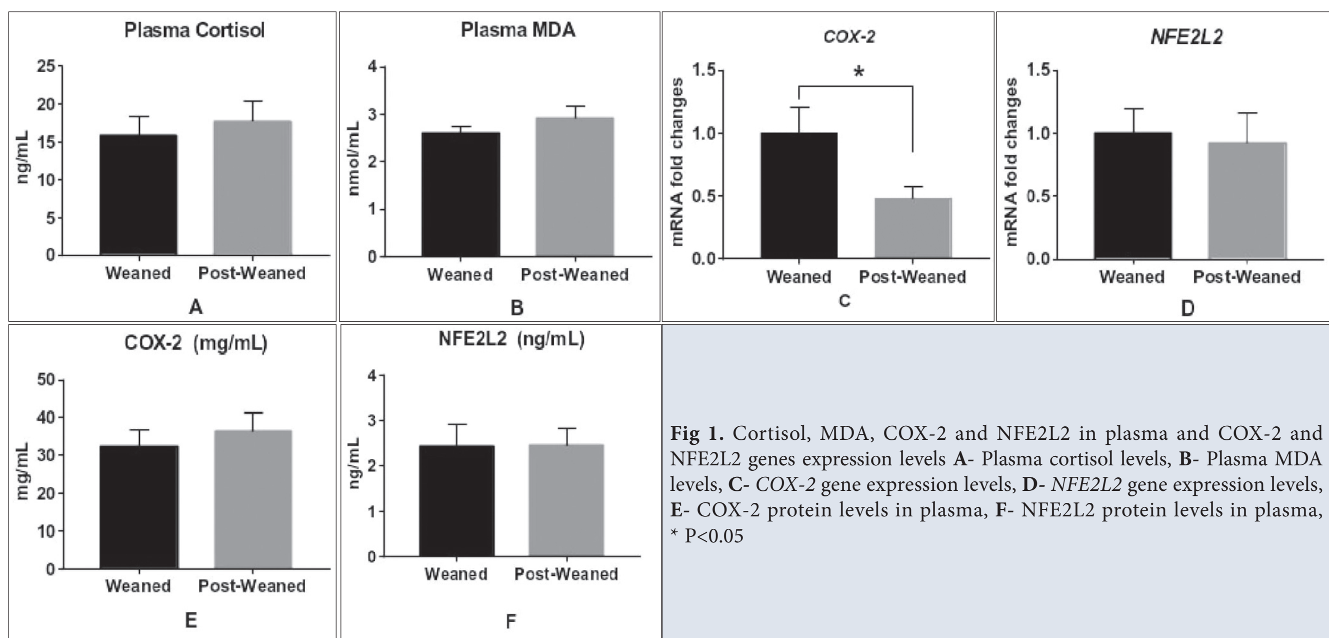

Fig 1. Cortisol, MDA, COX-2 and NFE2L2 in plasma and COX-2 and NFE2L2 genes expression levels **A-** Plasma cortisol levels, **B-** Plasma MDA levels, **C-** *COX-2* gene expression levels, **D-** *NFE2L2* gene expression levels, **E-** COX-2 protein levels in plasma, **F-** NFE2L2 protein levels in plasma, * P<0.05

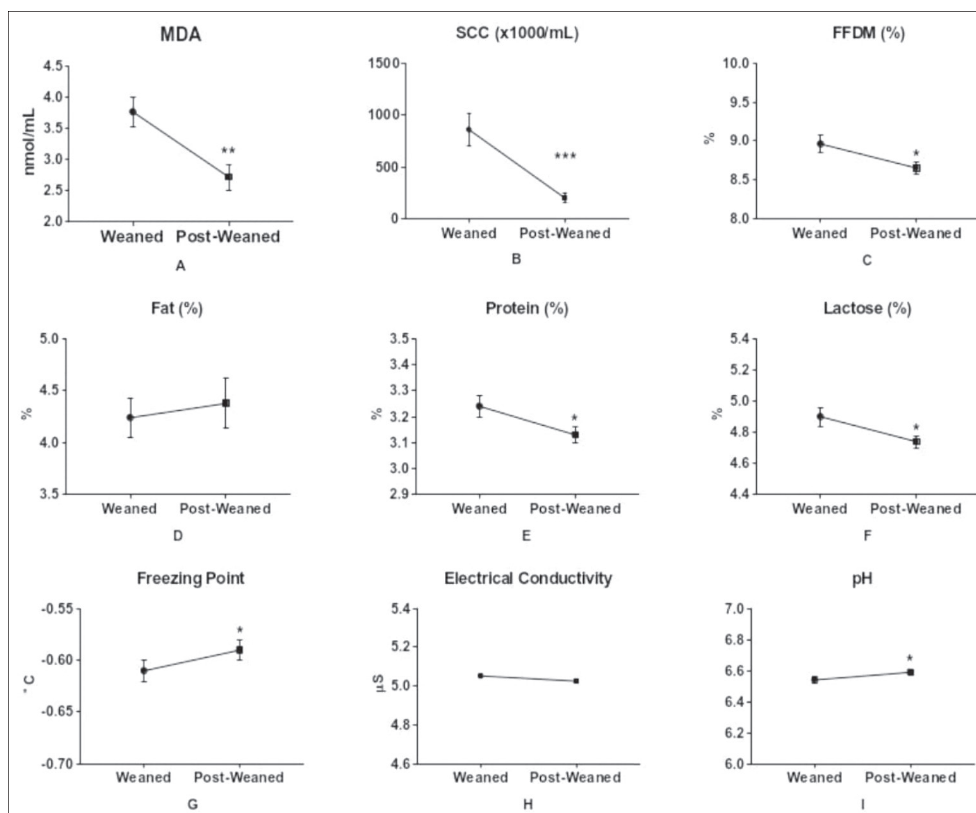

Fig 2. Changes in compositional parameters of milk in weaning process, * P<0.05, ** P<0.01, P<0.001

Discussion

Goat milk has recently become a valuable animal product that attracts tremendous attention due to the realization of its positive effects on health. Therefore, goat breeding is mainly done for milk production [17,18]. Even if the milk yield is a major factor affecting profitable production, sucking the kids is an inevitable application for developing their immunity and sustainable health. Weaning is applied at different periods among breeders depending on the type of breeding, breed of animal, and environmental factors [17,19]. After weaning, researchers mainly focus on the health status and developmental changes of kids [20]. However, there is limited knowledge at the molecular

Table 2. *Correlations between studied parameters*

Item	Cortisol	COX-2	NFE2L2	MDA	Milk MDA	SCC	Fat	FFDM	Protein	Lactose	Freezing Point	Electrical Conductivity	pH
Cortisol	1.000	-0.178	-0.015	0.089	0.091	-0.056	0.133	0.239	0.219	0.270	0.264	-0.230	-0.099
COX-2		1.000	-0.102	0.084	0.130	-0.084	0.049	-0.116	-0.051	-0.108	-0.076	-0.144	-0.212
NFE2L2			1.000	-0.169	-0.142	-0.225	-0.320*	-0.093	-0.097	-0.114	-0.159	0.210	-0.109
MDA				1.000	-0.041	-0.169	-0.025	-0.037	-0.091	-0.029	-0.056	0.129	0.014
Milk MDA					1.000	0.251	0.115	0.153	0.129	0.137	0.193	-0.232	-0.153
SCC						1.000	0.163	0.245	0.283*	0.230	0.296*	-0.074	-0.190
Fat							1.000	0.058	0.049	0.042	0.231	-0.472**	0.030
FFDM								1.000	0.967***	0.991***	0.975***	-0.532***	0.031
Protein									1.000	0.965***	0.956***	-0.579***	0.028
Lactose										1,000	0.969***	-0.532***	0.051
Freezing P.											1.000	-0.619***	0.020
Electrical C.												1.000	-0.133
pH													1.000

** P<0.05; ** P< 0.01; *** P<0.001,* ***FFDM:*** *Fat-Free Dry Matter,* ***Freezing P:*** *Freezing Point,* ***Electrical C:*** *Electrical Conductivity*

levels about stress and oxidative stress status of goats after weaning application.

During the weaning process, cortisol levels in plasma have been found similar in goats in this study. While plasma cortisol levels have been widely used as a potential biomarker for the determination of stress, it has been reported in several studies that the cortisol levels of plasma might vary depending on the endogenous and non-specific environmental factors such as circadian rhythm and season [6]. In addition, the hypothalamic-pituitary-adrenal (HPA) axis is also responsible for maternal stress in response to lactation in mammals [21]. However, considering the postpartum weaning time, the stable cortisol levels in plasma are thought to be due to physiological reasons. Indeed, the stress-related mechanisms become sensitive to various stress factors starting from late pregnancy [21]. It is believed that during the different lactation stages, molecular activities in physiological and biological pathways might affect these parameters [10,22].

MDA is the main product of oxidative stress, and it is widely used as a biomarker in tissues and biological liquids to detect oxidative stress status [23]. Although it has been reported that plasma MDA levels may change even depending on the differences of ratio ingredients, it has been found similar in both sampled days [24]. COX-2 and NFE2L2 are highly active transcription factors for maintaining oxidative balance. While COX-2 activity increases on the oxidative stress state, NFE2L2 has a significant role in response to oxidative stress [10,25]. NFE2L2 is also an antioxidant transcription factor in the organism [26]. It has been known that *COX-2* and *NFE2L2* are the most related genes with the oxidative status of the tissues and biological liquids [10]. While *COX-2* gene expression has been found almost 2-fold downregulated on the post-weaned day, there are no statistical differences between two different sampled days regarding *NFE2L2* gene expression levels. It has been thought that the response to weaning might be the main reason for the downregulation of *COX-2* gene expression levels.

On the other hand, post-transcriptional mediators such as miRNAs might be the reason for the similar levels of the proteins encoded by the *COX-2* gene [27]. As it is well known, miRNAs regulate the levels of proteins in several pathways. On the other hand, there are few studies on circulating and milk miRNAs in goats. Also, there are still newly identified miRNAs in ruminants [28,29].

MDA levels, which were at similar levels in plasma, were significantly reduced in post-weaned milk as somatic cells. SCC has known as an effective indicator of mammary gland health. Higher SCC in goat milk is generally considered physiological because of the apocrine type of secretion. However, it is reported that milk SCC should be below one million for the mammary glands to be considered healthy. In small ruminants, SCC shows a wide range [10,18]. Although SCC is within the physiological limits, it is thought that the number of somatic cells decreased with weaning may have occurred depending on the suckling behavior of the offspring [30,31]. In contrast to our findings, it has been found in a study conducted Murciano-Granadina goats that weaning does not affect SCC in milk [32]. Even if it has been reported that MDA and SCC are related parameters in milk, no correlation has been found between these two parameters [10,33]. The obtained results suggest that the oxidative balance in the mammary gland has a more complex molecular network

than anticipated. Furthermore, the pH value of milk was significantly increased. While it is known that the pH value of milk is strongly related to the composition and SCC of milk, it has been thought that other parameters such as minerals in milk are thought to be the reason for obtained results [34-36].

Although the contents of ration and the lactation period were similar, the compositional parameters significantly changed in post-weaned milk. While FFDM, protein, and lactose content of milk decreased, the freezing point of milk increased concerning these findings. During suckling, udder stimulation and evacuation affect milk composition in the mammary gland [37]. In addition, hormonal regulation and weaning period have also been thought to lead to variable changes in milk composition during the weaning process [38,39].

Even if variable correlations were detected between studied parameters, one of the most remarkable findings was the negative correlation between plasma *NFE2L2* protein levels and milk fat. *NFE2L2* has an influential role in cellular defense against elevated oxidative damage [40]. In addition to a crucial mission in response to oxidative stress, it has been reported that the *NFE2L2* is a potential regulator of fatty acid metabolism [41,42]. The study pointed out that deletion of *NFE2L2* gene has led to obesity in mice fed with high-fat diets [43]. Therefore, the negative correlation between *NFE2L2* and milk fat has been considered reasonable. While most researchers focus on the relation between *NFE2L2* and oxidative status, energy metabolism has gained importance with this transcription factor. According to the results obtained from the study, it is thought that the decrease in milk fat of goats may be an indicator of increased oxidative stress in lactating goats due to the increase in *NFE2L2* protein.

In conclusion, it has been thought that notable findings on several parameters on milk and plasma of goats after the weaning process have been obtained with this study. The mechanism of suckling-related stress and molecular changes in farm animals, particularly in small ruminants, is still largely unknown. According to the obtained results about the relation between *NFE2L2* and milk fat, it is thought that possible regulators of *NFE2L2* should be investigated at the molecular levels in ruminants. Due to the particular importance of goat milk, it is thought that further studies are required at the molecular level, particularly in the weaning process.

Availability of Data and Materials

Datasets analyzed during the study are available from the corresponding author (H. Özkan) on reasonable request.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Statement

The study was approved by Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee (Approval no: 2021/02-08).

Author Contributions

HÖ, MY, and AY conceived and investigated the study. HÖ, UK, and MY collected samples. HÖ, SD, and İK determined the milk parameters. HÖ and BÇ performed RNA isolation, gene expression and ELISA applications. HÖ, AY, and UK analyzed the results. All authors read and approved the final manuscript.

References

1. Yurchenko S, Sats A, Tatar V, Kaart T, Mootse H, Jõudu I: Fatty acid profile of milk from Saanen and Swedish Landrace goats. *Food Chem*, 254, 326-332, 2018. DOI: 10.1016/j.foodchem.2018.02.041

2. Crisà A, Ferrè F, Chillemi G, Moioli B: RNA-sequencing for profiling goat milk transcriptome in colostrum and mature milk. *BMC Vet Res,* 12 (264): 1-21, 2016. DOI: 10.1186/s12917-016-0881-7

3. FAO: Food and Agriculture Organization Corporate Statistical Database. http://www.fao.org/faostat/en/#data; *Accessed:* 12.12.2021.

4. Jasper J, Weary DM: Effects of ad libitum milk intake on dairy calves. *J Dairy Sci*, 85 (11): 3054-3058, 2002. DOI: 10.3168/jds.S0022-0302(02)74391-9

5. Izuddin WI, Loh TC, Samsudin AA, Foo HL, Humam AM, Shazali N: Effects of postbiotic supplementation on growth performance, ruminal fermentation and microbial profile, blood metabolite and GHR, IGF-1 and MCT-1 gene expression in post-weaning lambs. *BMC Vet Res,* 15 (1): 1-10, 2019. DOI: 10.1186/s12917-019-2064-9

6. Magistrelli D, Aufy AA, Pinotti L, Rosi F: Analysis of weaning-induced stress in Saanen goat kids. *J Anim Physiol Anim Nutr,* 97 (4): 732-739, 2013. DOI: 10.1111/j.1439-0396.2012.01315.x

7. Redondo E, Franco A, Garcia A, Masot AJ: Changes in concentrations of cortisol and melatonin in plasma, expression of synaptophysin, and ultrastructural properties of pinealocytes in goat kids in situations of stress due to early weaning: The effect of melatonin. *N Z Vet J*, 58 (3): 160-167, 2010. DOI: 10.1080/00480169.2010.67518

8. Ugur F, Atasoglu C, Tolu C, Diken F, Pala A: Effects of different weaning programs on growth of Saanen kids. *Anim Sci J,* 78 (3): 281-285, 2007. DOI: 10.1111/j.1740-0929.2007.00436.x

9. Zobel G, Freeman H, Watson T, Cameron C, Sutherland M: Effect of different milk-removal strategies at weaning on feed intake and behavior of goat kids. *J Vet Behav*, 35, 62-68, 2020. DOI: 10.1016/j.jveb.2019.10.004

10. Yakan A, Özkan H, Çamdeviren B, Kaya U, Karaaslan İ, Dalkiran S: Expression patterns of major genes in fatty acid synthesis, inflammation, oxidative stress pathways from colostrum to milk in Damascus goats. *Sci Rep*, 11 (1): 1-10, 2021. DOI: 10.1038/s41598-021-88976-0

11. Lim JH, Goh LPW, Chong ETJ, Lee PC: High integrity total RNA isolation from human peripheral blood that is as competitive to commercialize kits. *Res J Pharm Biol Chem Sci*, 7 (4): 1937-1942, 2016.

12. Rio DC, Ares M, Hannon GJ, Nilsen TW: Purification of RNA using TRIzol (TRI reagent). *Cold Spring Harb Protoc,* 2010 (6):pdb-prot5439, 2010. DOI: 10.1101/pdb.prot5439

13. Deng S, Yu K, Jiang W, Li Y, Wang S, Deng Z, Yao Y, Zhang B, Liu G, Liu Y, Lian Z: Over-expression of Toll-like receptor 2 up-regulates heme

oxygenase-1 expression and decreases oxidative injury in dairy goats. *J Anim Sci Biotechno*, 8 (1): 1-10, 2017. DOI: 10.1186/s40104-016-0136-2

14. Modesto P, Peletto S, Pisoni, G, Cremonesi P, Castiglioni B, Colussi S, Caramelli M, Bronzo V, Moroni P, Acutis PL: Evaluation of internal reference genes for quantitative expression analysis by real-time reverse transcription-PCR in somatic cells from goat milk. *J Dairy Sci,* 96 (12): 7932-7944, 2013. DOI: 10.3168/jds.2012-6383

15. Esterbauer H, Cheeseman KH: Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal. *Methods Enzymol,* 186, 407-421, 1990. DOI: 10.1016/0076-6879(90)86134-h

16. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods*, 25 (4): 402-408, 2001. DOI: 10.1006/meth.2001.1262

17. Belanche A, Palma-Hidalgo JM, Nejjam I, Jiménez E, Martín-García AI, Yáñez-Ruiz DR: Inoculation with rumen fluid in early life as a strategy to optimize the weaning process in intensive dairy goat systems. *J Dairy Sci*, 103 (6): 5047-5060, 2020. DOI: 10.3168/jds.2019-18002

18. Yakan A, Özkan H, Eraslan Şakar A, Ateş CT, Ünal N, Koçak Ö, Doğruer G, Özbeyaz C: Milk yield and quality traits in different lactation stages of Damascus goats: Concentrate and pasture based feeding systems. *Ankara Üniv Vet Fak Derg*, 66, 117-129, 2019. DOI: 10.33988/auvfd.547470

19. Khan MA, Bach A, Weary DM, Von Keyserlingk MAG: Invited review: Transitioning from milk to solid feed in dairy heifers. *J Dairy Sci*, 99 (2): 885-902, 2016. DOI: 10.3168/jds.2015-9975

20. Liao R, Lv Y, Zhu L, Lin Y: Altered expression of miRNAs and mRNAs reveals the potential regulatory role of miRNAs in the developmental process of early weaned goats. *PloS One*, 14 (8):e0220907, 2019. DOI: 10.1371/journal.pone.0220907

21. Ralph CR, Tilbrook AJ: The hypothalamo-pituitary-adrenal (HPA) axis in sheep is attenuated during lactation in response to psychosocial and predator stress. *Domest Anim Endocrinol*, 55, 66-73, 2016. DOI: 10.1016/j.domaniend.2015.11.003

22. Kapusta A, Kuczyńska B. Puppel K: Relationship between the degree of antioxidant protection and the level of malondialdehyde in high-performance Polish Holstein-Friesian cows in peak of lactation. *PLoS One,* 13:e0193512, 2018. DOI: 10.1371/journal.pone.0193512

23. Özkan H, Kutlu T: The relationship of fructose consumption with MDA levels in rat liver and its effect on the expression levels of COX-2 and NRF-2 genes. *Ankara Üniv Vet Fak Derg*, 67 (4): 387-392, 2020. DOI: 10.33988/auvfd.645713

24. Yonny ME, García EM, Lopez A, Arroquy JI, Nazareno MA: Measurement of malondialdehyde as oxidative stress biomarker in goat plasma by HPLC-DAD. *Microchem J*, 129, 281-285, 2016. DOI: 10.1016/j.microc.2016.07.010

25. Pérez-Rodríguez L, Romero-Haro AA, Sternalski A, Muriel J, Mougeot F, Gil D, Alonso-Alvarez C: Measuring oxidative stress: The confounding effect of lipid concentration in measures of lipid peroxidation. *Physiol Biochem Zool*, 88 (3): 345-351, 2015. DOI: 10.1086/680688

26. Moretti DB, Santos CB, Alencar SM, Machado-Neto R: Colostrum from primiparous Holstein cows shows higher antioxidant activity than colostrum of multiparous ones. *J Dairy Res,* 87, 356-359, 2020. DOI: 10.1017/S0022029920000813

27. Harper KA, Tyson-Capper AJ: Complexity of COX-2 gene regulation. *Biochem Soc Trans*, 36 (3): 543-545, 2008. DOI: 10.1042/BST0360543

28. Do DN, Dudemaine PL, Mathur M, Suravajhala P, Zhao X, Ibeagha-Awemu EM: MiRNA regulatory functions in farm animal diseases, and biomarker potentials for effective therapies. *Int J Mol Sci*, 22 (6):3080, 2021. DOI: 10.3390/ijms22063080

29. Bourdon C, Boussaha M, Bardou P, Sanchez MP, Le Guillou S, Tribout T, Larroque H, Boichard D, Rupp R, Le Provost F, Tosser-Klopp G: In silico identification of variations in microRNAs with a potential impact on dairy traits using whole ruminant genome SNP datasets. *Sci Rep*, 11 (1): 1-13, 2021. DOI: 10.1038/s41598-021-98639-9

30. Antonič J, Jackuliaková L, Uhrinčať M, Mačuhová L, Oravcová M, Tančin V: Changes in milk yield and composition after lamb weaning and start of machine milking in dairy ewes. *Slovak J Anim Sci*, 46 (3): 93-99, 2013.

31. Bergonier D, Berthelot X: New advances in epizootiology and control of ewe mastitis. *Livest Prod Sci,* 79 (1):1-16, 2003. DOI: 10.1016/S0301-6226(02)00145-8

32. Fernández N, Palomares JL, Pérez-Baena I, Rodríguez M, Peris C: Effect of the rearing system on financial returns from Murciano-Granadina breed goats. *Animal*, 13 (8): 1730-1735, 2019. DOI: 10.1017/S1751731118003336

33. Andrei S, Matei S, Rugină D, Bogdan L, Ştefănuţ **C:** Interrelationships between the content of oxidative markers, antioxidative status, and somatic cell count in cow's milk. *Czech J Anim Sci*, 61 (9): 407-413, 2016. DOI: 10.17221/70/2015-CJAS

34. Nian Y, Chen BY, Aikman P, Grandison A, Lewis M: Naturally occurring variations in milk pH and ionic calcium and their effects on some properties and processing characteristics of milk. *Int J Dairy Technol*, 65 (4): 490-497, 2012. DOI: 10.1111/j.1471-0307.2012.00861.x

35. Stocco G, Pazzola M, Dettori ML, Paschino P, Summer A, Cipolat-Gotet C, Vacca GM: Effects of indirect indicators of udder health on nutrient recovery and cheese yield traits in goat milk. *J Dairy Sci*, 102 (10): 8648-8657, 2019. DOI: 10.3168/jds.2019-16369

36. Singh M, Yadav P, Garg VK, Sharma A, Singh B, Sharma H: Quantification of minerals and trace elements in raw caprine milk using flame atomic absorption spectrophotometry and flame photometry. *J Food Sci Technol,* 52 (8): 5299-5304, 2015. DOI: 10.1007/s13197-014-1538-9

37. McKusick BC, Thomas DL, Romero JE, Marnet PG: Effect of weaning system on milk composition and distribution of milk fat within the udder of East Friesian dairy ewes. *J Dairy Sci*, 85 (10): 2521-2528, 2002. DOI: 10.3168/jds.S0022-0302(02)74335-X

38. Panzuti C, Mandrile G, Duvaux-Ponter C, Dessauge F: Early weaning and high feeding level in post-weaning period did not impact milk production in Alpine dairy goats. *J Dairy Res,* 85 (3): 277-280, 2018. DOI: 10.1017/S0022029918000377

39. Lamb GC, Miller BL, Lynch JM, Grieger DM, Stevenson JS, Lucy MC: Suckling reinitiated milk secretion in beef cows after an early postpartum hiatus of milking or suckling. *J Dairy Sci*, 82 (7): 1489-1496, 1999. DOI: 10.3168/jds.S0022-0302(99)75376-2

40. Vomhof-DeKrey EE, Picklo Sr MJ: The Nrf2-antioxidant response element pathway: A target for regulating energy metabolism. *J Nutr Biochem,* 23 (10): 1201-1206, 2012. DOI: 10.1016/j.jnutbio.2012.03.005

41. Ludtmann MHR, Angelova PR, Zhang Y, Abramov AY, Dinkova-Kostova AT: Nrf2 affects the efficiency of mitochondrial fatty acid oxidation. *Biochem J*, 457 (3): 415-424, 2014. DOI: 10.1042/BJ20130863

42. da Costa RM, Rodrigues D, Pereira CA, Silva JF, Alves JV, Lobato NS, Tostes RC: Nrf2 as a potential mediator of cardiovascular risk in metabolic diseases. *Front Pharmacol*, 10 (382): 1-12, 2019. DOI: 10.3389/fphar.2019.00382

43. Chartoumpekis DV, Ziros PG, Psyrogiannis AI, Papavassiliou AG, Kyriazopoulou VE, Sykiotis GP, Habeos IG: Nrf2 represses FGF21 during long-term high-fat diet–induced obesity in mice. *Diabetes*, 60 (10): 2465-2473, 2011. DOI: 10.2337/db11-0112

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RESEARCH ARTICLE

Determination of the Effects of Silage Type, Silage Consumption, Birth Type and Birth Weight on Fattening Final Live Weight in Kıvırcık Lambs with MARS and Bagging MARS Algorithms

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Abstract: This study was carried out to determine the effect of silage type, silage consumption, birth type (single or twin) and birth weight on live weight at the end of fattening in Kıvırcık lambs. In the experiment, 40 male Kıvırcık lambs aged 2.5-3 months were used and the animals were fattened for 56 days. During the fattening period, the lambs fed with 5 different types of silage (100% sunflower silage, 75% sunflower + 25% corn silage, 50% sunflower + 50% corn silage, 25% sunflower + 75% corn silage, 100% corn silage) pure and mixed in different proportions and concentrate feed. Data on fattening results were analyzed with MARS and Bagging MARS algorithms. The main objective of this research is to predict fattening final live weight (FFLW) of lambs using Multivariate Adaptive Regression Splines (MARS) and Bagging MARS algorithms as a nonparametric regression technique. Live weight value was modeled based on factors such as birth type, birth weight, silage type and silage consumption. Correlation coefficient (r), determination coefficient (R^2), Adjust R^2, Root-mean-square error (RMSE), standard deviation ratio (SD ratio), mean absolute percentage error (MAPE), mean absolute deviation (MAD), and Akaike Information Criteria (AIC) values of MARS algorithm predicting live weight were as follows: 0.9986, 0.997, 0.977, 0.142, 0.052, 0.2389, 0.086 and -88 respectively. Like statistics for Bagging MARS algorithm were 0.754, 0.556, 0.453, 1.8, 0.666, 3.96, 1.47 and 115 respectively. It was observed that MARS and Bagging MARS algorithms have revealed correct results according to goodness of fit statistics. In this study it has been determined that the MARS algorithm gives better results in live weight modeling.

Keywords: *Kıvırcık lamb, Silage type, Birth weight, Birth type, Data mining*

Kıvırcık Kuzularda Silaj Tipi, Silaj Tüketimi, Doğum Tipi ve Doğum Ağırlığının Besi Sonu Canlı Ağırlık Üzerine Etkilerinin MARS ve Bagging MARS Algoritmaları ile Saptanması

Öz: Bu çalışma, Kıvırcık kuzularında silaj tipi, silaj tüketimi, doğum tipi (tek veya ikiz) ve doğum ağırlığının besi sonu canlı ağırlığa etkisini belirlemek amacıyla yapılmıştır. Deneyde 2.5-3 aylık 40 erkek Kıvırcık kuzu kullanılmış ve hayvanlar 56 gün beslenmiştir. Kuzular besi döneminde 5 farklı silaj çeşidi (%100 ayçiçeği silajı, %75 ayçiçeği + %25 mısır silajı, %50 ayçiçeği + %50 mısır silajı, %25 ayçiçeği + %75 mısır silajı, %100 mısır) saf ve farklı oranlarda karıştırılmış ve konsantre yem ile beslenmiştir. Besi sonuçlarına ilişkin veriler MARS ve Bagging MARS algoritmaları ile analiz edilmiştir. Bu araştırmanın temel amacı, parametrik olmayan bir regresyon tekniği olarak Çok Değişkenli Uyarlanabilir Regresyon Splines (MARS) ve Bagging MARS algoritmalarını kullanarak kuzuların canlı ağırlığını tahmin etmektir. Canlı ağırlık değeri, doğum tipi, doğum ağırlığı, silaj tipi ve silaj tüketimi gibi faktörlere göre modellenmiştir. Canlı ağırlığı tahmin eden MARS algoritması için korelasyon katsayısı (r), belirleme katsayısı (R^2), Düzeltilmiş R^2, Hata Kareler Ortalamasının Karekökü (RMSE), standart sapma oranı (SD oranı), ortalama mutlak yüzde hatası (MAPE), ortalama mutlak sapma (MAD) ve Akaike Bilgi Kriterleri (AIC) değerleri sırasıyla 0.9986, 0.997, 0.977, 0.142, 0.052, 0.2389, 0.086 ve -88'dir. Bagging MARS algoritması için benzer istatistikler sırasıyla 0.754, 0.556, 0.453, 1.8, 0.666, 3.96, 1.47 ve 115'dir. MARS ve Bagging MARS algoritmalarının uyum iyiliği istatistiklerine göre doğru sonuçlar ortaya koyduğu gözlemlenmiştir. Bu çalışmada, MARS algoritmasının canlı ağırlık modellemesinde daha iyi sonuçlar verdiği ortaya çıkmıştır.

Anahtar sözcükler: *Doğum tipi, Doğum ağırlığı, Veri madenciliği, Kıvırcık kuzusu, Silaj*

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Introduction

Approximately 70% of the expenses of the enterprises engaged in animal production are roughage and intensive feed costs [1]. This is very important in terms of showing how effective and decisive the feed is in the development of livestock. Today, where the demand for animal products is increasing, more and more roughage and concentrate feed production is needed for more animal food production.

In order to obtain high efficiency from animals, it is necessary to meet the nutrient needs in a balanced and sufficient level, and for this purpose, it is necessary to use quality roughage and concentrate feed sources. Roughage is generally divided into two groups as dry and watery roughage. Dry roughage, hay, straw and products with a crude cellulose content of 18% or higher, roughage consists of green fodder plants such as alfalfa, sainfoin, vetch, silage, roots and tubers.

One of the main problems of animal husbandry is the difficulties in obtaining good quality, cheap and sufficient amount of roughage. Many countries are faced with important problems, especially in terms of meeting the need for quality roughage [2]. Problems in the supply of quality roughage is one of the most important reasons for the low yield per animal in many countries. It is only possible to reduce the amount of intensive feed used in animal feeding, which is expensive, by using quality roughage. In this context, one of the important sources to refer to is silage [3].

It is not possible to meet the nutritional requirements of ruminant animals only with concentrate feeds. It is possible to realize both economical and rational feeding by adding silage as well as concentrate and roughage to the rations. It is possible to meet the green feed requirements of animals fresh from nature only in certain periods of the year due to vegetation conditions. In countries located in the Mediterranean climate zone, the vegetation period is approximately 200 days. Therefore, the fresh and green roughage needs of animals have to be met from different sources during the rest of the year. Green and fresh roughage given to animals by grazing or mowing during vegetation periods cannot be stored for a long time without spoiling due to the high water content they contain. For this reason, water-rich roughage should be stored until the period of use with different methods.

Among the forage crops produced for silage, cereals such as corn, wheat and sorghum, which have high water-soluble carbohydrate content and low buffer capacity, come first, but in many countries, corn silage constitutes a very large part of the total silage production [4]. However, sunflower, which is an annual industrial plant in some regions, is thought to be one of the plants that can be an alternative to corn in silage production. Although sunflower is mostly cultivated as a second crop after grains, it is currently used as a source of roughage by ensiling or grazing. Although sunflower is grown for different purposes (oil, pulp and snack food, etc.) around the world, it is also grown as a silage plant in many countries. Sunflower cultivation is easier than corn, and it can be used for silage as an alternative to corn, especially in regions that do not receive much precipitation and irrigation facilities are limited. It is possible to benefit from sunflower as an important forage plant, thanks to its ability to be silage in a shorter time than corn, its tolerance to high and low temperatures, and its high adaptability to various soil conditions [5].

Although silage is one of the most important roughage sources used in the feeding of sheep and goats as well as cattle in countries with developed livestock, silage production and use are still insufficient in some countries. Especially, the use of silage is very low in small ruminant. However, it has been reported that silage feed has started to be used in the rations of small ruminant in recent years [6].

In recent years, it has been reported that there has been a decrease in the number of Kıvırcık sheep in the province of Balıkesir - Türkiye, due to the conversion of meadow and pasture lands into field crops production area, and sheep breeding tends towards intensive breeding [7]. Addition of yeast or malic acid to the feed did not have a statistically significant effect on performance in Kıvırcık lambs fed with high concentrate feed. At the end of the 60th day, the average live weight was 35.5 kg in those fed with yeast addition and 35.8 kg in those fed with malic acid addition [8]. The change in live weight until the adult period in sheep breeding gives an idea about whether the breeding programs and production system are appropriate.

In a study conducted at Istanbul University Faculty of Veterinary Medicine, 52 Kıvırcık lambs were included in the trial. It has been reported that the lambs reared with the concentrate-based system grew 30% faster than pasture-based system during the trial period [9]. Fifty-five Kıvırcık lambs selected from Balıkesir Sheep Breeding Research Institute herds in Türkiye was determined that birth weight, weaning weight and final body weight were significantly affected by gender and birth type [10].

In Kıvırcık lambs reared intensively, 211 g live weight gain and 33 kg body weight after fattening were measured during the 68-day fattening period [11]. In an another study, the daily live weight gains of Kıvırcık lambs raised by intensive method were reported as 276 g during the 63-day fattening period and 44.68 kg at the end of fattening [12].

The main hypothesis of this study is that factors such as silage type, silage consumption, birth type and birth weight significantly affect the live weight of lambs.

In this study, it was aimed to investigate the effects of different silage type, silage consumption, birth type (single or twin) and birth weight on the live weight of Kıvırcık lambs at the end of fattening by using some data mining methods.

Material and Methods

Ethical Statement

This study was carried out with the approval of Bursa Uludağ University Animal Experiments Local Ethics Committee dated 07.01.2020 and numbered 2020-01/02.

Material

This study was carried out in a semi-open barn in a sheep farm belonging to Bursa Uludağ University Agricultural Application and Research Center. In the study, 40 Kıvırcık male lambs aged 2.5-3 months and an average live weight of 23-25 kg were used as animal material. The fattening lambs were housed in individual compartments during the experiment and individual feeding was applied to the animals during the 56-day fattening period. During the trial period, the live weights and feed consumptions of the lambs were determined individually and in 2-week periods.

During the experiment, lambs were fed 5 different silages (100% sunflower silage, 75% sunflower + 25% corn silage, 50% sunflower + 50% corn silage, 25% sunflower + 75% corn silage, 100% corn silage) as pure and mixed. Lambs housed in individual chambers consumed the silage mixtures of their groups *ad libitum*. In addition to the silage mixtures consumed by the lambs, 700 g of concentrate feed per animal was given in the first 4 weeks of the experiment. Later, this amount was increased to 900 g for 4 weeks, and to 1400 g in the last 2 weeks of the experiment, taking into account the daily nutrient needs of the lambs.

The lambs were fed once a day at 09:00 in the morning. The remaining feed from the feeders in the individual compartments was collected and weighed daily before new feeding was made the next day, and the amount of silage mixture and concentrated feed consumed by each animal daily was determined. Fresh and clean drinking water was always available in front of the lambs. During the fattening period, the live weights of the lambs were determined by control weighing made every 14 days. Weights of the animals at the beginning of fattening and other control weights were made on an empty stomach.

Method

Data on fattening results were analyzed with MARS and Bagging MARS algorithms. MARS (Multivariate Adaptive Regression Splines) algorithm was proposed by Friedman [13] in order to study the non-linear relationships between independent variables and dependent variable(s). For the MARS algorithm, no assumptions about functional relationships between dependent and independent variables are needful. It is a nonparametric statistical method that takes a basis for a divide.

The MARS model is highly flexible with the combination of hinge functions and two of them multiplied together, allowing for bends, thresholds, and other departures from typical linear functions [14,15].

The optimization procedure of the MARS model primarily consists of forward and backward phases. During this process, the forward phase generates basis functions, and finds the location of potential knots in a stepwise manner, leading to overfitting and complexity. Thereby, the backward phase intends to increase the generalization ability of the model by calculation. Piecewise functions are divided into three: These are a constant, a hinge function and a product of two or more hinge functions for different predictors. A hinge function is as follows [13].

$$\max(0, x - t) = \begin{cases} x - t, & x \geq t \\ 0, & otherwise \end{cases}$$

here t location t is called knot for the basis function [16]. MARS model is established as a linear combination of basis functions and interrelation, explained as follow [13].

$$f(x) = \beta_0 + \sum_{i=1}^{N} \beta_i B_i(x)$$

here each *Bi(x)* is the ith basis function. The coefficient β_0 is a constant, while β_i is the coefficient of the ith basis function, determined by the least-squares method, and f(x) produces the predicted value. The basis function, which demonstrates the largest decline in the training error, will be added to the model up to the specified maximum number of basis functions are achieved.

Model subsets are compared using generalized cross-validation (GCV). The GCV is a shape of regularization that trades off the goodness of-fit against the model complexity. The GCV of a model is defined as follows [17].

$$GCV = \frac{\frac{1}{n}\sum_{i=1}^{n}(y_i - f(x_i))^2}{\left[1 \frac{M + d(M-1)/2}{n}\right]^2}$$

here M is the number of basis functions, and d is the penalizing parameter. The optimal value of d usually falls in the range of $2 \leq d \leq 4$, and generally $d = 3$ is used [13].

The residuals are the difference between the values (x) predicted by the model and corresponding response values y. The residual sum of squares (RSS) is the sum of the squared values of residuals:

$$RSS = \sum_{i=1}^{n} (y_i - f(x_i))^2$$

The total sum of squares (TSS) is calculated as the sum over all squared differences between the response $\bar{y}$ and its mean :

$$TSS = \sum_{i=1}^{n} (y_i - \bar{y})^2$$

Generalized R^2 or GRSq is the generalization performance of the model estimated using the MARS algorithm. GRSq can be explained as follows [18].

$$GRS_q = 1 - \frac{GCV}{RSS}$$

GCV is important a statistic for MARS algorithm because it is used to evaluate model subsets in the backward pass.

Bagging (Bootstrap aggregating) MARS algorithm uses bootstrapping among resampling techniques. Bagging models can ensure their own internal estimate of predictive accuracy correlating well with either cross-validation estimates or test set estimates [19]. Bagging method is used as a tool to shape a more stable classifier. Bagging predictor is a method to generate multiple versions of predictors and use them for aggregate predictors [20]. Bagging is used for the purpose of improve the classification accuracy of the MARS method. Thus, this study is expected to obtain better modelling and classification functions through bagging MARS method [21].

To comparatively test the estimate criteria of all the models, the following goodness of fit criteria were determined [22-25]:

1. Pearson correlation coefficient (r) between the observed and predicted dependent variable values.

2. Coefficient of determination

$$R^2 = 1 - \frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{\sum_{i=1}^{n}(y_i - \bar{y})^2}$$

3. Adjusted coefficient of determination

$$Adj.R^2 = 1 - \frac{\frac{1}{n-k-1}\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{\frac{1}{n-1}\sum_{i=1}^{n}(y_i - \bar{y})^2}$$

4. Root-mean-square error (RMSE) given by the following formula.

$$RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}$$

5. Standard deviation ratio (SD_{ratio})

$$SD_{ratio} = \sqrt{\frac{\frac{1}{n-1}\sum_{i=1}^{n}(\varepsilon_i - \bar{\varepsilon})^2}{\frac{1}{n-1}\sum_{i=1}^{n}(y_i - \bar{y})^2}}$$

6. Mean absolute percentage error (MAPE)

$$MAPE = \frac{1}{n}\sum_{i=1}^{n}\left|\frac{y_i - \hat{y}}{y_i}\right|.100$$

7. Mean absolute deviation (MAD)

$$MAD = \frac{1}{n}\sum_{i=1}^{n}|y_i - \hat{y}|$$

8. Akaike Information Criteria (AIC)

$$AIC = nln\left[\sum_{i=1}^{n}\frac{(y_i - \hat{y}_i)^2}{n}\right] + 2k$$

If its standard ratio value is 0.40 or 0.10, then a regression model applied had a good fit or a very good fit was underlined that by Grzesiak and Zaborski [26].

In order to building MARS and Bagging MARS predictive models, the earth package proposed by Milborrow [27,28] in RStudio software was used [25]. Also, the ehaGoF package was used to measure the predictive quality of the evaluated MARS models [25].

Results

Introductory statistics on birth weight, silage consumption and final live weight in Kıvırcık lambs according to birth type are given in *Table 1*.

***Table 1.** Descriptive statistics for birth type*

Statistics	BT	BW	SC	FFLW
N	Single	13	13	13
	Twin	27	27	27
$\bar{X}$	Single	4.64	917	37.5
	Twin	4.03	932	36.6
$S\bar{x}$	Single	0.188	107	0.889
	Twin	0.084	72.3	0.478
s	Single	0.678	388	3.21
	Twin	0.438	375	2.48
Min	Single	3.6	479	34.1
	Twin	3.4	320	32.4
Max	Single	6	2049	45.3
	Twin	5	1972	41.3

***BT:** Birth type, **BW:** Birth weight (kg), **SC:** Silage consumption (g), **FFLW:** Fattening final live weight (kg)*

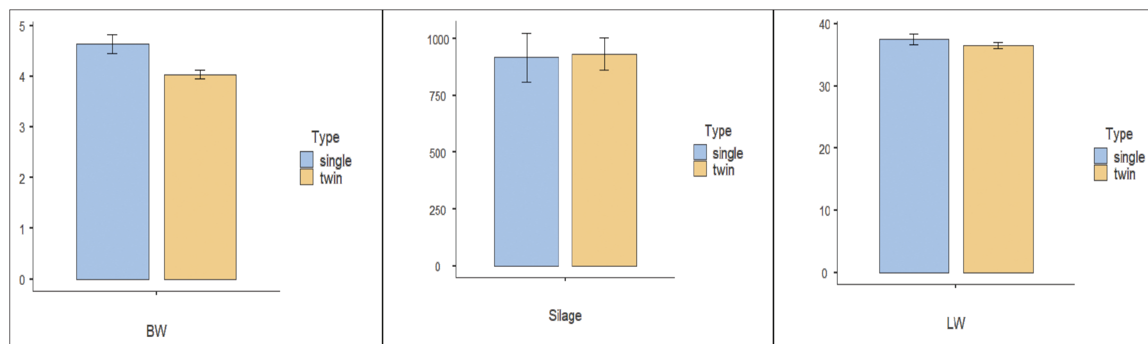

Fig 1. Silage consumption, live weight at birth and fattening final live weight at the end of the fattening period by birth type

As seen in *Table 1,* 13 of the 40 Kıvırcık lambs were born as singles and 27 of them were twins. The mean birth weight of the lambs was 4.64 kg in single and 4.03 kg in twins. While single lambs consumed an average of 917 g of silage per day, twin lambs consumed 932 g of silage. The average live weight at the end of fattening is 37.5 kg in single lambs and 36.6 kg in twin lambs. The values belonging to single and twin lambs is presented in *Fig. 1*.

In *Table 2,* introductory statistics for silage consumption, birth weight and post-fattening live weight are presented according to silage type.

When evaluated according to silage type, lambs fed 50% corn and 50% sunflower and average birth weight (4.56 kg) consumed the most feed. The body weight at the end of fattening was found to be higher at 37.4 kg (feeding with 100% corn, 75% maize + 25% sunflower, 50% maize + 50% sunflower) in the first 3 groups *(Table 2, Fig. 2)*.

Multivariate Adaptive Regression Splines (MARS) and Bagging MARS algorithms, which are data mining methods, were applied in order to examine the effects of factors affecting the end of fattening body weight Kıvırcık lambs.

Fattening body weight (LW) variable is the dependent variable, while delivery type (BT), silage type (ST), birth weight (BW) and daily average silage consumption (SC) variables are also independent variables. Goodness of fit statistics calculated for MARS and Bagging MARS algorithms are given in *Table 3*.

Predictive performances of MARS and Bagging MARS were assessed comparatively in predicting FFLW. Their goodness-of-fit-criteria outcomes are summarized in *Table 3*. The superiority order in the predictive accuracy of the mentioned algorithms was MARS > Bagging MARS according to the estimated model evaluation criteria. Inasmuch as, greater in the first criteria is better, whereas smaller in the remaining criteria is better. The predictive performance of the MARS algorithm was found better than Bagging MARS. Results of the MARS algorithm for Kıvırcık lambs are presented in *Table 4*. The GCV value of the MARS model was 0.0201. For the Kıvırcık lambs,

Table 2. *Descriptive statistics for silage type*

Statistics	Silages	BW	SC	FFLW
N	Corn100	8	8	8
	Corn75-Sunflower25	8	8	8
	Corn50-Sunflower50	8	8	8
	Corn25-Sunflower75	8	8	8
	Sunflower100	8	8	8
$\overline{X}$	Corn100	4.1	763	37.4
	Corn75-Sunflower25	3.96	834	37.4
	Corn50-Sunflower50	4.56	1099	37.4
	Corn25-Sunflower75	4.38	755	35.9
	Sunflower100	4.15	1186	36.3
$S\bar{x}$	Corn100	0.227	87.7	0.808
	Corn75-Sunflower25	0.134	72.6	0.828
	Corn50-Sunflower50	0.239	148	1.35
	Corn25-Sunflower75	0.154	101	0.922
	Sunflower100	0.249	171	0.948
s	Corn100	0.641	248	2.29
	Corn75-Sunflower25	0.378	205	2.34
	Corn50-Sunflower50	0.676	420	3.83
	Corn25-Sunflower75	0.437	286	2.61
	Sunflower100	0.705	485	2.68
Min	Corn100	3.4	484	34.4
	Corn75-Sunflower25	3.4	524	34.1
	Corn50-Sunflower50	3.8	703	34.5
	Corn25-Sunflower75	3.8	320	32.4
	Sunflower100	3.4	751	32.8
Max	Corn100	5	1215	41.3
	Corn75-Sunflower25	4.4	1077	41.1
	Corn50-Sunflower50	6	2049	45.3
	Corn25-Sunflower75	5	1225	40.7
	Sunflower100	5.5	1972	40

BW: *Birth weight (kg),* ***SC:*** *Silage consumption (g),* ***FFLW:*** *Fattening final live weight (kg)*

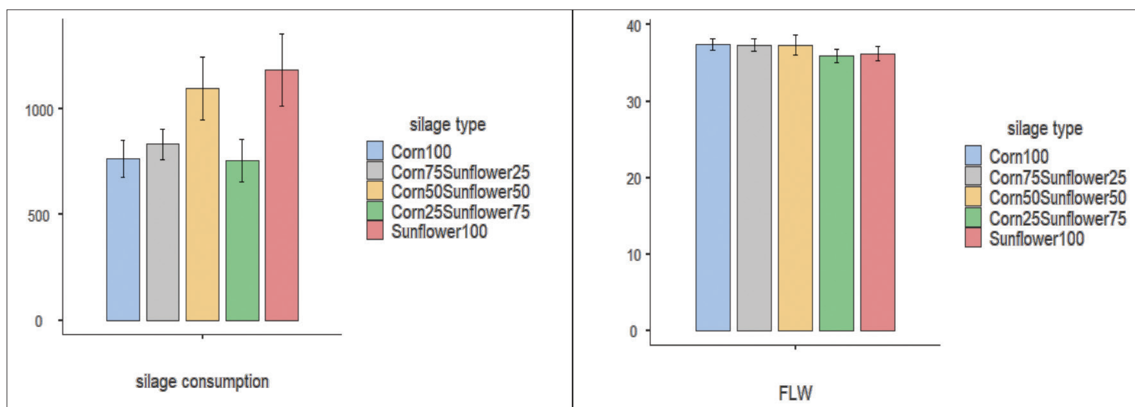

Fig 2. Silage consumption and final live weight according to silage type. Here, the graph of the descriptive statistics of these variables is shown

Table 3. *Predictive performance of MARS and Bagging MARS algorithms*

Methods	r	R^2	Adj. R^2	RMSE	SD Ratio	MAPE	MAD	AIC
MARS	0.9986	0.997	0.977	0.142	0.052	0.239	0.086	-88
Bagging MARS	0.754	0.556	0.453	1.8	0.666	3.960	1.47	115

RMSE: *Root-mean-square error,* ***SD ratio:*** *Standard deviation ratio,* ***MAPE:*** *Mean absolute percentage error,* ***MAD:*** *Mean absolute deviation,* ***AIC:*** *Akaike Information Criteria*

the observed FFLW values of the MARS model with the interaction order of 3 displayed much better fit.

The model equation of the MARS algorithm is as follows.

FFLW = 42.9 - 33 * Typetwin - 2.75 * SilageCorn50Sunflo50 + 8.06 * SilageCorn75Sunflo25 - 4.5 * SilageSunflower100 + 33.5 * max(0, 4.1 - BW) - 26.7 * max(0, BW - 4.1) - 0.0406 * max(0, silage - 745) + 0.034 * max(0, silage - 774) + 0.0647 * max(0, silage - 838) - 0.0875 * max(0, silage - 960) - 0.266 * max(0, 1024 - silage) + 0.0265 * max(0, silage - 1024) - 5.89 * Typetwin * SilageCorn25Sunflo75 -1.27 * Typetwin * SilageCorn50Sunflo50 + 6.13 * Typetwin * BW-0.0204 * SilageCorn75Sunflo25 * silage + 7 * Typetwin * max(0, BW- 4.1) + 0.0243*Typetwin*max(0,1024-silage)-4.38*SilageCorn25Sunflo75 * max(0, 4.1-BW) - 34.7 * SilageCorn25Sunflo75 * max(0, BW - 4.1) + 0.00549 * SilageCorn25Sunflo75 * max(0, 1024 - silage) + 14 * SilageCorn50Sunflo50 * max(0, 4.1 - BW) + 0.000967 * SilageCorn50Sunflo50 * max(0, silage - 1024) + 42.9 * SilageCorn75Sunflo25 * max(0, BW - 4.1) - 0.137 * SilageCorn75Sunflo25*max(0,silage-1024)-41*SilageSunflower100 * max(0, 4.1 - BW) + 0.00515 * SilageSunflower100 * max(0, 1024 - silage) + 0.0598 * BW * max(0, 1024-silage) + 0.0139 * max(0, BW - 4.1) * silage-6.39 * Typetwin * SilageCorn25Sunflo75 * max(0, BW - 4.1) + 0.00468 * Typetwin * SilageCorn75Sunflo25 * max(0, 1024 - silage) + 30.1 * Typetwin * SilageSunflower100 * max(0, 4.1 - BW) + 0.049 * SilageCorn25Sunflo75 * max(0, BW - 4.1) * silage

Among independent variables, the most important and highest positive effects SilageCorn75Sunflo25 * max(0, BW - 4.1), max(0, 4.1 - BW) and Typetwin * SilageSunflower100 * max(0, 4.1 - BW) explained the variability in FFLW in the MARS algorithm, successfully. Likewise, highest negative effects SilageSunflower100 * max(0, 4.1-BW), SilageCorn25Sunflo75 and Typetwin defined the variability in FFLW in the MARS algorithm.

The relative importance of the independent variables is presented in *Table 5*.

As seen in *Table 5*, the greatest importance order was obtained for silage (100%), BW (96.7%), SilageSunflower100 (83%), SilageCorn25Sunflo75 (74.9%), SilageCorn75Sunflo25 (72%), Type twin (67.4%) and SilageCorn50Sunflo50 (67.4%).

The distribution graphs of observed predicted values of FLW was indicated in *Fig. 3*.

In this study, it was observed that there was a bilateral interaction between the variables. The graph representing the three-dimensional surface of the analysis results and the relationship between a pair of predictor variables and the objective variable is presented in *Fig. 4*.

The prediction equation of the Bagging MARS algorithm as below.

FFLW = (32.87505 +17.8484 * max(0, 4-BW) + 13.0234 * max(0, BW-4) - 18.91613 * max(0, BW-4.4) - 0.008153931 * max(0, 1020.86 - silage) + 0.005381609 * max(0, silage - 1020.86) + 3.473147 * Typesingle * max(0, BW-4) + 2.749936 * SilageCorn25Sunflo75 * max(0, BW-4) + 21.03818 * SilageCorn50Sunflo50 * max(0, 4-BW) + 0.01457022 * SilageCorn75Sunflo25 * max(0, 1020.86-silage) - 14.40757 * SilageSunflower100 * max(0, 4-BW) + 50.10852 - 3.484945 * SilageCorn50Sunflo50 - 20.27059 * max(0, BW-3.6) + 19.93425 * max(0, BW-4.2) - 17.32094 * max(0, 4.4-BW) + 8.801585 * SilageCorn50Sunflo50 * max(0, 4.4-BW) - 2.844158 * SilageSunflower100 * max(0, 4.4-BW) + 0.003617947 * max(0, 4.4-BW) * silage + 0.003469904 * max(0, BW-4.4) * silage + 38.08742 - 0.005349635 * max(0, 1024.79-silage) - 0.01436351 * SilageSunflower100 * max(0, 1024.79-silage)/3

Table 4. Results of the MARS algorithm for Kıvırcık lambs

Coefficients	Estimate	Std. Error	T Value	Pr(>|t|)
(Intercept)	4.291e+01	7.772e-01	55.204	2.37e-09 ***
bx[, -1]h(silage-1024.14)	2.648e-02	1.114e-02	2.377	0.055024
bx[, -1]h(1024.14-silage)	-2.659e-01	1.248e-02	-21.311	6.96e-07 ***
bx[, -1]SilageSunflower100	-4.501e+00	6.405e-01	-7.027	0.000415 ***
bx[, -1]h(BW-4.1)	-2.673e+01	1.495e+00	-17.882	1.97e-06 ***
bx[, -1]h(4.1-BW)	3.352e+01	2.053e+00	16.325	3.36e-06 ***
bx[, -1]SilageSunflower100*h(1024.14-silage)	5.145e-03	3.338e-03	1.541	0.174223
bx[, -1]SilageCorn25Sunflo75*h(4.1-BW)	-4.376e+00	1.934e+00	-2.263	0.064282
bx[, -1]SilageCorn75Sunflo25*h(silage-1024.14)	-1.369e-01	1.752e-02	-7.814	0.000232 ***
bx[, -1]Typetwin*h(BW-4.1)	6.999e+00	1.700e+00	4.117	0.006236 **
bx[, -1]SilageCorn75Sunflo25	8.060e+00	1.783e+00	4.521	0.004014 **
bx[, -1]SilageSunflower100*h(4.1-BW)	-4.104e+01	3.085e+00	-13.305	1.11e-05 ***
bx[, -1]SilageCorn25Sunflo75*h(BW-4.1)	-3.467e+01	3.466e+00	-10.003	5.78e-05 ***
bx[, -1]Typetwin*h(1024.14-silage)	2.429e-02	2.127e-03	11.420	2.70e-05 ***
bx[, -1]Typetwin	-3.303e+01	5.659e+00	-5.837	0.001114 **
bx[, -1]BW*h(1024.14-silage)	5.983e-02	2.886e-03	20.732	8.20e-07 ***
bx[, -1]h(BW-4.1)*silage	1.392e-02	9.999e-04	13.924	8.55e-06 ***
bx[, -1]SilageCorn50Sunflo50*h(silage-1024.14)	9.671e-04	1.816e-03	0.533	0.613510
bx[, -1]SilageCorn75Sunflo25*h(BW-4.1)	4.289e+01	3.959e+00	10.833	3.66e-05 ***
bx[, -1]SilageCorn75Sunflo25*silage	-2.045e-02	2.338e-03	-8.745	0.000124 ***
bx[, -1]Typetwin*SilageSunflower100*h(4.1-BW)	3.007e+01	3.119e+00	9.642	7.13e-05 ***
bx[, -1]h(silage-773.857)	3.400e-02	2.408e-02	1.412	0.207629
bx[, -1]Typetwin*SilageCorn25Sunflo75*h(BW-4.1)	-6.394e+00	1.607e+00	-3.980	0.007282 **
bx[, -1]SilageCorn25Sunflo75*h(BW-4.1)*silage	4.903e-02	4.388e-03	11.172	3.07e-05 ***
bx[, -1]SilageCorn50Sunflo50	-2.750e+00	4.438e-01	-6.196	0.000815 ***
bx[, -1]Typetwin*SilageCorn25Sunflo75	-5.889e+00	6.425e-01	-9.166	9.50e-05 ***
bx[, -1]SilageCorn25Sunflo75*h(1024.14-silage)	5.495e-03	1.518e-03	3.619	0.011114 *
bx[, -1]SilageCorn50Sunflo50*h(4.1-BW)	1.401e+01	2.161e+00	6.485	0.000639 ***
bx[, -1]h(silage-744.786)	-4.062e-02	1.642e-02	-2.473	0.048233 *
bx[, -1]Typetwin*SilageCorn50Sunflo50	-1.266e+00	5.582e-01	-2.268	0.063803
bx[, -1]Typetwin*BW	6.127e+00	1.371e+00	4.468	0.004248 **
bx[, -1]h(silage-960)	-8.745e-02	1.890e-02	-4.628	0.003587 **
bx[, -1]h(silage-837.571)	6.474e-02	1.760e-02	3.678	0.010358 *
bx[, -1]Typetwin*SilageCorn75Sunflo25*h(1024.14-silage)	4.683e-03	2.036e-03	2.300	0.061106

*Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

According to this obtained equation, in the first boot strap, an increase of 13.02 kg in lambs with BW>4, 3.47 kg in singles with BW≤4, 2.75 kg for BW>4 fed 25% corn and 75% sunflower, 21.04 kg in BW≤4 fed 50% corn and 50% sunflower is expected. In the second bootstrap, an increase of 19.93 kg in lambs with BW>4.2, 8.8 kg in those fed with 50% corn and 50% sunflower BW≤4.4 is expected. In the third bootstrap, a small decrease of 0.005 kg in those with slage≤1024.79 and in body weight of 0.014 kg is expected in lambs with slage≤1024.79 g fed 100% sunflower is expected.

The plot between the predicted and observed FFLW values is showed in *Fig. 5* for Bagging MARS algorithm.

In the Bagging MARS model, there is a dual interaction between the variables. The graph showing the three-dimensional surface explaining the relationship between the independent variables and the dependent variable is given in *Fig. 6*.

Table 5. *Relative importance of model independent variables*		
Variables	**GCV**	**Number of Subsets**
Silage	100.0	33
BW	96.7	32
SilageSunflower100	83	31
SilageCorn25Sunflo75	74.9	28
SilageCorn75Sunflo25	72	27
Type twin	67.4	25
SilageCorn50Sunflo50	67.4	25

BW: *Birth weight,* ***GCV:*** *Generalized cross-validation,* ***Number of Subsets:*** *Variable importance using the "number of subsets" criterion. The number of subsets that include the variable*

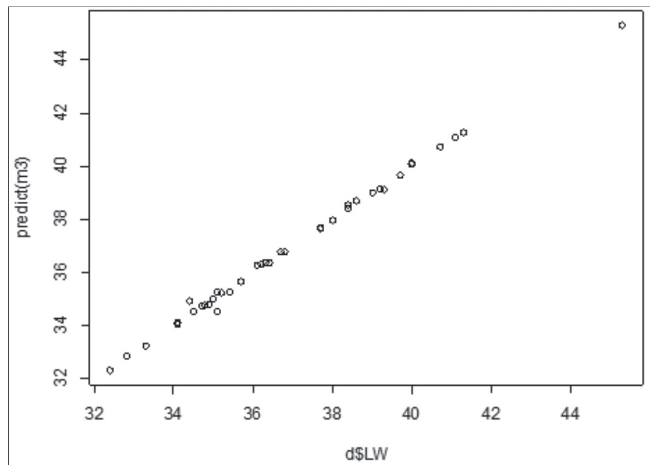

Fig 3. Observed versus predicted values of FFLW (for MARS algorithm)

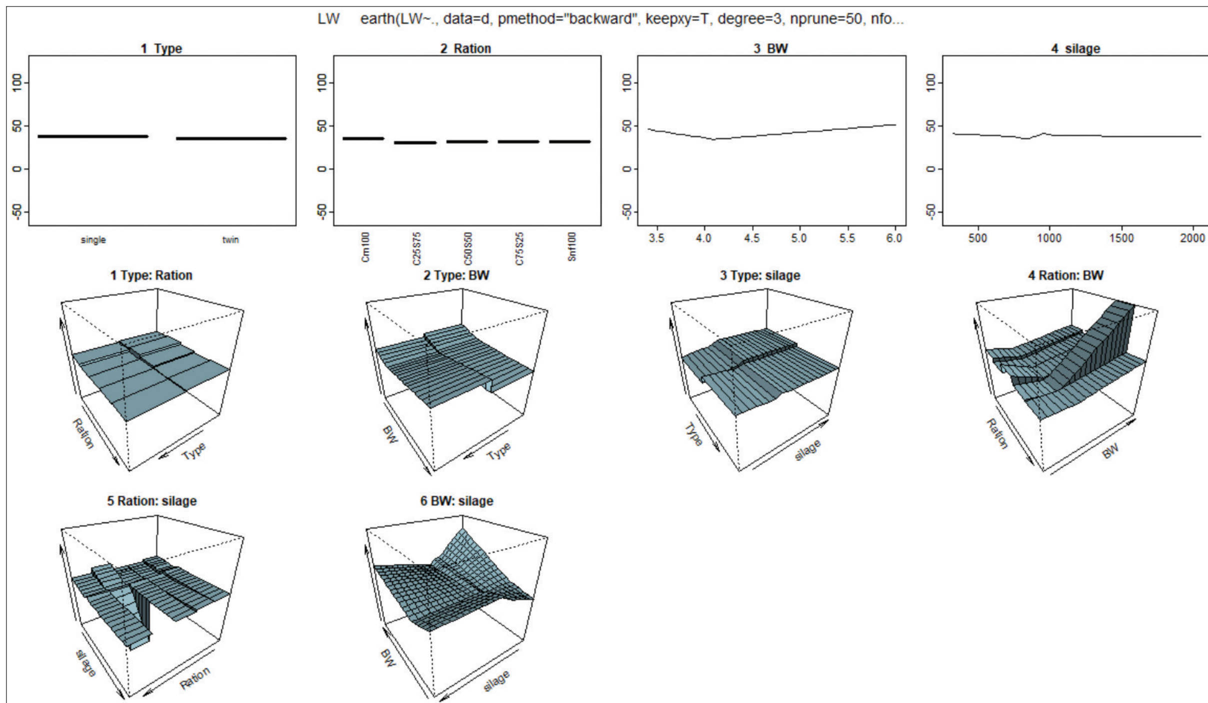

Fig 4. Model surface plots in MARS algorithm

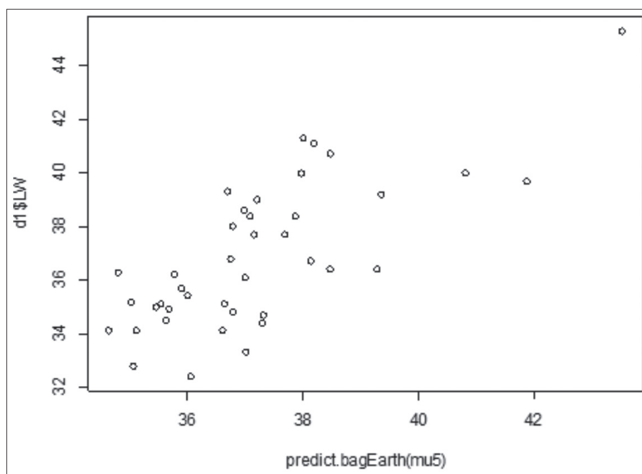

Fig 5. Observed and predicted values of FFLW (for Bagging MARS algorithm)

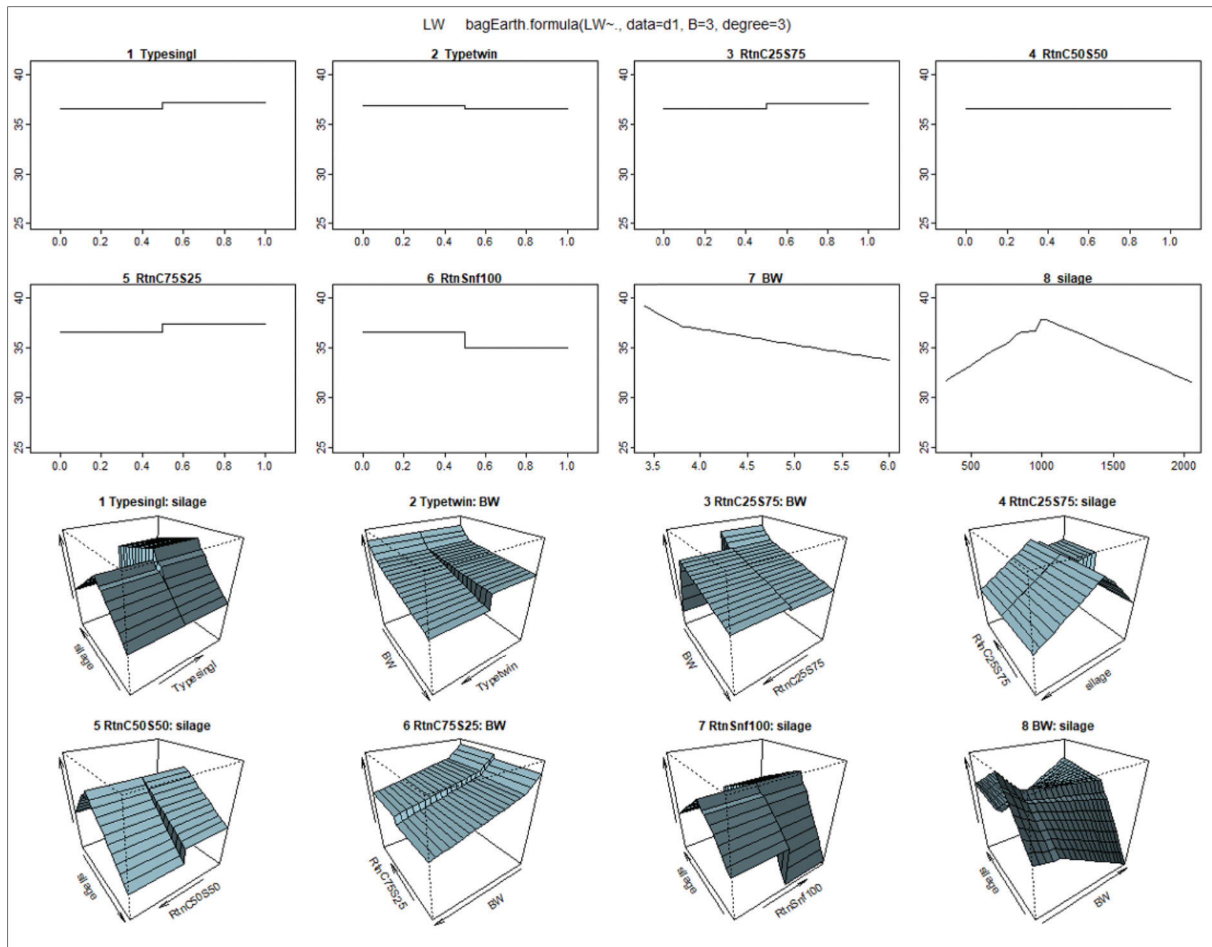

Fig 6. Model surface plots in Bagging MARS algorithm

Discussion

In the present study, two different techniques were used to determine the effect of silage type, silage consumption, birth type and birth weight on live weight in Kıvırcık lambs: MARS algorithm and Bagging MARS algorithm. In different studies, the values obtained regarding the performance of the fattening lambs show significant differences depending on the feeding method, fattening, duration, birth type and birth weight. In general, the results show that birth type and birth weight have an effect on final weight. Similarly, different feeding methods (consumption of concentrate feed, dry roughage and silage) can also have an effect on the final live weight of the lambs.

In the study, the effects of 4 different parameters (silage type, silage consumption, and birth type and birth weight) were found to be significant on the final body weight of lambs. Among these parameters, the most effective ones were determined as silage type, birth weight, silage consumption and birth type, respectively. When evaluated in terms of silage type, it was determined that the final live weight increased significantly due to the increase in sunflower silage in the ration. Altın et al.[29] determined the body weights of Kıvırcık and Karya breeds as 34.70 kg and 29.92 kg, respectively, in their study on live weight. In addition, with the regression analysis, it was determined that the effect of fattening starter live weight on the live weight at the end of the fattening was significant. The body weight values obtained are lower than the findings in this study. In the study of [30], live weights of 180-day-old Kıvırcık lambs were found to be 37.67 kg in singles and 35 kg in twins. The reported findings are in agreement with the results of this study. In addition, the researchers used the linear regression model to determine the effect of birth weight and daily age on body weight. With regression analysis, they found to be significant the effect of birth weight on live weight. In addition, the effect of birth type (single and twin) on birth weight and body weight was found to be significant. The results obtained were similar to the findings of this study. Ekiz et al.[31] found as 26.74 kg the body weight of Kıvırcık lambs at Marmara Animal Breeding Research Institute. The reported values differed from the results in this study. In another study, birth weights were investigated in different genotypes and growth periods. Birth weights in German Black Head

x Kıvırcık x Kıvırcık, German Black Head x Merino x Kıvırcık and Kıvırcık genotypes were 4.08, 4.32 and 3.85 kg, respectively, while their 75-day live weight was 19.33, 19.38 and 17.58 kg, respectively [32]. Birth weights were close to the results obtained in this study.

In a study, Kıvırcık lambs housed in cross-ventilated coops were fed with an average of 600 g/lamb concentrate, 100 g/lamb alfalfa grass and 300 g/lamb vetch-wheat mixed grass daily until the post-weaning period (135 days). Birth type significantly affected the birth weight and live weight of lambs [10]. Similarly, the effect of birth type on live weight was found to be significant in this study.

Goodness-of-fit statistics are important in comparing data mining and other statistical methods used to predict any trait in lambs as in all living things.

In a study, artificial neural network, multivariate adaptive regression splines (MARS), support vector regression and fuzzy neural network models were used to predict the serum Immunoglobulin G concentration from gamma-glutamyl transferase enzyme activity, total protein concentration and albumin in lambs. Correlation coefficient (r), root mean square error (RMSE) and mean absolute error (MAE) statistics were used to compare models. It has been observed that the fuzzy neural network is the most successful method for the prediction of Immunoglobulin G value [33]. Although the study in question is similar in terms of using the MARS method and calculating the correlation coefficient and RMSE statistics, it differs in terms of the results obtained with other methods and several different goodness-of-fit statistics used in estimating the dependent variable.

In another study on the use of goodness-of-fit statistics to compare models, different growth models were used for body weight modeling in Romanov lambs. Adjusted determination coefficient (R^2 adj.), mean square error (MSE), Akaike information criteria (AIC) and Durbin-Watson (DW) statistics were used to determine the most appropriate model [34]. Goodness-of-fit statistics (R^2 adj. and AIC) used by the authors in their study were also used in this study.

In the current research, final live weight of Kıvırcık lambs were evaluated to on the basis of Multivariate Adaptive Regression Splines (MARS) and Bagging MARS algorithms showing perfect performance as a robust algorithm without overfitting problem. MARS algorithm gave better results than Bagging MARS algorithm in modeling body weight in lambs. It is expected that good results can be achieved in data mining applications such as MARS and Bagging MARS algorithms in livestock data.

Ethical Statement

This study was carried out with the approval of Bursa Uludağ University Animal Experiments Local Ethics Committee dated 07.01.2020 and numbered 2020-01/02.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author (Ş. Çelik).

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Competing of Interest

The authors declared no competing interests.

Author Contributions

IA conceived and supervised the study. ÖŞ carried out animal experiments and made measurements. ŞÇ statistical analysis and writing the manuscript. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

References

1. Kara H, Eroğlu A: Tam sayılı doğrusal programlama metodu ile entansif hayvancılık işletmesinin kapasite planlaması: Konya (Ereğli) örneği. *Çukurova Tarım Gıda Bil Der,* 33 (2): 31-46, 2018.

2. Kutlu HR: Tüm yönleriyle silaj yapımı ve silajla besleme. ÇÜ Ziraat Fakültesi, Zootekni Bölümü, 2002. http://www.zootekni.org.tr/upload/file/silaj%20el%20ktabi.pdf. *Accessed:* 29.01.2022.

3. Alçiçek A: Silo yemi; önemi ve kalitesini etkileyen faktörler. E.Ü.Z.F. Tarımsal Uygulama ve Araştırma Merkezi Yayını No. 22, İzmir, 1995.

4. Yaylak E, Alçiçek A: Sığır besiciliğinde ucuz bir kaba yem kaynağı: Mısır silajı. *Hayvansal Üretim,* 44 (2): 29-36, 2003.

5. Yıldız S: Saanen x Kıl keçisi melezi (F_1) keçilerin rasyonlarında mısır silajı yerine ayçiçeği silajının kullanılmasının rumen ve kan parametreleri ile süt verimi ve bileşimine etkisi. *Doktora tezi*. Yüzüncü Yıl Üniv. Fen Bil. Enst., 2017.

6. Öztürk D: The characteristics of goat farming systems in Kahramanmaraş in the North Eastern Mediterranean region of Turkey. *7th International Conference on Goats*. 15-21 May 2000, Paris, France, pp.360-361, 2000.

7. Koyuncu M, Akgün H: Some fertility traits of Kıvırcık sheep in rural farms. *J Anim Prod*, 59 (1): 33-40, 2018. DOI: 10.29185/hayuretim.406696

8. Erdoğmuş Süer İN, Kocabağlı N: Yüksek düzeyde konsantre yemle beslenen kuzularda yeme maya *(Saccharomyces cerevisiae)* veya malik asit ilavesinin performans üzerine etkisi. *IJVAR*, 1 (1): 01-04, 2018.

9. Ekiz B, Yilmaz A, Yalcintan H, Yakan A, Kocak O, Ozcan M: The effect of production system and finish weight on carcass and meat quality of Kivircik lambs. *Ann Anim Sci*, 19 (2): 517-538, 2019. DOI: 10.2478/aoas-2019-0010

10. Kader Esen V, Elmacı C: The estimation of live weight from body measurements in different meat-type lambs. *J Agr Sci*, 27 (4): 469-475, 2021. DOI: 10.15832/ankutbd.719037

11. Karabacak A, Boztepe S: Yağlı kuyruklu ve ince yağsız ince koyun ırklarının besi performanslarının karşılaştırılması. *Selçuk Üniv Ziraat Fak Derg*, 21 (42): 89-95, 2007.

12. Canbolat Ö, Kara H, Filya İ, Kamalak A: Kuzu besi rasyonlarına

ilave edilen canlı mayanın besi performansı ile bazı rumen sıvısı ve kan parametreleri üzerine etkisi. *Uludağ Üniv Ziraat Fak Derg*, 29 (1): 73-85, 2015.

13. Friedman JH: Multivariate adaptive regression splines. *Ann Statist*, 19 (1): 1-67, 1991. DOI: 10.1214/aos/1176347963

14. Goh ATC, Zhang W, Zhang Y, Xiao Y, Xiang Y: Determination of earth pressure balance tunnel-related maximum surface settlement: A multivariate adaptive regression splines approach. *Bull Eng Geol Environ*, 77, 489-500, 2018. DOI: 10.1007/s100640160937-8

15. Zhang YZ, Ma W, Wang TL, Cheng BY, Wen A: Characteristics of the liquid and vapor migration of coarse-grained soil in an open-system under constant temperature freezing. *Cold Reg Sci Technol*, 165:102793, 2019. DOI: 10.1016/j.coldregions.2019.102793

16. Ju X, Chen VCP, Rosenberger JM, Liu F: Fast knot optimization for multivariate adaptive regression splines using hill climbing methods. *Expert Sys Appl*, 171:114565, 2021. DOI: 10.1016/j.eswa.2021.114565

17. Hastie T, Tibshirani R, Friedman J: The Elements of Statistical Learning: Data Mining, Inference, and Prediction. 2nd ed., Springer, New York, USA, 2009.

18. Milborrow S: Notes on the earth package 2021; http://www.milbo.org/doc/earthnotes.pdf. *Accessed:* 22.11.2021.

19. Kunn M, Johnson K: Applied predictive modeling. NY, 2013. https://doi.org/10.1007/978-1-46146849-3. *Accessed:* 17.10.2021.

20. Breiman L: Bagging Predictor Technical Report No. 421. Department of Statistics University of California, 1994.

21. Hasyim M, Rahayu DS, Muliawati NE, Hayuhantika D, Puspasari R, Anggreini D, Hastari RC, Hartanto S, Utomo FH: Bootstrap aggregating multivariate adaptive regression splines (Bagging MARS) to analyze the lecturer research performance in private university. *J Physi Conf Ser*, 1114:012117, 2018. DOI: 10.1088/1742-6596/1114/1/012117

22. Willmott CJ, Matsuura K: Advantages of the mean absolute error (MAE) over the root mean square error (RMSE) in assessing average model performance. *Clim Res*, 30, 79-82, 2005. DOI: 10.3354/cr030079

23. Liddle AR: Information criteria for astrophysical model selection. *Mon Not R Astron Soc,* 377: L74-L78, 2007. DOI: 10.1111/j.1745-3933.2007.00306.x

24. Takma C, Atil H, Aksakal V: Çoklu doğrusal regresyon ve yapay sinir ağı modellerinin laktasyon süt verimlerine uyum yeteneklerinin karşılaştırılması. *Kafkas Univ Vet Fak Derg*, 18, 941-944, 2012. DOI: 10.9775/kvfd.2012.6764

25. Eyduran E, Akın M, Eyduran SP: Application of multivariate adaptive regression splines in agricultural sciences through R software. Nobel Bilimsel Eserler Sertifika, p.20779, 2019.

26. Grzesiak W, Zaborski D: Examples of the use of data mining methods in animal breeding. **In,** Karahoca A (Ed): Data Mining Applications in Engineering and Medicine. 303-324, InTech, Rijeka, Croatia. 2012.

27. Milborrow S: Derived from Mda: Mars by Trevor Hastie and Rob Tibshirani. Uses Alan Miller's Fortran utilities with Thomas Lumley's leaps wrapper. earth: Multivariate Adaptive Regression Splines. R package version 4.4. 4. 20164; 2016.

28. Milborrow MS: Package 'earth'. R Software package, 2019.

29. Altın T, Karaca O, Cemal İ, Yılmaz M, Yılmaz O: Kıvırcık ve Karya kuzularda besi ve karkas özellikleri. *Hayvansal Üretim*, 46 (1): 19-29, 2005.

30. Alarslan E, Aygün T: Yalova'da yetiştirilen Kıvırcık kuzularında büyüme-gelişme ve bazı morfolojik özelliklerinin belirlenmesi. *J Anim Prod*, 60 (1): 39-50, 2019. DOI: 10.29185/hayuretim.556669

31. Ekiz B, Yılmaz A, Özcan M, Kaptan C, Hanoğlu H, Erdoğan I, Yalcıntan H: Carcass measurements and meat quality of Turkish Merino, Ramlic, Kıvırcık, Chios and Imroz lambs raised under an intensive production system. *Meat Sci*, 80, 64-70, 2009. DOI: 10.1016/j.meatsci.2008.12.001

32. Ekiz B, Altınel A: The growth and survival characteristics of lambs produced by commercial crossbreeding Kıvırcık ewes with F_2 rams with the German Black-Headed Mutton genotype. *Turk J Vet Anim Sci*, 30, 507-512, 2006.

33. Cihan P, Gökçe E, Atakişi O, Kırmızıgül AH, Erdoğan HM: Prediction of immunoglobulin G in lambs with artificial intelligence methods. *Kafkas Univ Vet Fak Derg*, 27 (1): 21-27, 2021. DOI: 10.9775/kvfd.2020.24642

34. Tahtali Y, Sahin M, Bayyurt L: Comparison of different growth curve models in Romanov lambs. *Kafkas Univ Vet Fak Derg*, 26 (5): 609-615, 2020. DOI: 10.9775/kvfd.2020.23954

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Research Article

In Silico Analysis of the Structural and Functional Consequences of Polymorphic Amino Acid Substitutions in the Cattle HSF1 Protein

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Abstract: Heat stress causes a decrease in the productivity of livestock by negatively affecting some important economic features such as fertility, growth and milk production. The heat shock transcription factor 1 (HSF1) gene plays a key role in the regulation of the stress response. Therefore, the present study aimed to predict the most deleterious non-synonymous single nucleotide polymorphisms (nsSNP) on the cattle HSF1 gene via in silico analyses. Out of 170 nsSNPs in the HSF1 gene, 14 SNPs were predicted as deleterious by all the nine servers (PredictSNP, Mapp, PhDSNP, PolyPhen-1, PolyPhen-2, Sift, Snap, nsSNPAnalyzer, and Panther). Consurf analysis determined that the vast majority of SNPs predicted to be deleterious were evolutionary conserved. Protein structural analyses were performed I-Mutant, Mupro, Hope Project server, RaptorX and Swiss Model server. The 12 amino acid substitutions (V15G, F18L, L19R, K21M, I35T, V46E, V56G, F61L, A67D, Y76D, V81G, L112P) in the DNA binding region of the cattle HSF1 protein were predicted to be highly deleterious. The P112 variant was predicted to disrupt an α-helix structure. It was determined that the two amino acid changes (K21M, Y76D) on the surface of the protein were different in terms of hydrophobicity, charge, and size. These variants (M21, D76) might hamper the protein's interaction with the heat shock elements.

Keywords: *HSF1, Heat stress, Cattle, nsSNP, HSP*

Sığır HSF1 Proteinindeki Amino Asit Polimorfizmlerinin Yapısal ve Fonksiyonel Sonuçlarının *In Silico* Analizi

Öz: Sıcaklık stresi, hayvanların doğurganlık, büyüme, süt üretimi gibi bazı önemli ekonomik özelliklerini olumsuz etkileyerek verimde azalmaya neden olmaktadır. Isı şoku transkripsiyon faktörü 1 (HSF1) geni, stres yanıtının düzenlenmesinde önemli bir rol oynar. Bu çalışma, sığır HSF1 geni üzerindeki, en zararlı eş anlamlı olmayan tek nükleotid polimorfizmlerini (nsSNP) *in silico* analizler ile belirlemeyi amaçlamıştır. HSF1 geni üzerinde bulunan 170 nsSNP dokuz tahmin programı (PredictSNP, Mapp, PhDSNP, PolyPhen-1, PolyPhen-2, Sift, Snap, nsSNPAnalyzer ve Panther) ile değerlendirildi. 14 nsSNP tüm tahmin programları tarafından zararlı bulundu. Consurf analizi, zararlı olduğu tahmin edilen SNP'lerin büyük çoğunluğunun evrimsel olarak korunduğunu belirledi. Proteinin yapısal analizleri, I-Mutant, Mupro, Hope Project, RaptorX ve Swiss Model sunucuları kullanılarak gerçekleştirildi. Sonuç olarak, sığır HSF1 proteininin DNA bağlama bölgesindeki 12 amino asit ikamesinin (V15G, F18L, L19R, K21M, I35T, V46E, V56G, F61L, A67D, Y76D, V81G, L112P) oldukça zararlı olduğu tahmin edildi. P112 varyantının, bir α-sarmal yapısını bozduğu belirlendi. HSF1 proteininin yüzeyindeki iki amino asit değişiminin (K21M, Y76D), hidrofobiklik, yük ve boyut açısından farklılığa neden olduğu belirlendi. Proteinin, M21 ve D76 varyantlarının, ısı şoku elementleri ile etkileşimini engelleyerek, ısı şok proteinlerinin transkripsiyonunu azaltabileceği tahmin edildi.

Anahtar sözcükler: *HSF1, Sıcaklık stresi, Sığır, nsSNP, HSP*

Introduction

The most important abiotic stress factor for livestock is the ambient temperature [1]. High environmental temperatures cause organisms to absorb more heat than they can dissipate, resulting in heat stress [2,3]. Heat stress causes a decrease in the productivity of livestock by negatively affecting some important economic features such as fertility, growth and milk production [4,5]. It also causes adverse effects on the immune responses of livestock [6]. Therefore, developing cattle breeds that can both tolerate heat stress and maintain productivity has become an important goal of researchers and cattle breeders around the world [7].

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The heat shock response (HSR) begins to produce with the transcription of heat shock genes in response to stress signals triggered by proteotoxic conditions such as elevated temperatures, oxidative stress, heavy metals and infections [8]. The HSR activation allows the cells to increase the expression of heat shock protein (HSP) genes known as molecular chaperones. The HSPs whose essential role is in protein folding, maintain cellular proteome homeostasis by assisting in the refolding of misfolded proteins or driving them to degradation [9,10]. The HSR is regulated by the heat shock transcription factor family (HSF) consisting of five-member (HSF1, HSF2, HSF3, HSF4, HSF5) in mammalians [11]. The evolutionarily highly conserved HSF1 acts as a key regulator of HSPs expression [12,13].

The HSF1 gene (ENSBTAG00000020751) is located on chromosome 14 in cattle and consists of 13 exons that encode 525 amino acids [14]. The bovine HSF1 protein (UniProtKB ID: Q08DJ8) contains several functional domains according to UniProtKB; DNA binding domain (position; 15-120 amino acids), N-terminal oligomerization domains-hydrophobic repeat (HR)-A/B (position; 130-203 amino acids), D domain (position; 203-224 amino acids), regulatory domain (position; 221-310 amino acids), oligomerization domain HR-C (position: 380-405 amino acids), transactivation domain and disordered region (position; 367-525 amino acids). The best-conserved region in the HSF family is the DNA binding domain (DBD) [15]. The HSF1 binds to DNA as a trimer. The trimer recognizes a specific nGAAn sequence in DNA known as heat shock elements (HSE) [16]. In the absence of stress, the majority of HSF1 exists in a monomeric conformation with a little affinity for the HSE. Spontaneous trimerization of the HSF1 is suppressed by the HR-C [17,18]. In stress conditions, the HSF1 is converted into a trimeric form that is a transcriptionally active form. The trimerization is regulated by the HR-A/B. The D domain interacts with JNK1 and MAPK3 and is involved in the translocation of HSF1 to the nucleus. The HSF1 is transported to the nucleus and post-transcriptional modifications occur. The regulatory domain is necessary for transcriptional activation through its phosphorylation. The transactivation domain is involved in directing HSF1 to specific target genes and regulating the extent of its activation [16,17].

It has been reported in previous studies that the HSF1 gene variants are associated with tolerance to heat stress [19], meat quality traits [20], reproductive traits [21] and milk yield [19]. The present study was designed to identify the deleterious nsSNPs in the bovine HSF1 gene using *in silico* analyses. These nsSNPs that are detrimental to the structure and function of the protein may be associated with susceptibility to heat stress in cattle.

Material and Methods

SNP Dataset

The SNPs in the HSF1 gene (ENSBTAG00000020751) were obtained from the Ensembl genome browser (www.ensemble.org). The amino acid sequence of HSF1 protein (Transcript ID: ENSBTAT00000083220.1) in the fasta format was retrieved from the Ensembl.

Prediction of Functional Effect

PredictSNP [22] was used to predict the effect of missense mutations in the bovine HSF1 gene. This tool is a consensus classifier that comprises scores from different predictors (MAPP, PhDSNP, PolyPhen-1, PolyPhen-2, SIFT, SNAP, nsSNPAnalyzer, and PANTHER) and classifies the variants as "Deleterious" and "Neutral". In addition, it converts the individual confidence scores of each estimator into a comparable scale that represents the expected percentage of accuracy, ranging from 0-100%. MAPP, PhDSNP, PolyPhen-1, PolyPhen-2, SIFT, SNAP, nsSNPAnalyzer, and PANTHER tools use different classification methods; physicochemical properties and alignment score, support vector machine, expert set of empirical rules, Bayesian classification, alignment score, neural network, random forest, and alignment score respectively [23]. SNPs that were found to be predicted as neutral by one tool have been excluded from the study.

Prediction of Protein Stability and Amino Acid Conservancy

The protein stability changes resulting from missense variants were predicted using I-Mutant 3.0 [24] and Mupro [25] servers. I-Mutant, a support vector machine-based automated web server, estimates the effect of an amino acid substitution effect on protein stability by calculating the free energy change value (DDG) of the native and mutant protein. The range of $-0.5 \leq DDG \leq 0.5$ values is classified as neutral mutation, while <-0.5 as a large decrease and $0.5<$ as a large increase. The Mupro is a server based on support vector machines and neural networks machine learning methods, which predict how single amino acid substitution affects protein stability. A negative DDG score indicates that amino acid substitution decreases protein stability, while a positive score indicates that protein stability increases. The bigger the DDG score, the more confident the result.

Conservation analysis of the HSF1 protein was performed using the Consurf webserver [26]. Utilized the phylogenetic relationships between homologous sequences, Consurf calculates the conservation scores of amino acid positions and determines functional regions. These conservations scores (1-3 is variable, 4-6 is average and 7-9 is highly conserved) are organized in color-coded regions depicted in the structure of the protein for representation.

Prediction of Protein 3-D Structure

The 3-D structural analysis and modeling studies of HSF1 protein were performed using the HOPE server [27], RaptorX [28], Swiss-PdbViewer v4.1 [29], and PyMOL v2.5 (The PyMOL Molecular Graphics System, Version 2.5 Schrödinger, LLC). The Hope server was used to investigate the effects of missense variants on protein structure. Hope server utilizes Protein Data Bank (PDB), UniProt databases, What IF web services, Reprof, Yasara programs, and Distributed Annotation System servers to estimate the effect of substitution amino acid on protein structure. It predicts hydrophobicity, charge, size change, and modeling of 3-D structure for natural and mutant amino acids.

The full-length 3-D structure of bovine HSF1 protein is not available in PDB. Hence, RaptorX was used to make a 3-D structural model for HSF1. It is a web server predicting the structure property of a protein sequence without using any template. Furthermore, the Swiss-Model server was used to predict the 3-D structure of the DNA binding domain of the HSF1 gene. The quality of models was evaluated according to Ramachandran Plot and MolProbity score by Swiss-Model Structure Assessment. PyMOL v2.5 and Swiss PDB Viewer v4.1 were used to visualize the HSF1 protein 3-D structures.

Results

Retrieval of nsSNPs and Function Prediction

The nsSNPs (n: 170) of the HSF1 gene were retrieved from the Ensembl SNP database. The retrieved nsSNPs were analyzed using PredictSNP, Mapp, PhD-SNP, PolyPhen-1, PolyPhen-2, Sift, Snap, and Panther. PredictSNP classified 51 out of 170 missense mutations to be deleterious, Mapp predicted 46 out of 170 missense mutations to be deleterious, PhD-SNP predicted 40 out of 170 missense mutations to be deleterious, PolyPhen-1 predicted 57 out of 170 missense mutations to be deleterious, PolyPhen-2 predicted 80 out of 170 missense mutations to be deleterious, Sift predicted 85 missense mutations to be deleterious, Snap predicted 44 out of 170 missense mutations to be deleterious, and Panther predicted 22 out of 170 missense mutations to be deleterious. Forty-four missense mutations were classified as "unknown" by the Panther tool. The effect of 14 SNPs *(Table 1)* categorized as "deleterious" in all tools on the protein structure and stability was investigated.

Prediction of Protein Stability and Amino Acid Conservancy

I-Mutant 3.0 and Mupro were used to predict changes in protein stability caused by functionally damaging 14 nsSNPs. The DDG value and binary classification estimate in I-Mutant 3.0 showed that thirteen amino acid substitutions (V15G, F18L, L19R, I35T, V46E, V56G, F61L, A67D, Y76D, V81G, L112P, V172E, F496C) were able to cause largely decreased stability (<-0.5 DDG) of the protein. The K12M mutation was classified as neutral (DDG= -0.14) by I-Mutant 3.0 *(Table 2)*. The Mupro made similar predictions for amino acid substitutions other than K21M. It predicted that the K21M mutation could

Table 1. *Results of nsSNPs predicted with I-Mutant, Mu-Pro and Consurf servers*

rsIDs	Allele	Residue Change	I-Mutant DDG Value (kcal/mol)	Mu-Pro DDG Value (kcal/mol)	Consurf Results		
					Functional/ Structural	Score	Domain
rs481839785	A/C	V15G	-2.43	-2.12	Structural	9	DNA binding
rs474682793	G/T	F18L	-1.10	-0.97	Structural	9	DNA binding
rs459488723	A/C	L19R	-1.75	-1.43	Structural	9	DNA binding
rs451876582	T/A	K21M	-0.14	0.18	Functional	9	DNA binding
rs445864779	A/G	I35T	-2.49	-1.97	Structural	9	DNA binding
rs454788300	A/T	V46E	-0.95	-1.22	Structural	9	DNA binding
rs442669059	A/C	V56G	-2.19	-2.25	Structural	9	DNA binding
rs432002899 rs453627348	G/C A/G	F61L	-1.21	-1.05	Structural	9	DNA binding
rs456086940	G/T	A67D	-0.76	-0.77	Structural	9	DNA binding
rs470395900	A/C	Y76D	-1.17	-1.20	Functional	9	DNA binding
rs477899807	A/C	V81G	-2.59	-1.98	Structural	9	DNA binding
rs443947366	A/G	L112P	-1.67	-1.87	Structural	9	DNA binding
rs481574682	A/T	V172E	-0.50	-0.81	-	8	N-terminal oligomerization domains
rs439067006	A/C	F496C	-1.34	-1.06	-	6	Disordered

Table 2. *The HOPE server analysis results for deleterious nsSNPs*

Amino Acid Substitutions	Size of Mutant Type a.a.[a] Relative to Wild Type a.a.[a]	Hydrophobicity of Wild Type a.a.[a] Relative to Mutant Type a.a.[a]	Wild Type a.a.[a] Charge	Mutant Type a.a. Charge
V15G	Smaller	More hydrophobic	-	-
F18L	Smaller	-	-	-
L19R	Bigger	More hydrophobic	Neutral	Positive
K21M	Smaller	Less hydrophobic	Positive	Neutral
I35T	Smaller	More hydrophobic	-	-
V46E	Bigger	More hydrophobic	Neutral	Negative
V56G	Smaller	More hydrophobic	-	-
F61L	Smaller	-	-	-
A67D	Bigger	More hydrophobic	Neutral	Negative
Y76D	Smaller	More hydrophobic	Neutral	Negative
V81G	Smaller	More hydrophobic	-	-
L112P	Smaller	-	-	-
V172E	Bigger	More hydrophobic	Neutral	Negative
F496C	Smaller	-	-	-

[a] *Amino acid*

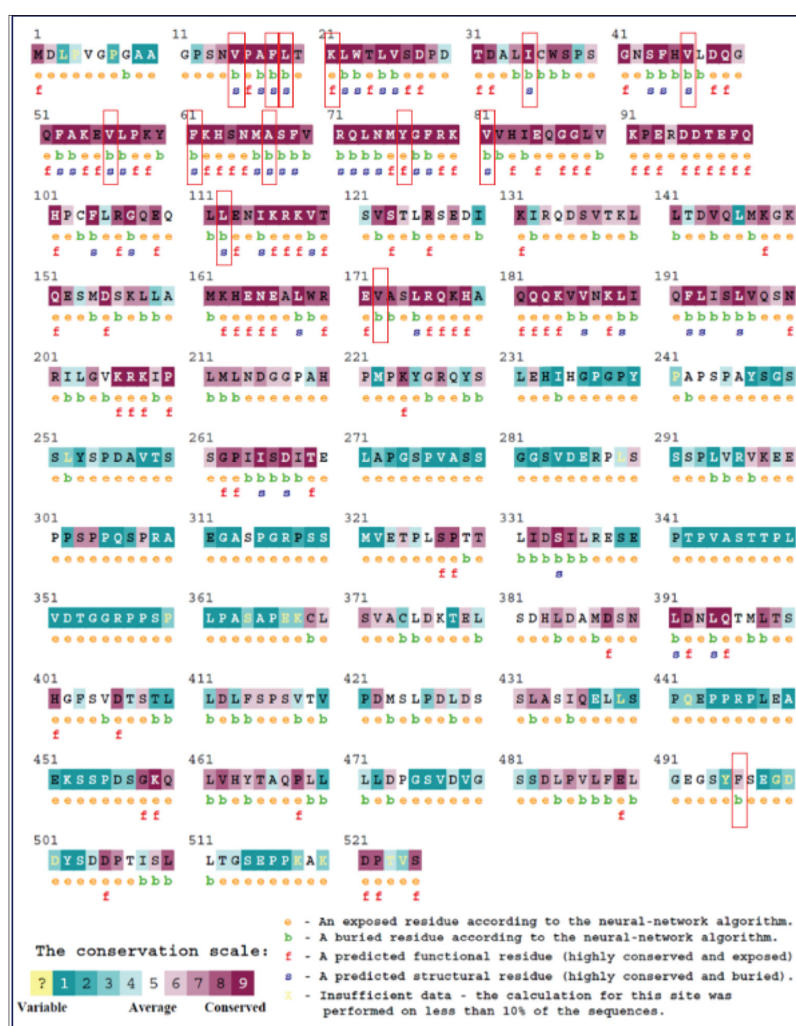

Fig 1. The Consurf analysis of the bovine HSF1 protein. The positions of the amino acid substitutions predicted to be most deleterious are marked with a red rectangle

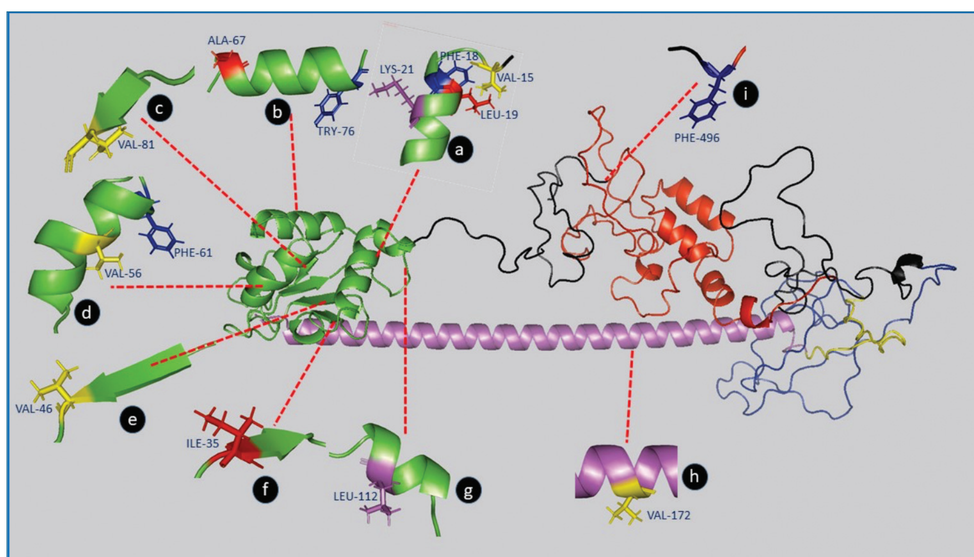

Fig 2. The 3-D structure of the bovine HSF1 protein generated by RaptorX; DNA binding domain (green), N-terminal oligomerization domain HR-A/B *(magenta),* D domain (yellow), transactivation domain *(red),* regulatory domain *(blue),* disordered regions *(black).* The locations of deleterious amino acid substitutions are shown on the structure; **a-** The three mutations (F18L, L19R, K21M) on the first α-helix of the DBD and a mutation (V15G) on the coil, **b-** The two mutations (A67D, Y76D) on the fourth α-helix of the DBD, **c-** A mutation (V81G) on the third beta-sheet of the DBD, **d-** Two mutations (V56G, F61L) in the third α-helix of the DBD, **e-** A mutation (V46E) on the second beta-sheet of the DBD, **f-** The mutation (I35T) on the first beta-sheet of the DBD, **g-** The mutation (L112P) on the last α-helix of the DBD, **h-** The mutation (V172E) on the N-terminal oligomerization domain, **i-** The mutation (F496C) on the disordered region

result in increased protein stability (DDG= 0.18). Since the DDG values predicted by the I-Mutant (DDG= -0.14) and Mupro (DDG= 0.18) programs for the K21M were less than 0.5, it was accepted as neutral *(Table 2)*.

The fasta sequence of the HSF1 protein was analyzed on the Consurf server. Of 14 amino acid residues, twelve (V15G, F18L, L19R, I35T, V46E, V56G, F61L, A67D, V81G, L112P, V172E, F496C) were categorized as structural residues, and two (K21M, Y76D) as functional residues *(Fig. 1)*. It was determined that 13 (V15G, F18L, L19R, K21M, I35T, V46E, V56G, F61L, A67D, Y76D, V81G, L112P, V172E) of them had a high conservation score and one (F496C) of them had an average conservation score. According to the Consurf analysis results, the most conserved region of the HSF1 protein is the DBD region. Out of 106 residues in the DBD region, 93 residues (87.74%) are in the range of 7-9 conserved scores *(Fig. 1)*.

Hope Result and 3-D Structure Prediction

The fourteen amino acid substations were also submitted for the HOPE project analysis. According to the hope results it was found that, out of the 14 mutations, 10 have altered hydrophobicity, 6 differed in charge, and all mutated amino acids differed in size *(Table 2)*. Hope server determined that the L112P mutation will disrupt the last α-helix of the DBD *(Fig. 3)*. The structural effect of this mutation was examined using the Swiss PDB viewer. It was observed that the H-bond formed by L122 with E109 was not formed by P122. The proline residue can disrupt the α-helix in the absence of the hydrogen bond (P122 with E109), and this can have serious effects on the structure and function of the protein.

The HSF1 protein fasta sequence (525 amino acids) from the Ensemble genome browser was the input for the RaptorX server. The five 3-D models were generated by RaptorX. The obtained structures were assessed according to Ramachandran Plot and MolProbity score in the Swiss-Model server. The 3-D model of the HSF1 protein with the best score (MolProbity Score: 3.26, Ramachandran Favored: 90.06) was visualized using the PyMOL *(Fig. 2)*. The positions of the amino acid mutations predicted to be deleterious were shown in this model *(Fig. 2)*.

Fasta sequence of the DBD (between 15-200 amino acids) was submitted as the input file for Swiss-Model. The human HSF1 protein (PDB ID: 5D5U.1.b) having sequence coverage of 80% and sequence identity of 98.11% was selected as a template for bovine HSF1 protein and the 3-D model was constructed automatically in Swiss-Model. The amino acid substitutions in DBD were evaluated using this model in Swiss PDB viewer *(Fig. 3, Fig. 4)*.

Discussion

The HSF1, the main regulator of the HSPs expression, plays an important role in cell survival under stress [30]. The HSPs are the major molecular chaperones that modulate

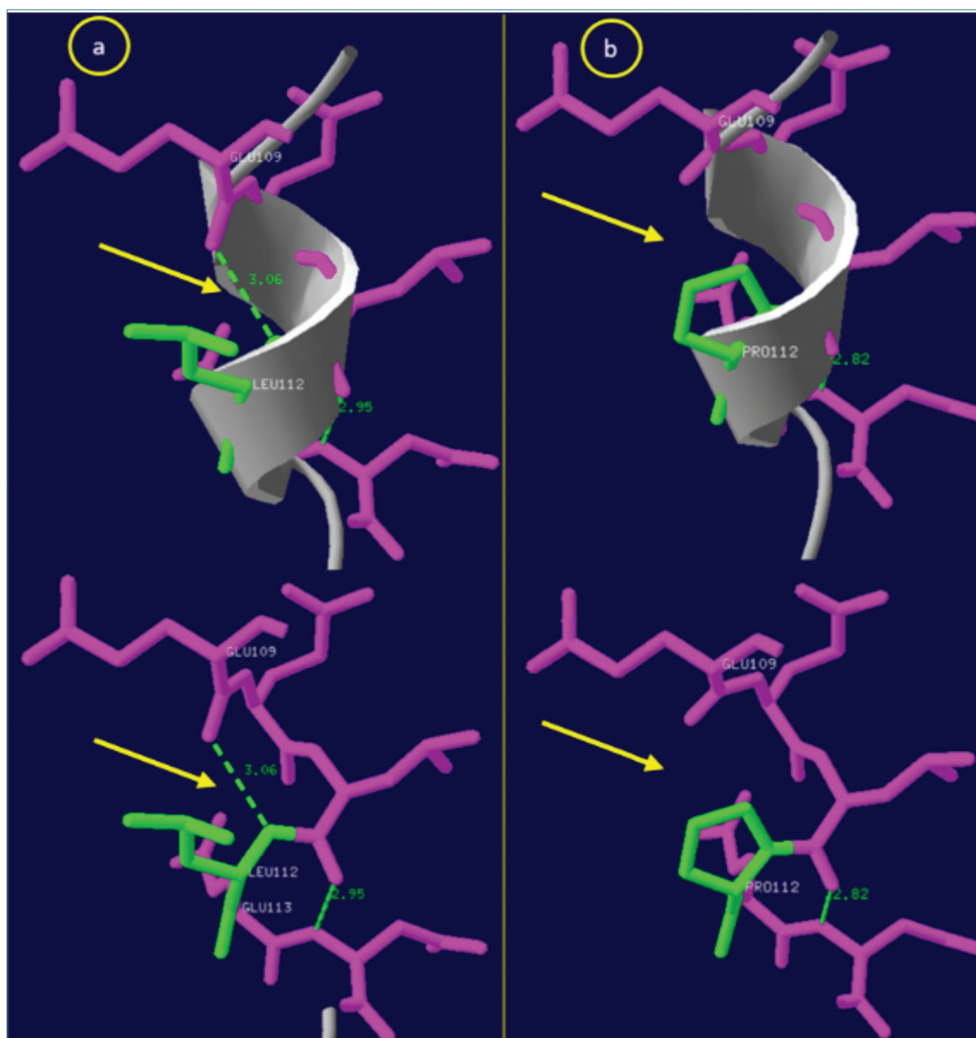

Fig 3. Structural effect of the L112P mutation on the native structure of HSF1 protein visulated by Swiss PDB Viewer. The backbone, sidechain and ribbon presentation structures are presented in the upper pictures, and the backbone and sidechain structures are presented in the lower pictures. H-bonds are indicated by green discontinuous lines. **a-** In the native HSF1 protein, L112 forms two H-bonds with E109 (3.06 Å) and E113 (2.95 Å), **b-** In the mutant HSF1 protein, P122 forms one H-bond with E113 (2.82 Å)

protein folding. They maintain protein homeostasis under stress factors such as increasing temperatures, infectious diseases, and heavy metals [31,32]. The expression of the HSF1 gene is increased in cattle under heat stress [30]. It has been suggested that the HSF1 gene expression level may be an indicator of thermotolerance in cattle [4]. Moreover, SNPs in the HSF1 gene are associated with susceptibility to heat stress in cattle [5,33].

SNPs cause alteration of a single base pair in both coding and non-coding regions. SNPs in non-coding regions can affect gene expression at the transcriptional and posttranscriptional levels. nsSNPs alter amino acid sequences, affect protein structure and function, and have potentially deleterious effects [34]. Today, thanks to rapidly developing sequencing technologies, there are many nsSNPs in variation databases. However, determining the effects of nsSNPs by molecular genetic experiments is a time-consuming and laborious task [35]. The fastest and cheapest way to predict the potential consequences of an nsSNP is to perform bioinformatics analysis [36,37]. Using multiple bioinformatics tools that evaluate different parameters helps to identify results with higher confidence levels [38].

In this study, 13 bioinformatics tools (PredictSNP, MAPP, PhDSNP, PolyPhen-1, PolyPhen-2, SIFT, SNAP, nsSNPAnalyzer, PANTHER, Consurf and Hope project, I-Mutant, MUpro) were used for the prediction of deleterious nsSNPs in the HSF1 gene. Fourteen nsSNPs were predicted to be deleterious SNPs by these tools. Of these nsSNPs, twelve are in the DBD, one in the N-terminal oligomerization domain, and one in the disordered region *(Table 1)*. After activation by stress, HSF1 trimerizes and binds to HSEs in the promoter regions of HSPs. DBD, the most conserved region of HSF1,

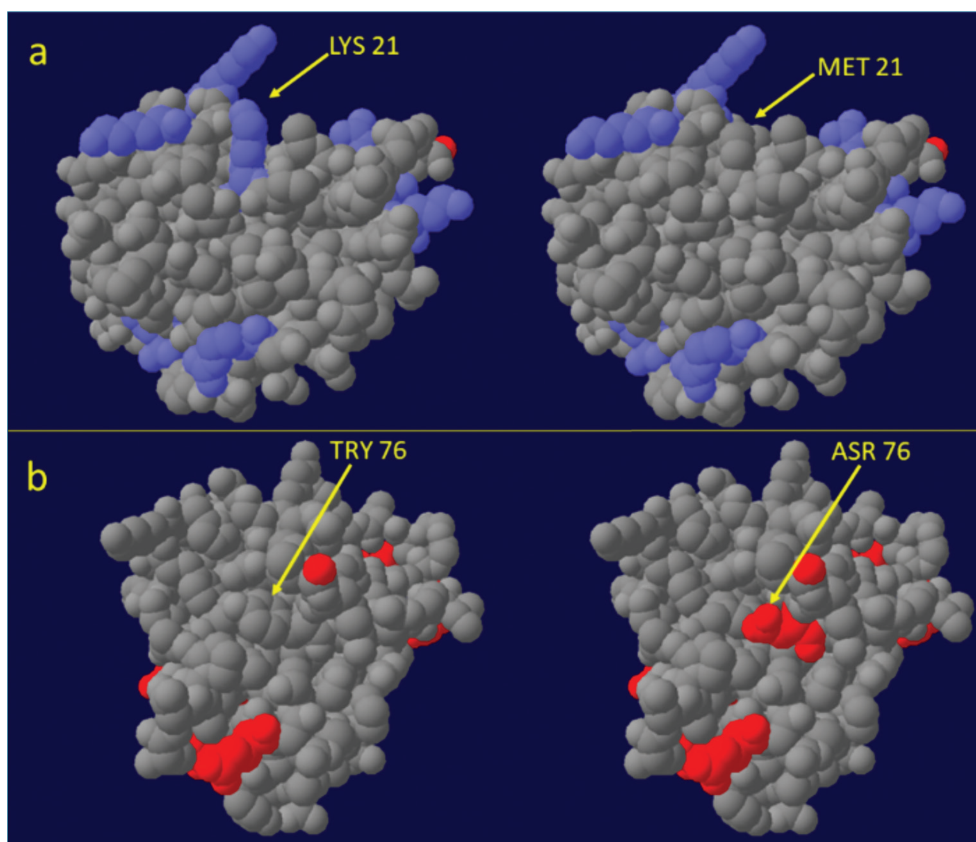

Fig 4. The 3-D surface structure of the DNA binding domain of the bovine HSF1 protein. K21M and Y76D mutations cause charge changes on the protein surface. **a-** Positively charged amino-acid residues are shown in blue, **b-** Negatively charged amino-acid residues are shown in red

plays a major role in recognizing and binding to HSEs [12]. The N-terminal oligomerization domain is responsible for the trimerization of HFS1. The binding affinity of the HSF1 trimer to HSE is significantly increased relative to the monomer HSF1 [39]. The disordered regions that remain unstructured have important roles in determining the function and structure of the protein [40]. Thus, the mutations in these three domains may reduce the affinity of HSF1 to HSE and result in decreased stability of HSF1 structure [41].

Evolutionarily conserved protein residues are the most important parts of protein folding, function, and structure. Mutations in conserved amino acids can cause changes in the 3-D structure of the protein and its interaction with other molecules [42]. Therefore, evolutionary conservation analysis was done with the Consurf server. All twelve nsSNPs predicted by the bioinformatic tools to be potentially deleterious in the DBD were found to have high conservation (scores=9) *(Table 1)*. Two (K21 and Y76) of them were categorized as functional residues (exposed) and ten (V15, F18, L19, I35, V46, V56G, F61, A67, V81, L112, V172, F496) as structural residues (buried). Conservation scores for the residues in the N-terminal oligomerization domain (V172) and disordered region (F496) residues were estimated as 8 and 6, respectively. These results are similar to previous studies [42,43], which suggest that nsSNPs that cause substitution in evolutionarily conserved residues may change the function of the protein.

For a protein to perform its functions efficiently, it must be folded correctly. Interactions between hydrophobic amino acids have an important role in accurately folding a protein chain [44]. The Hope Project server determined that wild type 9 amino acids (V15G, L19R, I35T, V46E, V56G, A67D, Y76D, V81G, V172E) in the HSF1 protein are more hydrophobic than the mutant type. Seven of these residues are buried and one (Y76D) is on the surface of the protein. These mutations will cause a loss of hydrophobic interactions either in the core of the protein or on the surface. It is known that hydrophobic residues on the surface and core of the protein support protein stability [45].

Prolines are known to have a very rigid structure, sometimes forcing the backbone in a specific conformation [46] and it may disturb the α-helix structure [47]. L112P mutation in the last α-helix of the DBD in HSF1 may cause a significant deterioration in the function and structure of the protein by disrupting this α-helix structure *(Fig.*

3). It is also predicted that mutation results in loss of interaction in the core of the protein because proline is smaller than leucine. When the mutant residue is smaller than the wild type residue, it causes an empty space in the core of the protein [48].

For the exposed Y76D variation, tyrosine is more hydrophobic than aspartic acid. In addition, tyrosine (Y76-mutant type) is neutral and aspartic acid (D76-wild type) is negatively charged *(Fig. 4)*. This mutation can both decrease protein stability and significantly hamper interaction with other molecules. Another amino acid on the surface, lysine (K21-wild type) is positively charged, while methionine (M21-mutant type) is neutral *(Fig. 4)*. The difference in charge disrupts the ionic attractions that are important for protein structure and function [49]. For these two exposed variations (K21M, Y76D), mutant residues are smaller than native residues. Smaller residues may cause a loss of external interactions [50]. Considering these physicochemical changes, K21M and Y76D mutations can cause a significant decrease in binding HSF1 to HSEs.

The results of this study suggest that the 14 nsSNPs in the HSF1 in cattle might represent associated with heat stress susceptibility. The deleterious nsSNPs on the biological function of the HSF1 protein provide a starting point for genetic marker-assisted selection against heat stress of cattle. These findings need to be validated by performing wet-lab experiments.

Availability of Data and Materials

Datasets analyzed during the current study are available in the author on reasonable request.

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Competing Interest

The author declare that have no conflict of interest.

References

1. Mishra SR: Significance of molecular chaperones and micro RNAs in acquisition of thermo-tolerance in dairy cattle. *Anim Biotechnol*, 1-11, 2020. DOI: 10.1080/10495398.2020.1830788

2. Strandén I, Kantanen J, Russo IRM, Orozco-terWengel P, Bruford MW, the Climgen Consortium: Genomic selection strategies for breeding adaptation and production in dairy cattle under climate change. *Heredity,* 123 (3): 307-317, 2019. DOI: 10.1038/s41437-019-0207-1

3. Tesema Z, Taye M, Ayichew D: The role of phenotypic and genetic basis of livestock selection for climate change adaptation and mitigation: A review. *J Appl Adv Res,* 4 (2): 66-77, 2019. DOI: 10.21839/jaar.2019.v4i2.251

4. Rocha RFB, Baena MM, de Cássia Estopa A, Gervásio IC, Ibelli AMG, Gionbelli TRS, Gionbelli MP, de Freitas RTF, Meirelles SLC: Differential expression of HSF1 and HSPA6 genes and physiological responses in Angus and Simmental cattle breeds. *J Therm Biol,* 84, 92-98, 2019. DOI: 10.1016/j.jtherbio.2019.06.002

5. Rong Y, Zeng M, Guan X, Qu K, Liu J, Zhang J, Chen H, Huang B, Lei C: Association of HSF1 genetic variation with heat tolerance in Chinese cattle. *Animals,* 9 (12): 1027, 2019. DOI: 10.3390/ani9121027

6. Bagath M, Krishnan G, Devaraj C, Rashamol VP, Pragna P, Lees AM, Sejian V: The impact of heat stress on the immune system in dairy cattle: A review. *Res Vet Sci,* 126, 94-102, 2019. DOI: 10.1016/j.rvsc.2019.08.011

7. Scharf B, Carroll JA, Riley DG, Chase Jr CC, Coleman SW, Keisler DH, Weaber RL, Spiers DE: Evaluation of physiological and blood serum differences in heat-tolerant (Romosinuano) and heat-susceptible (Angus) *Bos taurus* cattle during controlled heat challenge. *J Anim Sci,* 88 (7): 2321-2336, 2010. DOI: 10.2527/jas.2009-2551

8. Balasubramaniam B, Balamurugan K: Role of Heat Shock Factors in Diseases and Immunity. **In,** Asea AAA, Kaur P (Eds): Heat Shock Proteins in Human Diseases. 197-210, Springer, Cham, 2020. DOI: 10.1007/7515_2020_21

9. Zeng L, Qu K, Zhang J, Huang B, Lei C: Genes related to heat tolerance in cattle-A review. *Anim Biotechnol*, 1-9, 2022. DOI: 10.1080/10495398.2022.2047995

10. Onasanya GO, Thiruvenkadan AK, Sreekumar C, Okpeku M, Tirumurugaan GK, Msalya GM, Sanni MT, Olowofeso O, Fafiolu AO, Decampos JS, Obadire FO, Abdullahi AA, Ikeobi CON: Molecular characterization of hsp 70 gene using single nucleotide polymorphism in nigerian breeds of zebu cattle. *Fuw Trends Sci Technol J,* 4, 714-720, 2019.

11. Jaiswal L, De S, Singh RK, Baithalu RK: Molecular characterization and protein structure prediction of heat shock transcriptional factors in goat *(Capra hircus)* and sheep *(Ovis aries)*. *Anim Biotechnol,* 31 (5): 432-439, 2020. DOI: 10.1080/10495398.2019.1615497

12. Barna J, Csermely P, Vellai T: Roles of heat shock factor 1 beyond the heat shock response. *Cell Mol Life Sci,* 75 (16): 2897-2916, 2018. DOI: 10.1007/s00018-018-2836-6

13. Kovács D, Sigmond T, Hotzi B, Bohár B, Fazekas D, Deák V, Vellai T, Barna J: HSF1Base: A comprehensive database of HSF1 (heat shock factor 1) target genes. *Int J Mol Sci,* 20 (22): 5815, 2019. DOI: 10.3390/ijms20225815

14. Howe KL, Achuthan P, Allen J, Allen J, Alvarez-Jarreta J, Amode MR, Armean IM, Azov AG, Bennett R, Bhai J, Billis K, Boddu S, Charkhchi M, Cummins C, Fioretto LDR, Davidson C, Dodiya K, El Houdaigui B, Fatima R, Gall A, Giron CG, Grego T, Guijarro-Clarke C, Haggerty L, Hemrom A, Hourlier T, Izuogu OG, Juettemann T, Kaikala V, Kay M, Lavidas I, Le T, Lemos D, Martinez JG, Marugán JC, Maurel T, McMahon AC, Mohanan S, Moore B, Muffato M, Oheh DN, Paraschas D, Parker A, Parton A, Prosovetskaia I, Sakthivel MP, Abdul Salam AI, Schmitt BM, Schuilenburg H, Sheppard D, Steed E, Szpak M, Szuba M, Taylor K, Thormann A, Threadgold G, Walts B, Winterbottom A, Chakiachvili M, Chaubal A, De Silva N, Flint B, Frankish A, Hunt SE, IIsley GR, Langridge N, Loveland JE, Martin FJ, Mudge JM, Morales J, Perry E, Ruffier M, Tate J, Thybert D, Trevanion SJ, Cunningham F, Yates AD, Zerbino DR, Flicek P: Ensembl 2021. *Nucleic Acids Res,* 49 (D1): D884-D891, 2021. DOI: 10.1093/nar/gkaa942

15. Anckar J, Sistonen L: Regulation of HSF1 function in the heat stress response: Implications in aging and disease. *Annu Rev Biochem,* 80, 1089-1115, 2011. DOI: 10.1146/annurev-biochem-060809-095203

16. Sharma C, Seo YH: Small molecule inhibitors of HSF1-activated pathways as potential next-generation anticancer therapeutics. *Molecules,* 23 (11): 2757, 2018. DOI: 10.3390/molecules23112757

17. Gomez-Pastor R, Burchfiel ET, Thiele DJ: Regulation of heat shock transcription factors and their roles in physiology and disease. *Nat Rev Mol Cell Biol,* 19 (1): 4-19, 2018. DOI: 10.1038/nrm.2017.73

18. Hentze N, Le Breton L, Wiesner J, Kempf G, Mayer MP: Molecular mechanism of thermosensory function of human heat shock transcription factor Hsf1. *eLife*, 5:e11576, 2016. DOI: 10.7554/eLife.11576

19. Badri TM, Chen KL, Alsiddig MA, Li L, Cai Y, Wang GL: Genetic polymorphism in *Hsp90AA1* gene is associated with the thermotolerance in Chinese Holstein cows. *Cell Stress Chaperones,* 23 (4): 639-651, 2018. DOI:

10.1007/s12192-017-0873-y

20. Malheiros JM, Enríquez-Valencia CE, da Silva Duran BO, de Paula TG, Curi RA, de Vasconcelos Silva JAII, Dal-Pai-Silva M, de Oliveira HN, Chardulo LAL: Association of *CAST2*, *HSP90AA1*, *DNAJA1* and *HSPB1* genes with meat tenderness in Nellore cattle. *Meat Sci,* 138, 49-52, 2018. DOI: 10.1016/j.meatsci.2018.01.003

21. Shergojry SA, Ganayi BA, Ramesha KP, Rengarajan K, Srihari GV, Das DN, Kataktalware MA: Association of single nucleotide polymorphisms (SNPS) of HSP90AA1 gene with reproductive traits in Deoni cattle. *Int J Livest Res,* 1 (1): 17-29, 2011. DOI: 10.5455/ijlr.20120204082821

22. Bendl J, Stourac J, Salanda O, Pavelka A, Wieben ED, Zendulka J, Brezovsky J, Damborsky J: PredictSNP: Robust and accurate consensus classifier for prediction of disease-related mutations. *Plos Comp Biol,* 10 (1):e1003440, 2014. DOI: 10.1371/journal.pcbi.1003440

23. Mueller SC, Backes C, Haas J, the INHERITANCE Study Group, Katus HA, Meder B, Meese E, Keller A: Pathogenicity prediction of non-synonymous single nucleotide variants in dilated cardiomyopathy. *Brief Bioinform,* 16 (5): 769-779, 2015. DOI: 10.1093/bib/bbu054

24. Capriotti E, Fariselli P, Casadio R: I-Mutant2. 0: Predicting stability changes upon mutation from the protein sequence or structure. *Nucleic Acids Res,* 33 (Suppl. 2): W306-W310, 2005. DOI: 10.1093/nar/gki375

25. Cheng J, Randall A, Baldi P: Prediction of protein stability changes for single-site mutations using support vector machines. *Proteins Struct Funct Bioinf,* 62 (4): 1125-1132, 2006. DOI: 10.1002/prot.20810

26. Ashkenazy H, Erez E, Martz E, Pupko T, Ben-Tal N: ConSurf 2010: Calculating evolutionary conservation in sequence and structure of proteins and nucleic acids. *Nucleic Acids Res,* 38 (Suppl. 2): W529-W533, 2010. DOI: 10.1093/nar/gkq399

27. Venselaar H, Te Beek TAH, Kuipers RKP, Hekkelman ML, Vriend G: Protein structure analysis of mutations causing inheritable diseases. An e-Science approach with life scientist friendly interfaces. *BMC Bioinform,* 11 (1): 1-10, 2010. DOI: 10.1186/1471-2105-11-548

28. Wang S, Li W, Liu S, Xu J: RaptorX-Property: A web server for protein structure property prediction. *Nucleic Acids Res,* 44 (W1): W430-W435, 2016. DOI: 10.1093/nar/gkw306

29. Guex N, Peitsch MC: SWISS-MODEL and the Swiss-Pdb Viewer: An environment for comparative protein modeling. *Electrophoresis,* 18 (15): 2714-2723, 1997. DOI: 10.1002/elps.1150181505

30. Mohapatra S, Kundu AK, Mishra SR, Senapati S, Jyotiranjan T, Panda G: HSF1 and GM-CSF expression, its association with cardiac health, and assessment of organ function during heat stress in crossbred Jersey cattle. *Res Vet Sci,* 139, 200-210, 2021. DOI: 10.1016/j.rvsc.2021.07.018

31. Wankhede NL, Kale MB, Upaganlawar AB, Taksande BG, Umekar MJ, Behl T, Abdellatif AAH, Bhaskaran PM, Dachani SR, Sehgal A, Singh S, Sharma N, Makeen HA, Albratty M, Dailah HG, Bhatia S, Al-Harrasi A, Bungau S: Involvement of molecular chaperone in protein-misfolding brain diseases. *Biomed Pharmacother,* 147:112647, 2022. DOI: 10.1016/j.biopha.2022.112647

32. Höhfeld J, Benzing T, Bloch W, Fürst DO, Gehlert S, Hesse M, Hoffmann B, Hoppe T, Huesgen PF, Köhn M, Kolanus W, Merkel R, Niessen CM, Pokrzywa W, Rinschen MM, Wachten D, Warscheid B: Maintaining proteostasis under mechanical stress. *Embo Rep,* 22 (8):e52507, 2021. DOI: 10.15252/embr.202152507

33. Li QL, Ju ZH, Huang JM, Li JB, Li RL, Hou MH, Wang CF, Zhong JF: Two novel SNPs in *HSF1* gene are associated with thermal tolerance traits in Chinese Holstein cattle. *DNA Cell Biol,* 30 (4): 247-254, 2011. DOI: 10.1089/dna.2010.1133

34. Lee YS, Won K, Shin D, Oh JD: Risk prediction and marker selection in nonsynonymous single nucleotide polymorphisms using whole genome sequencing data. *Anim Cells Syst,* 24 (6): 321-328, 2020. DOI: 10.1080/19768354.2020.1860125

35. Kumar A, Rajendran V, Sethumadhavan R, Shukla P, Tiwari S, Purohit R: Computational SNP analysis: Current approaches and future prospects. *Cell Biochem Biophys,* 68 (2): 233-239, 2014. DOI: 10.1007/s12013-013-9705-6

36. Domené S, Scaglia PA, Gutiérrez ML, Domené HM: Applying bioinformatic platforms, *in vitro,* and *in vivo* functional assays in the characterization of genetic variants in the GH/IGF pathway affecting growth and development. *Cells,* 10 (8): 2063, 2021. DOI: 10.3390/cells10082063

37. Oliver GR, Zimmerman MT, Klee EW, Urrutia RA: "The molecule's the thing:" The promise of molecular modeling and dynamic simulations in aiding the prioritization and interpretation of genomic testing results. *F1000Res,* 5:766, 2016. DOI: 10.12688/f1000research.8600.3

38. Gassoum A, Abdelraheem NE, Elsadig N: Comprehensive analysis of rsSNPs associated with hypertension using *in-silico* bioinformatics tools. *Open Access Lib J,* 3 (7): 1, 2016. DOI: 10.4236/oalib.1102839

39. Feng N, Feng H, Wang S, Punekar AS, Ladenstein R, Wang DC, Zhang Q, Ding J, Liu W: Structures of heat shock factor trimers bound to DNA. *iScience,* 24 (9):102951, 2021. DOI: 10.1016/j.isci.2021.102951

40. Alshehri MA, Manee MM, Al-Fageeh MB, Al-Shomrani BM: Genomic analysis of intrinsically disordered proteins in the genus *Camelus*. *Int J Mol Sci,* 21 (11): 4010, 2020. DOI: 10.3390/ijms21114010

41. Bridges TM, Scheraga RG, Tulapurkar ME, Suffredini D, Liggett SB, Ramarathnam A, Potla R, Singh IS, Hasday JD: Polymorphisms in human heat shock factor-1 and analysis of potential biological consequences. *Cell Stress Chaperones,* 20 (1): 47-59, 2015. DOI: 10.1007/s12192-014-0524-5

42. Wang Z, Huang C, Lv H, Zhang M, Li X: *In silico* analysis and high-risk pathogenic phenotype predictions of non-synonymous single nucleotide polymorphisms in human *Crystallin beta A4* gene associated with congenital cataract. *Plos One,* 15 (1):e0227859, 2020. DOI: 10.1371/journal.pone.0227859

43. Ghani U, Ullah R, Anjum T, Ali Y, Hussain S, Shah S, Zulfiqar I, Sultan T, Tanveer MA, Sabar MF: Identification of most lethal non-synonymous SNPs in tollip gene-An *in-silico* analysis. 2021. DOI: 10.21203/rs.3.rs-488110/v1

44. Mirzaie M: Hydrophobic residues can identify native protein structures. *Proteins Struct Funct Genet,* 86 (4): 467-474, 2018. DOI: 10.1002/prot.25466

45. Kumar A, Biswas P: Effect of site-directed point mutations on protein misfolding: A simulation study. *Proteins Struct Funct Genet,* 87 (9): 760-773, 2019. DOI: 10.1002/prot.25702

46. Sudarshan S, Kumar M, Kaur P, Kumar A, Sethuraman G, Sapra S, Gulati S, Gupta N, Kabra M, Roy Chowdhury M: Decoding of novel missense TSC2 gene variants using *in-silico* methods. *BMC Med Genet,* 20 (1): 1-9, 2019. DOI: 10.1186/s12881-019-0891-y

47. Agrahari AK, Kumar A, Siva R, Zayed H, Doss CGP: Substitution impact of highly conserved arginine residue at position 75 in *GJB1* gene in association with X-linked Charcot–Marie-tooth disease: A computational study. *J Theor Biol,* 437, 305-317, 2018. DOI: 10.1016/j.jtbi.2017.10.028

48. Elnasri HA, Al Bkrye AM, Khaier MAM: *In silico* analysis of non synonymous SNPs in *DHCR7* gene. *Am J Bioinform Res*, 8 (1): 12-18, 2018.

49. Sedaghat A, Zamani M, Jahanshahi A, Ghaderian SB, Shariati G, Saberi A, Hamid M, Aminzadeh M, Galehdari H: Frequent novel mutations are causative for maple syrup urine disease from Southwest Iran. *Meta Gene,* 16, 96-104, 2018. DOI: 10.1016/j.mgene.2018.01.010

50. Abdelhalim DM, Albkrye AM, Salih MA, Abodlaa AEE, Elnasri HA, Khaier MAM: *In silico* analysis of a single nucleotide polymorphism (SNPS) in human GSTM1 gene associated with cancer development. *European J Biomed Pharm Sci,* 7 (1): 514-521, 2020.

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Research Article

Phenotypic Characterization of Hair and Honamli Goats Using Classification Tree Algorithms and Multivariate Adaptive Regression Spline (MARS)

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Abstract: Some morphological and physiological data are needed to scientifically describe animals and distinguish breeds from one another. Except for those who are not experts in the field, it is difficult to distinguish goat breeds from each other. Using data mining algorithms, this study aimed to develop a new phenotypic characterization for Honamli and Hair goats via some body measurement characteristics. In the study, some body characteristics of the Hair goat (65 animals) and the Honamli goat (83 animals) were used as independent variables. The dependent variable of the data mining algorithms, on the other hand, was defined as the binary response variable of Honamli and Hair breeds. The success of the CHAID, Exhaustive CHAID, CART, QUEST, and MARS algorithms in breed discrimination was determined at 87.80%, 85.80%, 87.80%, 77.00%, and 88.51%, respectively, while the area under the ROC curve was detected 0.880, 0.853, 0.868, 0.784, and 0.942, respectively, and Cohen's Kappa coefficient (κ) 0.755, 0.711, 0.749, 0.549 and 0.739, respectively. As a result, the phenotype characterization of Honamli and Hair goats, whose morphological distinctions could not be made exactly, in MARS and CHAID algorithms, achieved with high success compared to other methods. The present study showed that Honamli and Hair goats may be distinguished by suitable statistical algorithms based on morphological data, which can be integrated with goat breeding studies to detect the origin of breeding animals.

Keywords: *CART, CHAID, Classification, Exhaustive CHAID, MARS, QUEST*

Sınıflandırma Ağacı Algoritmaları ve Çok Değişkenli Uyarlanabilir Regresyon Uzanımları (MARS) Kullanılarak Kıl ve Honamlı Keçilerinin Fenotipik Karakterizasyonu

Öz: Hayvanları bilimsel olarak tanımlamak ve ırkları birbirinden ayırt etmek için bazı morfolojik ve fizyolojik verilere ihtiyaç vardır. Alanında uzman olmayanlar dışında keçi ırklarını birbirinden ayırt etmek güçtür. Bu çalışma, veri madenciliği algoritmaları kullanılarak bazı vücut özellikleri üzerinden Honamlı ve Kıl keçileri için yeni bir fenotipik karakterizasyon geliştirmeyi amaçlamıştır. Çalışmada, Kıl keçisi (65 hayvan) ve Honamlı keçisinin (83 hayvan) bazı vücut özellikleri bağımsız değişkenler olarak kullanılmıştır. Veri madenciliği algoritmalarının bağımlı değişkeni ise Honamlı ve Kıl ırkları ikili yanıt değişkeni olarak tanımlanmıştır. CHAID, Exhaustive CHAID, CART, QUEST ve MARS algoritmalarının ırk ayrımındaki başarısı sırasıyla %87.80, %85.80, %87.80, %77.00 ve %88.51 iken, ROC eğrisi altında kalan alan ise sırasıyla 0.880, 0.853, 0.868, 0.784 ve 0.942 ve Cohen's Kappa katsayıları (κ) 0.755, 0.711, 0.749, 0.549 ve 0.739 olduğu tespit edilmiştir. Sonuç olarak, morfolojik ayrımları tam olarak yapılamayan Honamlı ve Kıl keçilerinin MARS ve CHAID algoritmalarında fenotip karakterizasyonu diğer yöntemlere göre yüksek başarı ile gerçekleşmiştir. Bu çalışma, Honamlı ve Kıl keçilerinin morfolojik verilere dayalı uygun istatistiksel algoritmalarla ayırt edilebileceğini ve damızlık hayvanların kökenini tespit etmek için keçi ıslahı çalışmaları ile entegre edilebileceğini göstermiştir.

Anahtar sözcükler: *CART, CHAID, Sınıflama, Exhaustive CHAID, MARS, QUEST*

Introduction

Approximately 97% of the existing goats in Turkey consist of the Hair goats [1]. Hair and Honamli goats have some morphological similarities. Therefore, the breeds were not separated until the 2000s, and total numbers were evaluated as if all were the same breeds [2]. However, it is stated that the Honamli goat breed has higher productivity in terms of birth weight, live weight, lactation milk yield, and reproduction [3,4]. The lack of scientific research on

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the Honamli goat breed is possibly due to the continuous transhumance of the Turkish Yoruks (nomads) [5]. The Honamli goat, which is defined as a new goat breed in animal genetic resources, was taken under protection by the Turkish General Directorate of Agricultural Research and Policies in 2015 [6].

To date, the morphological characteristics of Honamli have been defined and numerous studies have aimed to compare Honamli and Hair goats. Generally, these studies indicate that the phylogenetic similarity of Honamli and Hair goats is over 85%, and these breeds cannot be distinguished via microsatellite markers [2,7,8]. Therefore, the phenotypic characterization may be useful to separate these breeds. Because of Honamli and Hair goats morphologically having body clours are similar to each other, they can be separated subjectively by experienced breeders [4]. The reliability of this separation should be tested by robust quantitative methods and these methods could fill an important gap in the literature. Considering both the conservation of genetic resources and economical aspects, discrimination against the Honamli breed should be beneficial for goat breeders. Due to their very different morphological and physiological characteristics, goat breeders in Turkey have preferred Honamli goats in recent years, which are reared within the scope of the improvement projects in breeder conditions, to Hair goats [9].

Identification and classification of breeds within a certain species according to phenotypic characteristics play a key role in the basis of breeding and conservation program strategies. The identification and classification of breeds are of great importance to separate and define the breeds. For this purpose, comparisons of some local goat genotypes reared worldwide in terms of morphology traits and breed discrimination have been made using multivariate statistical methods (MANOVA), principal components analysis (PCA), canonical analysis, hierarchical, k-means clusters and step-wise, linear and nonlinear discriminate analysis [10-14]. To use these traditional statistical methods, the data must be multivariate normally distributed, the covariance matrix must be equal across all groups, the independent variables must be independent of each other, and the number of observations must be at least 10 times the number of independent variables included in the model [15]. The most significant distinction between data mining algorithms and traditional statistical methods is that they are not subject to any preconditions and can control classification through cross-validation using the sampling method [16]. As a result, it will be possible to classify with much greater accuracy and reliability [17].

This study, it was aimed to determine the phenotypic characterization of Hair and Honamli goats using data mining algorithms and MARS algorithm together on morphological characteristics.

Material and Methods

Ethical Statement

Ethical rules were considered by following all applicable international, national, and institutional guidelines for the care and use of animals. In the study, there is no need for ethical approval due to the lack of blood sampling from the animals and the absence of any surgical procedures. All data were collected with the approval of the breeder.

Animals

The animal material of the study consists of 65 Hair goats (45 female, 20 male) and 83 Honamli goats (73 female, 10 male) at different ages (1, 2, 3, 4, 5, and 6 years and over), which were reared extensively on a private farm in the Çalca district of Kütahya province, Turkey. In 2015, Honamli goats with pedigree records were brought from a farm that is a member of the Antalya Sheep and Goat Breeding Association. Hair goat breeding has been carried out on the farm, where the study was conducted since 2008. Pedigree records of the breeds were checked through the TurkVet system. During the heavy winter season or in adverse weather conditions, goats were fed in the barn. Concentrated feed was given for one month before the breeding season for flushing, and in addition to pasture, straw, alfalfa, and fescue grass are given as roughage sources.

Measurement of Morphological Characteristics of Breeds

A special scale designed for weighing small ruminants was used in determining the live weight of animals. All body characteristics of the goats were measured as described by [18], and live weight (LW) was taken with 0.1 kg precision. All body measurements were taken after the animals had adapted to the environment on a flat platform and the stress factors were minimized. Withers height (WH), back height (BH), rump height (RH), body length (BL), and chest depth (CD) were taken using a measuring stick and body circumference measurements (chest girth (CG), and leg girth (LG)) were taken using a measuring tape. Head length (HL), nose length (NL), ear length (EL), and tail length (TL) values were measured using calipers. All animals were measured by the same expert.

Statistical Analysis

Regression Tree-Based Data Mining Algorithms

The data structure created by using all arguments and dividing the data into subgroups is termed a classification tree. The root node, which does not contain any fragmentation and contains only the dependent variable, is at the top of the classification tree. First, this root node is divided into two or more parts. These separated parts are called parent branches. The breaking up of parent branches created child nodes or subsets [19]. The node, which is appeared

when the fragmentation is complete in additional nodes and there is no more branching is called a terminal node [20]. By testing the independent variables in the model, the cut-off values of the explanatory variable are determined in a way to provide the specified category in the new node to be formed [21].

In all algorithms of this study, LW, WH, BH, RH, CD, BL, CG, LG, HL, NL, EL, TL variables and sex, age, and ear type factors were taken, while the dependent variable was binary goat breeds such as Honamli and Hair. In literature, many algorithms were used to create classification trees. In the study, tree-based data mining algorithms were used Classification and Regression Tree (CART), Chi-Square Automatic Interaction Detector (CHAID), Exhaustive CHAID, Quick Unbiased, Efficient Statistical Tree (QUEST), and Multivariate Adaptive Regression Spline (MARS) algorithm, which is a not tree-based algorithm. The main reason for using these algorithms is that they were simple to understand and allow for the determination of the cut-off points of the independent variables. In the classification trees, the maximum tree depth was used as CHAID (3), Exhaustive CHAID (3), CART (5), and QUEST (3), respectively. In the formation of classification trees, the minimum number of parent and daughter (child) nodes were taken as 10 and 5. Also, the multicollinearity problem was not detected to exist due to the pearson correlation coefficients and variance inflation factor (VIF) values between the independent variables used by the classification tree algorithms being smaller than the critical values specified in the literature [22].

CART (Classification and Regression Trees)

The CART algorithm is a non-parametric regression method developed by some researchers [23]. The CART is a tree-based data mining algorithm that reveals the relationship between the dependent variable and the independent variable, as well as the relationships between the independent variables. The branching in the tree structure is based on the division into two sub-homogeneous groups. As the split criterion in the CART algorithm, impurity and Gini index variability are taken into account in the selection of the best independent variable in the classification. The Gini index takes values between 0 and 1 and provides assignments to classes. The Gini index is calculated by subtracting the sum of the squares of the probabilities of each class from one and is obtained using equation 1.

$$\text{Gini Index (L)} = 1 - \sum_{i=1}^{j} p_i^2 \qquad (1)$$

j: number of class; L: a data set with j th class; pi: relative frequency if class 'i' in 'L'

CHAID (Chi-squared-Automatic-Interaction-Detection)

Some researchers [24] developed the CHAID algorithm, which is a non-parametric regression method in the tree structure created by taking statistical significance ratios and cross tables into account. Branching in the tree structure is based on the split of two or more sub-homogeneous groups. CHAID algorithm with merge, split, and stop stages iteratively creates homogeneous nodes starting from the root node, increasing/decreasing variance between/within nodes [25]. Because the whole population can be split into stable sub-nodes using a strong translation algorithm, a regression equation to be obtained is kept independent of classical assumptions (normality, linearity, homogeneity, etc.) in CHAID analysis. With this process, normality and homogeneity can be achieved in the distribution of the data.

Exhaustive CHAID (Exhaustive Chi-squared-Automatic-Interaction-Detection)

It is a modified version of the CHAID algorithm that looks into all possible splits for each predictor developed by some researchers [26]. As a result, transactions take longer than with the CHAID algorithm. Exhaustive CHAID keeps combining the prediction variable's categories until only two supercategories remain. It identifies the category with the strongest relationship to the dependent variable and computes the adjusted p-value for these. Although it depends on the data, it can be said that there will be no significant difference between the results of the CHAID algorithms.

QUEST (Quick Unbiased, Efficient Statistical Tree)

Some researchers [27] created QUEST as a classification algorithm, and the branching in the tree structure, like the CART algorithm, is based on the separation of two sub-homogeneous groups. Unlike CHAID and CART, it handles variable selection and split point selection separately. In the QUEST algorithm, the association between each independent and dependent variable for each separation is found by calculating the F test, Levene test, or Pearson Chi-square value. In the algorithm, the variable with a small p-value is selected for explanatory variable selection.

MARS (Multivariate Adaptive Regression Splines)

The MARS algorithm, developed by some researchers [28], is a non-parametric regression technique used to examine complex relationships between the dependent variable and a set of independent variables. In order to apply this non-linear technique, there is no need for any assumptions between the dependent variable and predictor variables. The MARS algorithm, which is a modified version of the CART algorithm, makes better predictions than binary logistic regression thanks to the hinges function in its structure [29]. Using appropriate transformation techniques, the MARS technique converts nonlinear relationships between dependent and independent variables into linear ones. The MARS method can calculate the best trans-

formations and interactions of variables, as well as analyze complex relationships in high-dimensional data. To prevent these relationships from causing multicollinearity problems in the MARS algorithm, it is recommended that a model be created in the earth package of the R software with penalty=2 [17].

k-Fold Cross-Validation

Cross-validation is a popular method for assessing the effectiveness of a machine learning model. This method is used for small datasets and is based on the resampling procedure. It can also be done using cross-validation instead of dividing the data into training and test sets because it works based on validation in machine learning algorithms [30]. Cross-validation is used to train and validate the model by dividing all the data into k multiples, also known as subsamples. In this way, it reduces overfitting and determines the model's hyperparameters. For this purpose, usually 10-fold or 5-fold cross-validation is used. In this study, after all data set (148 records) was randomly divided into 10 parts, nine parts of the training set of the models were created, while the model was validated 5 times with the remaining part in this study.

Model Evaluation Criteria

CART, CHAID, Exhaustive CHAID, QUEST, and MARS data mining algorithms were utilized to compare in terms of accuracy, sensitivity, specificity, Matthews correlation (Phi), Cohen's Kappa coefficient (κ), and area under Receiver Operating Characteristics (ROC) curve. The Phi coefficient was used to determine the relationship between the real classes and the classes estimated by the algorithms, and Cohen's Kappa coefficient was used to determine the concordance. Accuracy is the proportion at which a classification algorithm correctly separates Honamli and Hair goats. Sensitivity is the proportion at which the algorithm correctly classifies Honamli goats, while specificity is the proportion at which the algorithm correctly classifies Hair goats. *Table 1* presents the confusion table for classifying algorithms.

The expressions T+, T-, F+, and F- used in the accuracy, sensitivity and specificity equations represent numbers of true positive, true negative, false positive, and false negative, respectively. The formula is used below to determine the area under the ROC curve (AUC) and the area under the ROC curve's standard error (AUCse) as developed by [31].

***Table 1.** Confusion table for the classifier algorithms*

Observed	Predicted as Breeds	
	Honamli	Hair
Honamli	T^+	F^+
Hair	F^-	T^-

Accuracy = $(T^+ + D)/(T^+ + F^+ + F^- + T^-)$

Sensitivity = $T^+/(T^{+-} + F^+)$

Specificity = $T^- / (F^- + T^-)$

Error proportion = 1 - Accuracy

$$se_{AUC} = \sqrt{\frac{AUC(1-AUC)+(n_A-1)(q1-AUC^2)+(n_B-1)(q2-AUC^2)}{n_A n_B}} \quad (2)$$

$n_A = T^+ + F^-$ and $n_B = F^+ + T^-$

$q1 = \frac{AUC}{2-AUC}$ and $q2 = \frac{2AUC^2}{1+AUC}$

Statistical analyses of the classification trees, Phi and Cohen's Kappa (κ) coefficients were performed in IBM SPSS 23 package program [32]. Earth (v5.1.2) [33] and caret (v60.86) [34] packages of R software were used for MARS analysis [35]. The trial version (19.5.1) of the MedCalc package program was used to determine the areas under the ROC and to compare (z test) the area under the ROC curve of the algorithms.

Results

Categorical variables belonging to Honamli and Hair goats in the study are given in *Table 2*. Descriptive statistics of continuous variables obtained from Honamli and Hair goats are given in *Table 3*. Honamli females were larger than males because females in the herd were older *(Table 3)*. Young billy goats were preferred in the herd to reduce generation intervals.

***Table 2.** Categorical variables belonging to Honamli and Hair goats*

Factors	Levels		N	Percentage (%)
Breed-Sex	Honamli	Female	73	49.32%
		Male	10	6.76%
	Hair	Female	45	30.41%
		Male	20	13.51%
Breed-Age	Honamli	1	16	10.81%
		2	6	4.06%
		3	14	9.46%
		4	4	2.70%
		5	2	1.35%
		6	41	27.70%
	Hair	1	32	21.62%
		2	2	1.35%
		3	4	2.70%
		4	6	4.06%
		5	2	1.35%
		6	19	12.84%
Breed-Ear	Honamli	Comuk (native terms)	27	18.24%
		Lop	56	37.84%
	Hair	Comuk (native terms)	21	14.19%
		Lop	44	29.73%

Table 3. Descriptive statistics on live weight and some body measurements in Honamli and Hair goats of different age

Traits	Breed	Sex	N	Minimum	Maximum	Mean±SE	StdDev	CoefVar
LW	Honamli	Female	73	27.10	84.70	60.76±1.54	13.13	21.60
		Male	10	37.20	63.10	48.02±2.77	8.76	18.24
	Hair	Female	45	27.30	72.20	48.40±1.76	11.77	24.33
		Male	20	29.00	43.60	36.15±0.95	4.25	11.74
WH	Honamli	Female	73	51.90	89.50	75.78±1.04	8.90	11.75
		Male	10	66.00	87.00	76.25±2.37	7.51	9.84
	Hair	Female	45	51.70	85.00	70.63±1.20	8.05	11.39
		Male	20	63.50	77.50	68.70±0.94	4.22	6.15
BH	Honamli	Female	73	64.50	90.50	79.14±0.71	6.05	7.65
		Male	10	66.50	86.00	76.60±1.97	6.23	8.13
	Hair	Female	45	60.00	79.50	71.09±0.69	4.66	6.55
		Male	20	61.00	75.50	68.40±1.01	4.50	6.58
RH	Honamli	Female	73	65.50	89.00	79.14±0.66	5.63	7.12
		Male	10	70.00	86.50	77.45±1.69	5.36	6.92
	Hair	Female	45	60.50	81.00	71.82±0.72	4.80	6.68
		Male	20	61.00	77.00	68.08±0.98	4.39	6.45
CD	Honamli	Female	73	16.50	29.50	24.66±0.29	2.47	10.00
		Male	10	20.50	27.50	24.45±0.76	2.41	9.85
	Hair	Female	45	18.50	27.50	22.36±0.28	1.86	8.31
		Male	20	18.50	23.50	21.13±0.32	1.44	6.82
BL	Honamli	Female	73	46.00	91.50	80.57±0.97	8.25	10.24
		Male	10	62.50	88.00	75.65±2.70	8.54	11.29
	Hair	Female	45	60.00	92.00	74.63±1.12	7.50	10.05
		Male	20	58.00	76.50	68.30±0.83	3.69	5.41
CG	Honamli	Female	73	70.00	104.00	91.04±0.82	6.96	7.65
		Male	10	81.00	95.00	86.25±1.36	4.30	4.99
	Hair	Female	45	73.00	102.50	86.64±1.05	7.02	8.11
		Male	20	72.50	90.50	81.50±0.98	4.38	5.37
LG	Honamli	Female	73	35.00	67.00	51.21±0.74	6.29	12.28
		Male	10	46.00	65.00	55.90±2.05	6.48	11.59
	Hair	Female	45	39.00	77.50	50.87±1.13	7.58	14.90
		Male	20	46.50	61.00	55.00±0.75	3.35	6.09
HL	Honamli	Female	73	17.00	24.00	21.23±0.18	1.49	7.03
		Male	10	19.50	22.50	20.80±0.31	0.98	4.70
	Hair	Female	45	17.00	23.00	19.67±0.22	1.45	7.39
		Male	20	17.50	22.50	20.13±0.29	1.30	6.44
NL	Honamli	Female	73	11.00	21.00	14.26±0.25	2.13	14.95
		Male	10	12.00	16.00	13.95±0.46	1.46	10.48
	Hair	Female	45	11.00	17.00	13.37±0.24	1.58	11.79
		Male	20	11.50	23.00	14.03±0.53	2.37	16.86
EL	Honamli	Female	73	8.00	22.50	16.97±0.44	3.74	22.02
		Male	10	9.50	21.00	15.50±1.34	4.22	27.24
	Hair	Female	45	13.00	28.00	17.79±0.45	3.00	16.88
		Male	20	7.00	20.50	14.68±0.86	3.86	26.31
TL	Honamli	Female	73	13.00	27.50	19.11±0.39	3.37	17.61
		Male	10	15.00	29.00	20.35±1.35	4.28	21.04
	Hair	Female	45	11.00	22.00	15.97±0.35	2.31	14.50
		Male	20	14.00	20.00	16.43±0.42	1.88	11.44

Live weight (LW), Withers height (WH), Back height (BH), Rump height (RH), Chest Depth (CD), Body length (BL), Chest girth (CG), Leg girth (LG), Head length (HL), Nose length (NL), Ear length (EL), and Tail length (TL)

The MARS algorithm, which provided one of the best classifications of Honamli and Hair goats, takes the form of body characteristics "LW", "BH", "CD", "HG", and "HL" as independent variables in the prediction model. In addition, the model also includes "Age" variable and "Sex" factors that do not have body characteristics. The remaining characteristics were not included in the MARS model because they were found to be statistically non-significant (P<0.05). In the MARS model which was given below, GLM indicates the general linear model, while max denotes the basic function of the MARS.

$GLM_{HONAMLI}$ = -0.5232799 - 3.033782 * SexMale + 1.5192 * max(0, 4 - Age) - 1.068315 * max(0, 35.4 - LW) + 0.4609831 * max(0, BH - 72) + 25.86152 * max(0, BH - 82) - 0.6795643 * max(0, 25 - CD) + 1.559002 * max(0, 77.5 - HG) - 0.5741605 * max(0, 21.5 - HL). The probability of any goat being Honamli can be estimated by PHONAMLI = exp$GLM_{HONAMLI}$/(1+ exp $GLM_{HONAMLI}$). The "exp" value used in the equation refers to the base of the natural logarithm of 2.718. Using the basic MARS model, it is possible to derive a new prediction equation used in the classification of females. If the goats used in breed discrimination estimation are female animals older than four years old, the following equation can be used.

$GLM_{HONAMLI}$ = -0.5232799 - 1.068315 * max(0, 35.4 - LW) + 0.4609831 * max(0, BH - 72) + 25.86152 * max(0, BH - 82) - 0.6795643 * max(0, 25 - CD) + 1.559002 * max(0, 77.5 - HG) - 0.5741605 * max(0, 21.5 - HL).

For example, a 4-year-old female Honamli goat with body characteristics which was LW = 40 kg, BH = 78 cm, CD = 25 cm, HG = 75 cm, and HL = 20 cm in the dataset could be estimated discrimination of breed. As follows by the MARS estimation equation;

1- $GLM_{HONAMLI}$ = -0.5232799 - 3.033782 * SexMale (Female=0) + 1.5192 * max(0, 4 - 4) - 1.068315 * max(0, 35.4 - 40) + 0.4609831 * max(0, 78 - 72) + 25.86152 * max(0, 78 - 82) - 0.6795643 * max(0, 25 - 25) + 1.559002 * max(0, 77.5 - 75) - 0.5741605 * max(0, 21.5 - 20)

2- $GLM_{HONAMLI}$ = -0.5232799 + 0.4609831 * max(0, 78 - 72) + 1.559002 * max(0, 77.5 - 75) - 0.5741605 * max(0, 21.5 - 20)

3- $GLM_{HONAMLI}$ = -0.5232799 + 0.4609831 *6 + 1.559002 * 2.5 - 0.5741605 * 1.5

4- $GLM_{HONAMLI}$ = 5.27888295

5- $P_{HONAMLI}$ = exp$GLM_{HONAMLI}$/(1+ exp $GLM_{HONAMLI}$)

6- $P_{HONAMLI}$ = 2.7185.27888295/(1+ 2.7185.27888295)

$P_{HONAMLI}$ = 0.994924974

The estimated goat with a probability of 99.49% belongs to the Honamli breed.

Classification performances of data mining algorithms used for breed discrimination are shown in *Table 4*. The areas under the ROC curve (AUC) were statistically significant for all algorithms for breed discrimination (P<0.01).

The sensitivity and specificity values of the model's criteria were close to each other and the AUC values were close to 1, which indicated the accuracy of the classification *(Fig. 1)*. Models compared statistically with the z-test in terms of AUC could be mathematically expressed as MARS = CHAID = CART> = Exhaustive CHAID> = QUEST *(Table 4)*. When all data mining algorithms were compared among themselves in terms of AUC performance criteria, it was determined that the most successful algorithm used in breed discrimination were MARS, CHAID, CART, and Exhaustive CHAID. The performance of the MARS algorithm was determined as 0.916, 0.846, and 0.937 in terms of sensitivity, specificity, and general accuracy rate respectively. The MARS algorithm was able to classify 75 of 83 Honamli goats, 55 of 65 Hair goats, and 88.50% of all goats correctly. The MARS algorithm was found to have the highest breed discrimination diagnostic test with the area under the ROC curve of 0.942. Also, the concordance (κ) and correlation (Phi) coefficients between the breeds estimated by the MARS algorithm and the actual breeds were found to be 0.739. It was determined that the CHAID algorithm had the best diagnostic test performance and other performance criteria among the classification tree algorithms. The discrimination performances made by the CHAID algorithm had the values of sensitivity, specificity and accuracy respectively as 0.911, 0.841, and 0.878. The CHAID algorithm allocated 11 of 83 Honamli incorrectly and 72 correctly, while it separated 58 of 65 Hair goats correctly. CHAID has the second-largest AUC value as 0.880 after the MARS algorithm. In addition, among the

Table 4. *Classification performances of the data mining algorithms for each diagnosis test*

Algorithm	Sensitivity	Specificity	Matthews Correlation (Phi)	Cohen's Kappa Coefficient (κ)	AUC±SE	Accuracy of Model	Correctly Classify of Honamli Breed	Correctly Classify of Hair Breed	P-Value
MARS	0.916	0.846	0.739	0.739	0.942 ± 0.028^{a}	0.885	0.894	0.892	<0.001
CHAID	0.911	0.841	0.756	0.755	0.880 ± 0.027^{a}	0.878	0.867	0.892	<0.001
CART	0.849	0.927	0.756	0.749	0.868 ± 0.023^{a}	0.878	0.952	0.785	<0.001
Exhaustive CHAID	0.861	0.855	0.711	0.711	0.853 ± 0.030^{ab}	0.858	0.892	0.815	<0.001
QUEST	0.889	0.682	0.569	0.549	0.784 ± 0.032^{b}	0.770	0.675	0.892	<0.001

[a,ab,b] *The difference between AUC with letter in any data mining algorithm column is significant (P<0.05)*

tree-based data mining algorithms, the CHAID algorithm had the highest concordance with a Kappa (κ) value of 0.755, while it had the same correlation coefficient (Phi) as the CART algorithm with a value of 0.756. Although the CART algorithm correctly classified Honamli goats with a high rate (95.20%), the correct classifying percentage of Hair goats (78.50%) remained low. The performances of CART were determined as 0.849, 0.927, and 0.848 for sensitivity, specificity, and accuracy rate respectively. The CART algorithm estimated 79 of 83 Honamli goats, 51 of 65 Hair goats, and 87.80% of all goats by classifying them correctly. Moreover, the CART algorithm had the third-largest AUC (0.868), and the coefficient of agreement between actual breeds and breeds estimated by the CART algorithm was 0.749. The Exhaustive CHAID algorithm had performance values as 0.861 for sensitivity, 0.855 for specificity 0.855, and 0.858 for accuracy rate. While the Exhaustive CHAID algorithm classified 74 of 83 Honamli goats correctly, this algorithm assigned 12 of 65 Hair goats incorrectly. Exhaustive CHAID had the 4th largest area under ROC among algorithms, with an AUC of 0.853. The coefficient of concordance (κ) and correlation (Phi) between the predicted values of this algorithm and the actual values were 0.711. Although the Exhaustive CHAID algorithm correctly separated both breeds in close percentages, their performance values were a little low compared to other algorithms (MARS, CHAID, and CART). The QUEST algorithm correctly separated Hair goats with a high rate (89.20%) but, the separation percentage of Honamli goats (67.50%) remained quite low. It was also the algorithm with the worst prediction performance in terms of other performance criteria.

It has been determined that the CHAID algorithm was one of the best classifiers among classification trees for Honamli and Hair goat discrimination *(Table 4)*. When the CHAID diagram is examined, it was determined that the first order effective independent variable on breed discrimination was RH (Adj. P-value = 0.000, $\chi2$ = 59.332), second order was Age (Adj. P-value = 0.014, $\chi2$ = 9.981), and BH (Adj. P-value = 0.036, $\chi2$ = 6.313), and third-order independent variables were LG (Adj. P-value = 0.045, $\chi2$ = 13.362) and CD (Adj. P-value = 0.003, $\chi2$ = 12.577) *(Fig. 2)*. Branches generated by independent variables in the entire tree structure were statistically significant (P<0.05).

All goats considered in the study were divided into 3 sub-groups (nodes) in terms of RH variable. In the first node, 39 (83%) of the goats with RH = <71.00 cm shorter were Hair and 8 (17%) of them were Honamli. In the second node, 25 Hair (43.1%) and 33 Honamli (56.9%) of 58 goats were classified in a range of 71.0 <RH = <79.0. In the third node, it was determined that 42 of the goats (71.9 <RH) with RH traits more than 79 cm were Honamli (97.7%) and only one of them was Hair goat.

Goats (3rd node) with RH characteristics greater than 79 cm formed the 6th and 7th nodes in terms of GH characteristics. In the 6th node, 83.30% of the goats with the BH trait less or equal to 79.50 cm were classified as Honamli and 16.70% as Hair goat. All of the goats with the BH trait values greater than 79.50 belong to the Honamli breed (7th node).

While the 3rd and 4th nodes of the CHAID algorithm diagram showed a division according to the age variable, it did not have a direct effect on breed discrimination.

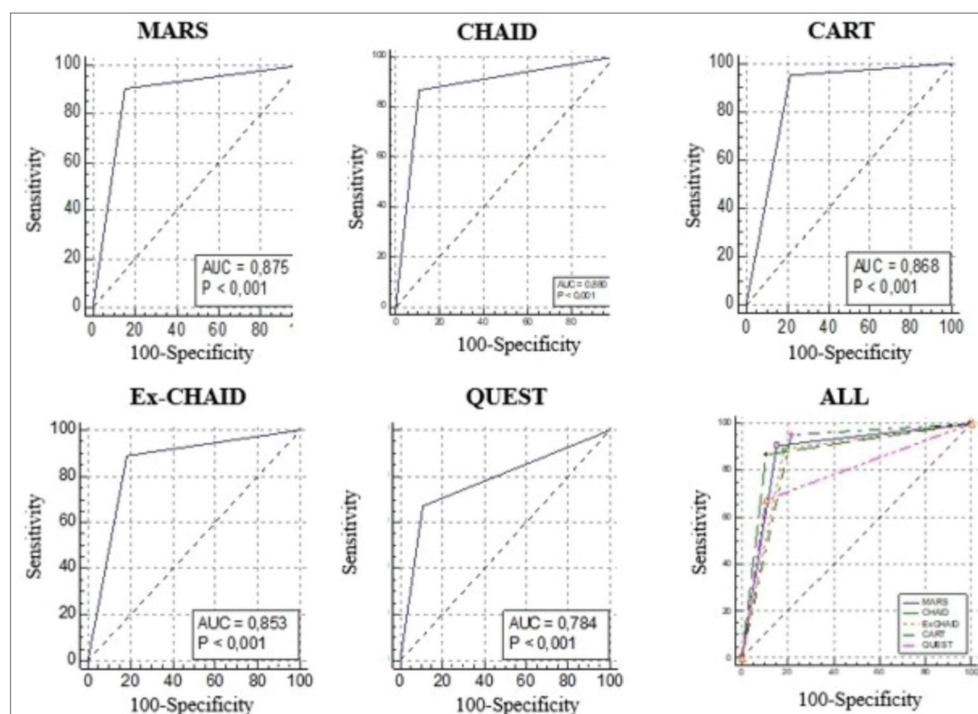

Fig 1. All and individual ROC curves of classifying algorithms for diagnostic tests of breed discrimination

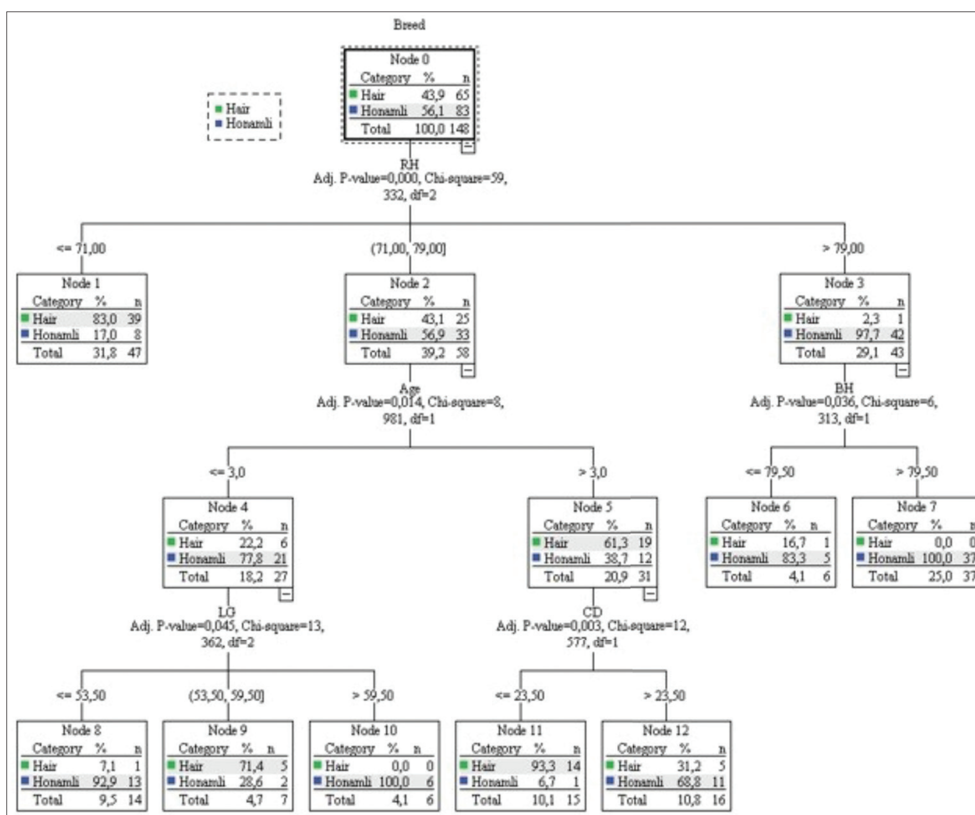

Fig 2. CHAID classification tree diagram of the diagnosis test of breed discrimination

Accordingly, it was determined that 13 of the goats aged 3 and under were Honamli (92.9%), and one of them was Hair goat (7.1%) (LG = <53.50) in terms of LG (8th node). In 9th node, the goats between 53.50 <LG = <59.50 were classified as 2 Honamli (28.6%) and 5 as Hair (71.4%). At the 10th node, 100% of all goats with the LG trait larger than 79 cm belong to the Honamli breed. In the 11th node, when the CD of goats older than three years was less than and equal to 23.50 cm, (CD = <23.50), 93.30% of goats are classified as Hair and 6.7% as Honamli. If the CD trait is greater than 23.50 cm (23.50 <CD), the probability of finding a Honamli goat is 68.80% and 31.20% is a Hair goat (12th node).

Discussion

The most successful data mining algorithms used in the phenotypic characterization of Honamli and Hair goats were MARS and CHAID. While the MARS algorithm used "LW", "BH", "CD", "CG" "Sex", "Age", and "HL" traits as independent variables in breed discrimination, the CHAID algorithm used "RH", "Age", "BH", "LG", and "CD". The reason why these two algorithms use different independent variables was their different working principles. While the CHAID algorithm created a more homogeneous subset, the MARS algorithm reveals the independent variables and coefficients of regression that affect the prediction model. Also, the CHAID algorithm determines the independent variables by using the Chi-square statistics and the Bonferroni corrected P-value after categorizing the independent variables and converting them into binary crosstabs [36,37]. The MARS algorithm, on the other hand, selects the independent variable using the generalized cross-validation error (GCV) method. The non-significant variables in the model are eliminated using the penalty function (λ) in the GCV term, and in this way, the multicollinearity problem is avoided [33].

It is claimed that the distinction between Honamli and Hair goats will be made by experienced breeders using HL, NL, and TL characteristics. This, however, is a subjective statement that has not been scientifically proven by any literature. Based on the findings of our study, it is understood that this is just a discourse with no scientific value. BH and CD features are common independent variables used by MARS and CHAID algorithms in the current study. It can be used in both algorithms to discriminate between these two breeds. However, because the CHAID algorithm uses fewer explanatory variables than the MARS algorithm, it may be preferred by breeders or researchers in terms of time and labor.

Essentially the same key variables can be used to describe closely related animal species [38]. Nsoso et al.[10] reported that the effect of age is important for the phenotypic characterization of indigenous Tswana goats reared in Bostwana. Body length (BL) and chest girth (CG) characteristics were reported to differ significantly in the distinction between Brown and Gray Bengal goats [39]. It

was emphasized that cannon bone circumference (CBC), chest girth (CG), chest depth (CD), rump height (RH), rump length (RL), and withers height (WH) traits are important for the distinction of five different indigenous goat breeds in Spain [12]. Gonzalez-Martinez et al.[40] reported that the chest depth (CD) and rump height (RH) characteristics of the Murciano-Granadina and Malagueña dairy goat breeds reared in Spain are important in breed discrimination. In Jordan, four indigenous breeds and crossbred goats were separated by simple, cluster, canonical, and stepwise discriminant analysis by using the morphological characteristics. The independent variables used in this distinction were reported as nose shape, withers height (WH), live weight (LW), ear type (ET), color, teat placement, chest width (CW), withers depth (WD), and rump width (RW) [41]. Although the statistical methods used in these studies were different, the goat breeds were reared in different environmental conditions, and their genetic structures are different, they were partially compatible with our study results.

Bourzat et al.[42] proposed two different indices for a simple classification of goats. The first index is the difference between withers height (WH), and chest depth (CD), while the other is the difference between ear length (EL) and chest depth (CD). Based on a univariate statistic for such a classification, it should not be discussed how successful these methods can be when all body characteristics of goats are considered together. The classification should be determined by a multivariate method and the methods used should be quite powerful. In the literature, they classified goats by using multivariate analysis methods such as discriminant, clustering, canonical, principal components analysis (PCA), multivariate statistical methods (MANOVA), etc. [10,11,13,41,43-45]. However, a strong classification could not be made since the multivariate classification methods have some prerequisites and the methods used do not have calibration (validation) capabilities [16]. In this context, it would be a more accurate approach to use data mining algorithms that are more powerful than the methods used and that can control the algorithm by cross-validation [33].

Orhan et al.[46] reported that there was a statistical difference in terms of strength, thickness, cuticle, medulla, and cortex characteristics of the hair structure of Honamli and Hair goats ($P<0.05$), but there was not any difference in terms of bulbous pili and scapus pili characteristics ($P>0.05$). Although they are phenotypically different and the individual comparison of the hair structure characteristics of goats is an important finding, it is not known what the result will be for breed discrimination when all the features are examined together. In our current study, although the data set has a very heterogeneous structure, it is seen that the characteristics of goats that are important for breed discrimination can be successfully made using data mining algorithms.

In this study results, showed that a new phenotypic characterization successfully allows distinguishing of Honamli and Hair goat breeds by using some body measurements and factors by data mining algorithms. Considering the successful performances of three different classification trees and MARS in breed distinction, CHAID and MARS methods can be used to make a more accurate classification. Moreover, data mining algorithms enable the discrimination of breed, which is phenotypically similar. In this way, the separation of phenotypically similar animals with powerful classification tools can be used as a preliminary step in selection programme. The results suggest that data mining algorithms could contribute to future studies about breed distinction of animals and might have a good potential for the protection of animal genetic resources. In addition, there is a need for studies that will be used in different species of animals by using data mining algorithms with both genetic and phenotype data. In this way, it is hoped that by doing so, a new quantitative method for the supply of breeding material can be developed.

Ethical Statement

In the study, there is no need for ethical approval due to the lack of blood sampling from the animals and the absence of any surgical procedures. All data were collected with the approval of the breeder.

Availability of Data and Materials

The author declares that data supporting the study findings are also available to the corresponding author.

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Competing Interests

The author declares no competing interests.

References

1. Atay O, Gökdal Ö, Eren V: Reproductive characteristics and kid marketing weights of hair goat flocks in rural conditions in Turkey. *Cuba J Agric Sci*, 44 (4): 353-358, 2010.

2. Karadag O: A study on the investigation of Honamli through some morphological characteristics fertility and casein genes polymorphism. *PhD Thesis*. Namık Kemal University Graduate School of Natural and Applied Sciences, 2016.

3. Varol M: Definition of morphological traits of Hair goats in Denizli province. *MSc Thesis,* Adnan Menderes University Graduate School of Natural and Applied Sciences, 2014.

4. Akbas AA, Saatci M: Growth, slaughter, and carcass characteristics of Honamlı, Hair, and Honamlı x Hair (F1) male goat kids bred under extensive conditions. *Turk J Vet Anim Sci*, 40 (4): 459-467, 2016. DOI: 10.3906/vet-1511-5

5. Taskaya H, Kale M: Investigation of Caprine Arthritis Encephalitis Virus (CAEV) infection in Honamlı goat breed. *MAE Vet Fak Derg*, 5 (2): 58-63, 2020. DOI: 10.24880/maeuvfd.682590

6. Elmaz Ö, Akbaş AA, Saatcı M: Effects of birth type on growth, fattening performance and carcass characteristics in Honamlı male kids. *Kafkas Univ Vet Fak Derg*, 23 (5): 749-755, 2017. DOI: 10.9775/kvfd.2017.17703

7. Bulut Z, Kurar E, Ozsensoy Y, Altunok V, Nizamlioglu M: Genetic diversity of eight domestic goat populations raised in Turkey. *BioMed Res Int*, 2016:2830394, 2016. DOI: 10.1155/2016/2830394

8. Karsli T, Demir E, Fidan HG, Aslan M, Karsli BA, Arik IZ, Semerci ES, Karabag K, Balcioglu MS: Determination of genetic variability, population structure and genetic differentiation of indigenous Turkish goat breeds based on SSR loci. *Small Ruminant Res*, 190:106147, 2020. DOI: 10.1016/j.smallrumres.2020.106147

9. Aytekin I: Effects of two different rearing systems on kid growth in Honamli goat. **In,** *Proceedings of International Human and Nature Sciences: Problems and Solution Seeking Congress*, 7-9 October, Sarajevo, Bosnia and Herzegovina, 2016.

10. Nsoso SJ, Podisi B, Otsogie E, Mokhutshwane BS, Ahmadu B: Phenotypic characterization of indigenous Tswana goats and sheep in Botswana: Continuous traits. *Trop Anim Health Prod*, 36 (8): 789-800, 2004. DOI: 10.1023/B:TROP.0000045979.52357.61

11. Jordana J, Ribo O, Pelegrin M: Analysis of genetic relationships from morphological characters in Spanish goat breeds. *Small Ruminant Res*, 12, 301-314, 1993. DOI: 10.1016/0921-4488(93)90065-P

12. Herrera M, Rodero E, Gutierrez MJ, Peña F, Rodero JM: Application of multifactorial discriminant analysis in the morphostructural differentiation of Andalusian caprine breeds. *Small Ruminant Res*, 22, 39-47, 1996. DOI: 10.1016/0921-4488(96)00863-2

13. Capote J, Delgado JV, Fresno M, Camacho ME, Molina A: Morphological variability in the Canary goat population. *Small Ruminant Res*, 27, 167-172, 1998. DOI: 10.1016/S0921-4488(97)00047-3

14. Herrera PSJI, Rodero E, Sànchez MD, Luque M: Raza caprina Moncaina. 1. Caracteres cuantitativos morfoestructurales. **In,** *Proceedings of Actas XXVIII Congreso de la SEOC,* 8-10 October, Granada, Spain, 2003.

15. Alpar C: Uygulamalı Çok Değişkenli Istatistiksel Yöntemler. 4th Ed., Detay Yayıncılık, Ankara, Turkey, 2017.

16. Maimon OZ, Rokach L: Data mining with decision trees: Theory and applications. **In,** Rocach L, Maimon O (Eds): Series In Machine Perception and Artificial Intelligence 2nd ed., World Scientific, Danvers, USA, 2014.

17. Akın M, Eyduran SP, Eyduran E: Mars algorithm in solving regression and classification type problems in agricultural sciences with R software. 1-264, Nobel Academic Publishing, Ankara, Turkey, 2020.

18. Ertugrul M: Ovine Breeding Practices. 2nd ed., Ankara University Faculty of Agriculture Publications Number: 1446, Ankara, Turkey, 1996.

19. Eyduran E, Keskin I, Erturk YE, Dag B, Tatliyer A, Tirink C, Aksahan R, Tariq MM: Prediction of fleece weight from wool characteristics of sheep using regression tree method (Chaid Algorithm). *Pak J Zool*, 48 (4): 957-960, 2016.

20. Orucoglu O: Determination of environmental factors affecting 305-day milk yield of Holstein cows by regression tree method. *MSc Thesis.* Suleyman Demirel University, Graduate School of Natural and Applied Sciences, 2011.

21. Aksahan R, Keskin I: Determination of the some body measurements effecting fattening final live weight of cattle by the regression tree analysis. *Selcuk J Agr Food Sci*, 2 (1): 53-59, 2015.

22. Kim SH, Kim CY, Seol DH, Choi JE, Hong SJ: Machine learning-based process-level fault detection and part-level fault classification in semiconductor etch equipment. *IEEE Trans Semicond Manuf,* 35, 174-185, 2022. DOI: 10.1109/TSM.2022.3161512

23. Breiman L, Friedman JH, Olshen RA, Stone CJ: Classification and regression trees. 1-368, Chapman & Hall/CRC, Wadsworth, New York, USA, 1984.

24. Kass GV: An exploratory technique for investigating large quantities of categorical data. *J R Stat Soc Ser C Appl Stat*, 29 (2): 119-127, 1980. DOI: 10.2307/2986296

25. Karakaya E, Çelik Ş, Taysi MR: CHAID algoritması ile balıketi tüketimini etkileyen faktörlerin incelenmesi. *JAFAG*, 35 (2): 85-93, 2018. DOI: 10.13002/jafag4381

26. Biggs D, De Ville B, Suen E: A method of choosing multiway partitions for classification and decision trees. *J Appl Stat*, 18 (1): 49-62, 1991. DOI: 10.1080/02664769100000005

27. Loh WY, Shih YS: Split selection methods for classification trees. *Stat Sin*, 7, 815-840, 1997.

28. Friedman JH: Multivariate adaptive regression splines. *Ann Statist*, 19, 1-67, 1991. DOI: 10.1214/aos/1176347963

29. Tırınk C, Eyduran E, Faraz A, Waheed A, Tauqir NA, Nabeel MS, Tariq MM, Sheikh IS: Use of multivariate adaptive regression splines for prediction of body weight from body measurements in Marecha *(Camelus dromedaries)* camels in Pakistan. *Trop Anim Health Prod*, 53 (3): 1-10, 2021. DOI: 10.1007/s11250-021-02788-y

30. Huma ZE, Iqbal F: Predicting the body weight of Balochi sheep using a machine learning approach. *Turk J Vet Anim Sci*, 43 (4): 500-506, 2019. DOI: 10.3906/vet-1812-23

31. Hanley JA, McNeil BJ: The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology*, 143, 29-36, 1982. DOI: 10.1148/radiology.143.1.7063747

32. IBM Corp. Released: IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp., 2015.

33. Eyduran E, Akin M, Eyduran SP: Application of multivariate adaptive regression splines in agricultural sciences through R software. 1-112, Nobel Academic Publishing, Ankara, Turkey, 2019.

34. Kuhn M: Caret: Classification and regression training. Retrieved from. https://CRAN.R-project.org/package=caret; *Accessed:* 07.12.2020.

35. R Core Team: R: A language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria. https://www.Rproject.org/; *Accessed:* 05.07.2020.

36. Altay Y, Boztepe S, Eyduran E, Keskin İ, Tariq MM, Bukhari FA, Ali I: Description of factors affecting wool fineness in Karacabey Merino sheep using chaid and mars algorithms. *Pak J Zool*, 53 (2): 691, 2021. DOI: 10.17582/journal.pjz/20190329150359

37. Olfaz M, Tırınk C, Önder H: Use of CART and CHAID algorithms in Karayaka sheep breeding. *Kafkas Univ Vet Fak Derg*, 25 (1): 105-110, 2019. DOI: 10.9775/kvfd.2018.20388

38. FAO: Phenotypic Characterization of Animal Genetic Resources. FAO Animal Production and Health Guidelines No: 11. Rome, Italy, 2012.

39. Mukeherjee DK, Singh CSP, Mishra HR: Anote on some phenotypic parameters in grey and brown Bengal goats. *Indian J Anim Sci*, 49, 671-671, 1979.

40. Gonzalez-Martinez A, Herrera M, Luque M, Rodero E: Influence of farming system and production purpose on the morphostructure of Spanish goat breeds. *Span J Agric Res*, 12 (1): 117-124, 2014. DOI: 10.5424/sjar/2014121-4673

41. Zaitoun IS, Tabbaa MJ, Bdour S: Differentiation of native goat breeds of Jordan on the basis of morphostructural characteristics. *Small Ruminant Res*, 56, 173-182, 2005. DOI: 10.1016/j.smallrumres.2004.06.011

42. Bourzat D, Souvenir Zafindrajoana P, Lauvergne JJ, Zeuh V: Comparaison morpho-biometrique de chevres au Nord Cameroun et au Tchad (Morphological and biometric comparison of goats in Northern Cameroon and Chad). *Rev Élev Méd Vét Pays Trop,* 46, 667- 674, 1993.

43. Dossa LH, Wollny C, Gauly M: Spatial variation in goat populations from Benin as revealed by multivariate analysis of morphological traits. *Small Ruminant Res*, 73, 150-159, 2007. DOI: 10.1016/j.smallrumres.2007.01.003

44. Chacón E, Macedo F, Velázquez F, Paiva SR, Pineda E, McManus C: Morphological measurements and body indices for Cuban Creole goats and their crossbreds. *R Bras Zootec*, 40, 1671-1679, 2011. DOI: 10.1590/S1516-35982011000800007

45. El Moutchou N, González Martínez AM, Chentouf M, Lairini K, Rodero E: Approach to morphological characterization of northern Morocco goat population. *Cah Options Mediterr*, 108, 427-432, 2014.

46. Orhan I, Duzler A, Alan A, Elmaz O, Ozgel O: Morphological investigations on the hairs of the Honamli and the Hair goat (Black goat). *Kocatepe Vet J*, 11 (2): 173-179, 2018. DOI: 10.30607/kvj.407473

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Research Article

Comparison of Bayesian Regularized Neural Network, Random Forest Regression, Support Vector Regression and Multivariate Adaptive Regression Splines Algorithms to Predict Body Weight from Biometrical Measurements in Thalli Sheep

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Abstract: In this study, it is aimed to compare several data mining and artificial neural network algorithms to predict body weight from biometric measurements for the Thalli sheep breed. For this purpose, the prediction capabilities of Bayesian Regularized Neural Network (BRNN), Support Vector Regression (SVR), Random Forest Regression (RFR) and Multivariate Adaptive Regression Splines (MARS) algorithms were comparatively investigated. To measure the predictive performances of the evaluated algorithms, body measurements such as body length, heart girth, ear length, ear width, head width, head length, withers height, rump length, rump width neck length, neck width of Thalli sheep were used for predicting the body weight. In this context, 270 female Thalli sheep were used to predict body weight. Model comparison criteria such as root-mean square error (RMSE), standard deviation ratio (SDR), performance index (PI), global relative approximation error (RAE), mean absolute percentage error (MAPE), Pearson's correlation coefficient (r), determination of coefficient (R^2) and Akaike's information criteria (AIC) were used to compare all algorithms. In conclusion, the MARS algorithm can be recommended to enable breeders to obtain an elite population of Thalli sheep breed.

Keywords: *Bayesian regularized neural network, Multivariate adaptive regression splines, Random forest regression, Support vector regression, Thalli sheep*

Thalli Koyunlarında Biyometrik Ölçümlerden Vücut Ağırlığı Tahmini İçin Bayesian Regularized Neural Network, Random Forest Regresyon, Support Vector Regresyon ve Çok Değişkenli Regresyon Uzanımları Algoritmalarının Karşılaştırılması

Öz: Bu çalışmada, Thalli koyun ırkı için biyometrik ölçümlerden vücut ağırlığını tahmin etmek için çeşitli veri madenciliği ve yapay sinir ağı algoritmalarının karşılaştırılması amaçlanmıştır. Bu amaçla BRNN, SVR, RFR ve MARS algoritmalarının tahmin performansları karşılaştırmalı olarak incelenmiştir. Değerlendirilen algoritmaların tahmin performanslarını ölçmek amacıyla vücut uzunluğu, göğüs çevresi, kulak uzunluğu, kulak genişliği, baş genişliği, baş uzunluğu, cidago yüksekliği, sağrı uzunluğu, sağrı genişliği boyun uzunluğu ve boyun genişliği gibi vücut ölçüleri canlı ağırlığını tahmin etmek için Thalli ırkı koyunlar kullanılmıştır. Bu kapsamda canlı ağırlık tahmini için 270 adet dişi Thalli koyunu kullanılmıştır. Tüm algoritmaların karşılaştırılmasında RMSE, SDR, PI, RAE, MAPE, r, R^2 ve AIC gibi model karşılaştırma kriterleri kullanılmıştır. Sonuç olarak, yetiştiricilerin elit bir Thalli koyun ırkı popülasyonu elde etmelerini sağlamak için MARS algoritması önerilebilir.

Anahtar sözcükler: *Bayesian regularized neural network, Çok değişkenli regresyon uzanımları, Random forest regresyon, Support vektör regresyon, Thalli koyunu*

Introduction

According to the FAO 2019-year database, there are 14.810.000 head meat sheep in Pakistan [1]. In total, Pakistan has 31 sheep breeds reared for meat, milk and wool products [2]. Among those, the Thalli breed is a thin-tailed sheep breed kept under tropical regions of Punjab province located in Pakistan, and medium size breed that has white body color, brown/black head with black spots on its legs. Sheep is an invaluable small ruminant breed that is used in many civilizations not only to obtain animal products such as meat, milk and fleece, but also to improve the rural economy [3]. Body weight is the major economical trait for all meat animals because income for farmers is directly gained by the weight of the animal. More sustained attention has been drawn to describe the relationship between body weight and linear biometric measurements (body measurements) for increasing meat production.

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Body measurements of sheep may reflect production performance and genetic characteristics as well as growth and development in sheep [4]. Some body measurements taken at early growth periods were reported to be beneficial for early selection to improve superior offspring in body weight in next generation and these measurements provide helpful in practice for sheep breeders who are willing to predict body weight, which is essential in flock management.

In rural conditions where there is no weighing scale, estimation of live weight in sheep by using body measurements provides an important advantage in flock management by making it easier to know the optimum feed amount per sheep in the herd, marketing price, medical doses, optimum slaughtering time [5,6]. Estimation based on body measurements used as one of the positive effects on body weight is considered as an indirect selection criterion in animal breeding [3]. In this framework, the best way to ascertain the effective body measurements are to implement reliable statistical techniques such as multivariate analysis methods and data mining algorithms for performing phenotypically breed description of sheep.

Many studies have been reported about the prediction of body weight from body measurements in different animal species such as sheep [7-9], cattle [10,11], rabbit [12], dog [13] and camel [14]. In the literature, there are many practical approaches to estimate body weight for sheep breeds by body measurements within the scope of multiple regression [15], Classification and Regression Tree (CART) and Chi-square automatic interaction detection (CHAID) and MARS algorithm [6] and artificial neural networks [16]. However, the application of Bayesian Regularized Neural Network, Random Forest Regression and Support Vector Regression is still rare for prediction of body weight in sheep breeds. For Thalli sheep, there is a dearth of information about revealing predictive performances of various data mining and artificial neural network algorithms to model the causal relationship between body measurements and body weight in Thalli sheep. Thalli sheep related further studies are necessary for developing economic situation of smallholder farms under tropical regions of Pakistan. Thalli sheep are a breed originating from the Thall region, with a black mouth, Roman nose, black long ears, and white colored other parts of the body.

In literature, there is an absence of information about Thalli sheep raised in Pakistan such as the description of the inbreeding effect on before weaning period of growth features, identification of influential environmental factors on before weaning time characteristics [17] and quantitative genetic evaluation of after weaning period characteristics [18]. Nevertheless, information on the body weight prediction in Thalli sheep by body measurements emphasize insufficiently. The prediction is of great importance for making much better decisions on flock management, breed standards, breeding schemes and conserving gene reserves of the Thalli sheep. In this respect, sophistical statistical techniques can help to produce more reliable estimates within the scope of indirect selection criteria to be applied in sheep and to reveal body measurements that affect body weight. In recent years, use of these techniques such as Artificial Neural Networks (ANNs), Classification and Regression Tree (CART), Exhaustive Chi-square Automatic Interaction Detector (Exhaustive CHAID), Chi-square Automatic Interaction Detector (CHAID) and Multivariate Adaptive Regression Splines (MARS) has gained importance for the prediction of body weight in various sheep breeds [3,9,16]. The current study has been carried out both to fill this gap in the literature and to compare the prediction performances of these algorithms.

Material and Methods

The research was carried out at Livestock Experiment Station in Punjab, Pakistan. The animals were sent to graze on the pasture between 9:00 am and 3:00 pm. The animals were given water twice a day. They were vaccinated for enteroxemia, peste des petits ruminants and pox. Wheat bran was daily given 250 g/animal during the pregnancy period. The rams were kept separately, and the natural mating methods were done in August or September. As a material, 270 female Thalli sheep were used. The age range of Thalli sheep used in the study is between 1-2 years old. The data on body measurements were recorded based on some phenotypic traits in Thalli sheep. A digital weighing device is used to determine body weight and a flexible measuring tape (special tape) to record different body measurements. Body measurements such as body length, heart girth, ear length, ear width, head width, head length, withers height, rump length, rump width, neck length, neck width were used to predict of body weight.

Statistical Analysis

The data set can be assumed normally distributed by the Kolmogorov-Smirnov normality test (P>0.05). Descriptive statistics of all quantitative characteristics for Thalli sheep breed were reported as mean, standard deviation. The Thalli sheep data was partitioned two different data sets, 75% - 25% training and test sets, respectively. Additionally, Bayesian Regularized Neural Network, MARS, Random Forest Regression and Support Vector Regression algorithms were used to estimate body weight from body measurements in Thalli sheep.

Statistical evaluations were made using the R software [19]. To provide information about the structure of the data, descriptive statistics were performed. Descriptive statistics for all variables were estimated by using “psych” package in R environment [20]. In addition, correlation plot drawn by

"corrplot" package in R software [21]. The "caret" packages in the R software were used to perform the analyzes of the BRNN and MARS algorithms [22]. The random forest regression was performed by using "randomForest" packages [23]. Also, support vector regression algorithm was performed by using "e1071" package in R software [24]. To display the performances of the constructed BRNN, MARS, Random Forest and Support Vector Regression models, the "ehaGoF" package was employed [25].

Bayesian Regularized Neural Network Algorithm (BRNN)

Artificial Neural Networks (ANNs) are known as mathematical models utilized in many scientific fields with the scope of solving prediction problems [26]. ANNs as one of the powerful artificial intelligence algorithms is structurally similar to the human brain and can be manipulated for sequential, nominal, scale-dependent variables. ANNs topologically consist of three layers such as input layer, hidden layer and output layer, respectively. The input layer consists of explanatory variables that the hidden layer depends on to start the process. The hidden layer consists of the activation functions and computes the weights of the explanatory variables in order to explore the effects of explanatory variables on the response variable [27]. Two types of ANNs algorithms such as radial basis functions neural networks (RBFNN) and bayesian regularized neural networks (BRNN) enable analysts to construct better models in predictive performance in comparison with linear models [26].

The BRNN function fits into a neural network that has input, hidden and output layers as described as given below [28,29].

$$y_i = g(x_i) + e_i = \sum_{k=1}^{s} w_k g_k \left(b_k + \sum_{j=1}^{p} x_{ij}\beta_k^{[k]}\right) + e_i,\ i = 1, \ldots, n \quad (1)$$

where, s is number of neurons for hidden layers, b_k is the bias for the kth neuron for k=1, …, s, w_k is the weight of the kth neurons for k=1, …, s, $\beta_k^{[k]}$ is the weight of the jth input of the network, x_{ij} is the input of jth predictor in ith observation, e_i is the error term of the model, g_k is the activation function that equation as given below:

$$g_k(x) = \frac{\exp(2x)-1}{\exp(2x)+1} \quad (2)$$

It uses Nguyen and Widrow algorithm to assign starting weights while performing this function and Gauss-Newton algorithm to provide optimization [30]. The Nguyen and Widrow initialization algorithm generates the initial weight and bias values to evenly distribute the active regions of neurons over the input area [31,32].

Support Vector Regression (SVR)

An important branch of the support vector machine, which is one of the machine learning algorithms, is the support vector regression (SVR) algorithm [33]. While the statistical method used in classification is called support vector classification (SVC), the statistical method used with modeling and prediction is called SVR [34-36]. Since SVR is a supervised learning method, the performance of SVR varies depending on the training and test dataset [37].

In linear SVR model, the main goal of SVR is to define a function f(x) that can have the maximum deviation (ε) from the training set and should be as straight as possible. Training data points are placed within the limit between −ε to +ε [37]. However, most of these studies cannot be modeled within the scope of linearity. Therefore, in the case of nonlinear SVR, the input data is matched to a higher dimensional Hilbert space $(\mathcal{H})$ so that the regression line can be linear [33].

The nonlinear regression hyperplane to be obtained is as follows.

$$\hat{y} = \langle w, \phi(x) \rangle + b \quad (3)$$

In this equation, w is a weight vector, $\phi(x)$ is non-linear kernel functions, $\langle .,. \rangle$ indicates vector inner product and is a bias term. There are many nonlinear kernel functions and one of these kernel functions is gaussian radial basis function kernel. The kernel function used in this study is the gausian radial basis function.

Random Forest Regression (RFR)

Random Forests is a popular method among multivariate statistical methods because of its easy applicability in classification and regression type problems. The Random Forest algorithm, which adds a layer of randomness to the bagging algorithm, was proposed by Breiman [38]. The RFR algorithm is a learning algorithm by combines sets of regression trees. A regression tree is represented as a set of constraints that are applied hierarchically from root to leaf of the tree [39,40]. The biggest advantage of this algorithm is that it can be easily used in the case of nonlinearity.

The algorithm requires a process that includes three stages [23]. The first procedure is to build the number of trees (n_{tree}) bootstrap samples from original data. The second procedure is to develop an un-pruned classification or regression tree for each sample. The last procedure is to estimate the new data from the tree. For the Thalli sheep data set, model parameters such as n_{tree} and the number of variables tried at each split are selected (m_{try}) 500 and 3, respectively.

Multivariate Adaptive Regression Splines Algorithm (MARS)

One of the tree-based algorithms are used to solve regression-

type problems while estimating based on quantitative traits [41-43]. To solve classification problems, The Multivariate Adaptive Regression (MARS) algorithm, which is a non-parametric regression technique that enables more effective identification of nonlinear and interaction effects between response and explanatory variables, was proposed by Friedman [44] and derived from the CART algorithm. In the algorithm, there is no need for any assumptions about both the distribution of variables and the relationships between variables [45,46]. The algorithm is a non-parametric regression technique in which various slopes in the training data set are divided into individual segmented linear segments (splines) [45]. Splines connect seamlessly and form connection points called "knot". Candidate nodes are randomly placed within the range of each estimator, so the model estimation to be made with the MARS algorithm is more flexible and interpretable with the help of piecewise linear regressions [45].

The algorithm consists of two different stages, a forward and backward pass stage [47]. The first stage for the algorithm is the forward pass stage. At this stage, the algorithm starts with an intercept in the first model and to improve the model recursively includes the basic function pairs with the least training error. The forward pass stage characteristically produces an over-fitted pattern that reaches maximum complexity [44]. The model constructed from the forward pass stage fits particularly good. Nevertheless, its generalization ability can be weak for a data set before an undetermined constructed model which means an overfitting problem. The basic functions that provide the least amount to the prediction model are eliminated in the backward pass stage and this situation is used in the solution of this problem [6,47].

At the beginning of the analysis, the multicollinearity between the explanatory variables was checked and it was found that there was no multicollinearity between the variables. To estimate BW using the training data set, the cross-validation method was used to decide the best MARS model among 324 MARS model with degree = 1:10 and nprune = 2:38 in determining the number of terms to be selected in the model. Ten-fold cross-validation was used for MARS model in the training data set.

RMSE, rRMSE, SDR, PI, RAE, MAPE, r, R^2 and Adj-R^2 criteria were used to compare the performance of the model. To compare the model performances were made according to the lowest RMSE, rRMSE, SDR, PI, RAE, MAPE values and the highest r, R^2 and Adj-R^2 value [48].

Results

Mean and standard deviation as the descriptive statistics of each trait in the present study for Thalli sheep are given in *Table 1*.

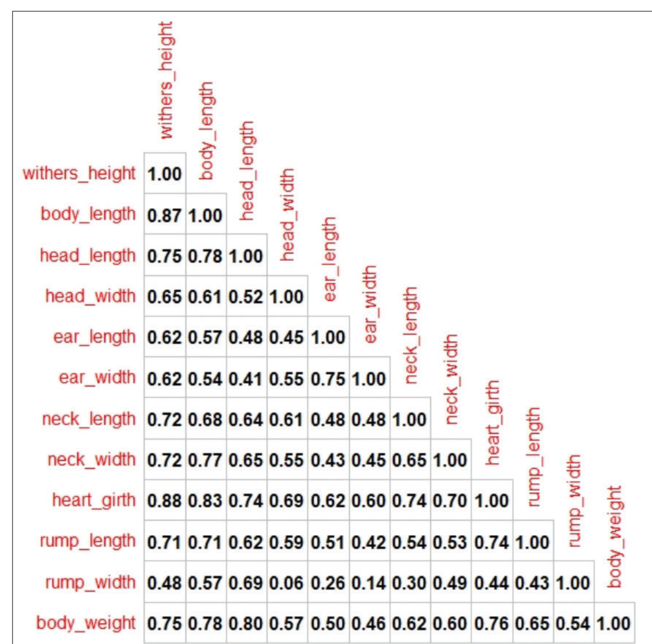
Fig. 1 showed that Pearson's correlation coefficient to determine the relationship between body measurements and BW. All correlation coefficients were determined to be statistically significant (P<0.01).

To compare the algorithms, some model comparison criteria were used for determining the performances of the algorithms in *Table 2*. Using metrics, the performances of all models at each stage were evaluated with test data from the respective stages and compared to determine the best model.

The metric methods used were evaluated for both the train and the test set, and the methods were compared to find the best method. According to *Table 2*, the performance in the test for each model was determined to be weaker than

Table 1. *Descriptive statistics for each measurement*

Variables	Mean	Standard Deviation
Withers height	63.92	7.66
Body length	63.71	9.08
Head length	24.63	4.31
Head width	9.53	1.64
Ear length	26.5	2.82
Ear width	11.69	1.31
Neck length	24.26	3.54
Neck width	15.62	2.25
Heart girth	68.15	9.58
Rump length	12.93	2.54
Rump width	18.16	4.36
Body weight	23.77	6.89



	withers_height	body_length	head_length	head_width	ear_length	ear_width	neck_length	neck_width	heart_girth	rump_length	rump_width	body_weight
withers_height	1.00											
body_length	0.87	1.00										
head_length	0.75	0.78	1.00									
head_width	0.65	0.61	0.52	1.00								
ear_length	0.62	0.57	0.48	0.45	1.00							
ear_width	0.62	0.54	0.41	0.55	0.75	1.00						
neck_length	0.72	0.68	0.64	0.61	0.48	0.48	1.00					
neck_width	0.72	0.77	0.65	0.55	0.43	0.45	0.65	1.00				
heart_girth	0.88	0.83	0.74	0.69	0.62	0.60	0.74	0.70	1.00			
rump_length	0.71	0.71	0.62	0.59	0.51	0.42	0.54	0.53	0.74	1.00		
rump_width	0.48	0.57	0.69	0.06	0.26	0.14	0.30	0.49	0.44	0.43	1.00	
body_weight	0.75	0.78	0.80	0.57	0.50	0.46	0.62	0.60	0.76	0.65	0.54	1.00

Fig 1. Correlation matrix

Table 2. Model comparison criteria for BRNN, SVR, RFR and MARS algorithms

Model Comparison Criteria	BRNN		SVR		RFR		MARS	
	Train	Test	Train	Test	Train	Test	Train	Test
Root mean square error (RMSE)	11.093	10.41	7.272	11.21	11.797	11.684	9.375	10.076
Standard deviation ratio (SDR)	0.477	0.476	0.386	0.496	0.492	0.507	0.439	0.460
Pearson's correlation coefficients (PC)	0.879	0.880	0.922	0.868	0.871	0.863	0.899	0.888
Performance index (PI)	7.467	7.197	5.909	7.514	7.733	7.694	6.793	7.05
Relative approximation error (RAE)	0.018	0.017	0.012	0.018	0.019	0.019	0.015	0.016
Mean absolute percentage error (MAPE)	12.063	13.177	7.764	12.537	11.615	12.884	10.39	12.086
Coefficient of determination (R^2)	0.772	0.758	0.851	0.74	0.758	0.729	0.808	0.766
Akaike's information Criterion (AIC)	490.881	154.620	404.737	159.511	503.435	162.245	474.571	170.467

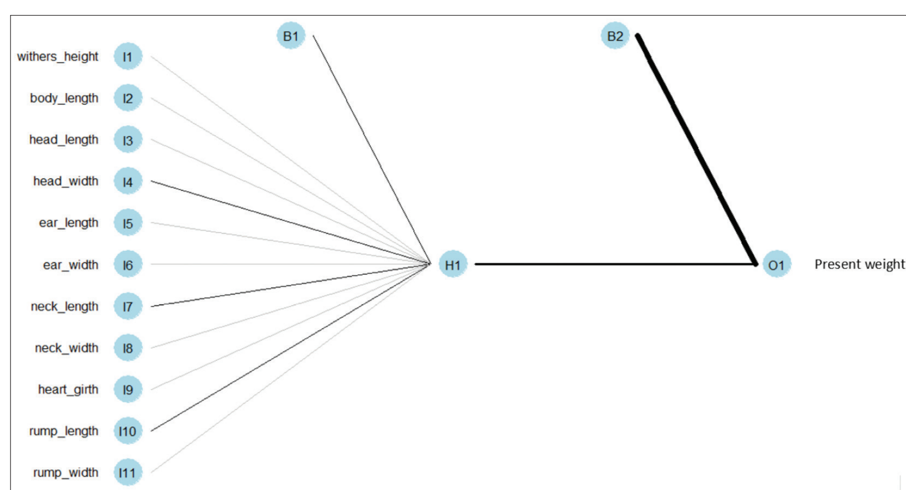


Fig 2. BRNN architecture to predict BW

the training dataset. When all metrics were evaluated, it turned out that the algorithm with the best fit for both the train and the test set was the MARS algorithm.

The Bayesian regularized neural network (BRNN) models were trained using the training data sets to predict the body weight. BRNN network algorithm produced a topologically 11-1-1 structure (the number of neurons in the input, hidden and output layers, respectively *(Fig. 2)* for the body weight prediction. In addition, the optimum BRNN model was achieved in the 12th epoch of the training phase. The network with one neuron gave the lowest RMSE value *(Fig. 3)*. The goodness of fit criteria revealed that BRNN algorithm produced the best fit among the candidate algorithms *(Table 2)*. Sensitivity analysis was carried out to estimate the relative importance values of the explanatory variables on body weight *(Fig. 4)*.

At the beginning of the SVR algorithm was trained training data. After the training process the SVR were examined to estimate the body weight for Thalli sheep breed. Gaussian radial basis kernel function for SVR estimation of body weight. The reliability of the model depends on the selection of parameters such as cost (C) and epsilon. These parameters were tested for various values and analysis was applied for C and epsilon values, which would give the most reliable model. Sensitivity analysis was carried out to estimate relative importance values of the explanatory variables on body weight for SVR *(Fig. 5)*.

For RFR, the model performance metrics were given in *Table 2*. In addition, sensitivity analysis was carried out to estimate relative importance values of the explanatory variables on body weight for RFR *(Fig. 6)*.

The optimum MARS model with 9 terms and degree: 1 is selected as the optimum model with the lowest cross-validated RMSE value among 324 candidate MARS models. The optimum MARS model obtained is as bellow in *Table 3*.

Discussion

Methods based on body measurements are widely used in determining the relationship between BW and the structure of the animal species. However, the validity of the statistical method used to estimate BW from these body measurements is also important. In this context, many studies have been carried out for different animal species. In multivariate statistics within the scope of data mining and artificial neural networks, the use of model

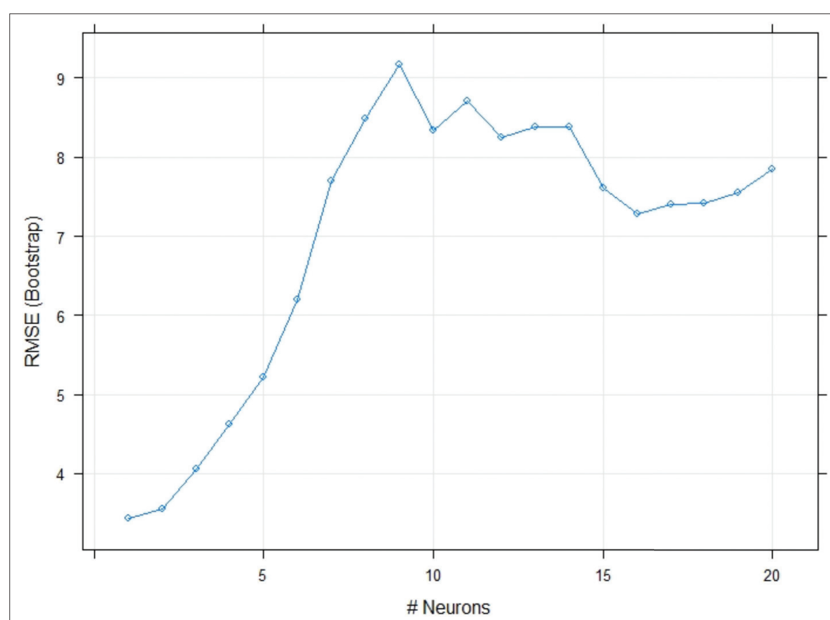


Fig 3. RMSE diagram for the bootsrap BRNN algorithm

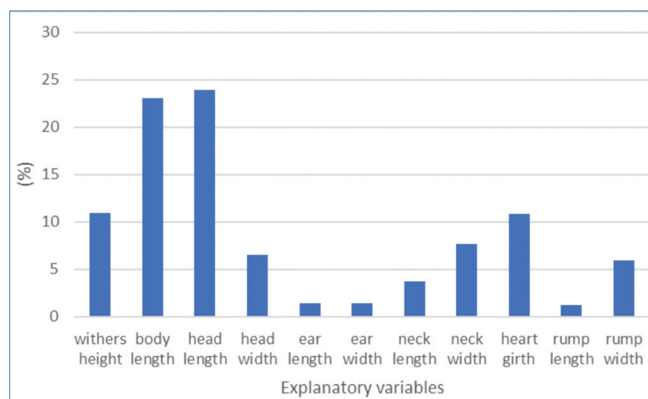


Fig 4. Sensitivity analysis for BRNN model

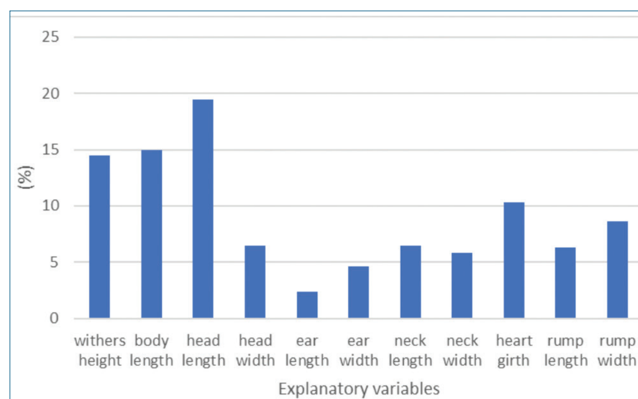


Fig 6. Sensitivity analysis for RFR model

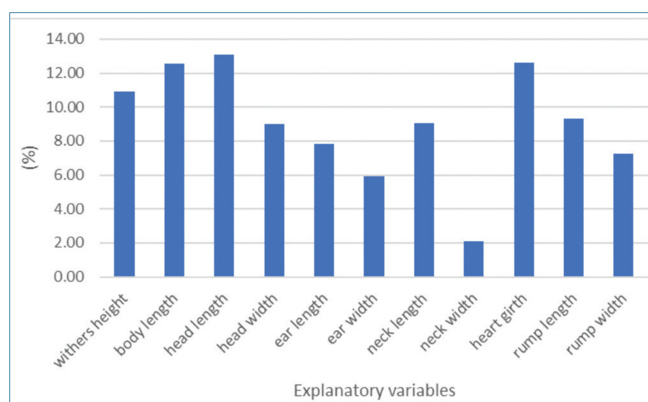


Fig 5. Sensitivity analysis for SVR model

Table 3. *Obtained optimum MARS model*

Explanatory Variables	Coefficients
Intercept	10.8827049
(body_length-48.26)	0.2558336
(19.05-head_length)	1.5792126
(head_length-19.05)	1.6836423
(head_length-22.86)	-1.2350427
(7.62-head_width)	-2.0680844
(neck_length-27.94)	0.6855337
(12.7-neck_width)	2.0226105
(heart_girth-53.34)	0.1611244

comparison criteria has been proposed in the selection of the best model [12]. In this context, model performances are compared according to the lowest RMSE, rRMSE, SDR, PI, RAE, MAPE values and the highest r, R^2 and Adj-R^2 values [48].

With the use of these algorithms, by determining the selection scheme in Thalli sheep, the variables that are effective in BW estimation can be determined, and this will lead to sustainable livestock breeding. However, there are deficiencies in the literature on studies using

these algorithms. In this context, it has been determined that only the MARS algorithm and some of the ANN algorithms are used in the literature.

Ali et al.[49] compared the ANN, CART, Exhaustive CHAID and CHAID algorithms in their study on the Harnai sheep breed. When the study was evaluated within the scope of R^2, Exhaustive CHAID was estimated 0.8421, CHAID as 0.8377, CART as 0.82644 and ANN as 0.81999. The proposed method was the Exhaustive CHAID method to define the standards of Harnai sheep breed. Our obtained R^2 values were found lower than the results in comparison of ANN algorithm which is comparable for both studies. Breed and evaluated traits may be the factors of these differences.

Eyduran et al.[50] used CART, CHAID and ANN (RBF, MLP1 and MLP2) algorithms for BW prediction for Beetal goat and this study, which was evaluated within the scope of the correlations found between predicted and actual values, the highest correlation was determined for RBF of the ANN algorithm. In this context, the current study is thought to have different results due to differences between species.

Celik et al.[3] aimed to compare CART, CHAID, Exhaustive CHAID, MARS, MLP, and RBF on Mengali rams. Within the scope of model comparison criteria R^2, SDratio and RMSE the best prediction model was determined as the CART algorithm. However, the MARS algorithm appears to have an R^2 value of 0.88. In the current study, it has been seen that the R^2 value for the train set is similar.

Compared to the results of previous studies, the breeds used in the studies, the age of the animals, the differences in flock management systems and the statistical methods used can be attributed to this wide variation. In this context, compared to results from other studies, it was determined that provide similar results according to the chosen model of evaluation criteria. However, different methods have been proposed in terms of the methods used. Suggesting different statistical methods for BW estimation using body measurements reveals that there is a need for more studies on this subject.

In the literature, variability in BW estimation in sheep may be due to differences in the number of samples used, breed, sex, flock management systems and statistical methods. For this purpose, the correct use of the factors that cause these differences is very important for a sustainable livestock breeding and selection. The correct use of statistical methods, which is one of the factors that cause variability, will create the make a right decision mechanism with more reliable estimates.

The results show that the BW estimation to be made with the MARS algorithm is more reliable within the scope of model comparison criteria. For the MARS algorithm, body length, head length, head width, neck length and neck width measurements were determined to be the most effective BW estimation.

There is no available information to estimate body weight from body measurements within the scope of BRNN, SVR, RFR and MARS algorithm for Thalli sheep. In the present study, body weight was estimated from body measurements by using these algorithms for Thalli sheep breed. Although all algorithms have their own advantages and disadvantages, the model performances obtained from the MARS algorithm were better determined.

In conclusion, the MARS algorithm can be recommended to enable breeders to obtain an elite population of Thalli sheep breed. In addition, it provides to increasing BW as a selection criterion for determining appropriate body measurements and flock management standards. The results of this study, based on model selection criteria for the selection of the most suitable model, showed that data mining and artificial neural network algorithms can be successfully applied to BW estimation based on measured body measurements. Even if there are some differences in the value of the comparison criteria, more reliable models can be obtained by conducting similar studies.

Availability of Data and Materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of Interest

The author declared that there is no conflict of interest.

References

1. FAO: FAOSTAT, 2019. http://www.fao.org/faostat/en/#data/QL; *Accessed*: 11.03.2021.

2. Tariq MM, Bajwa MA, Jawasreh K, Awan MA, Abbas F, Waheed A, Rafeeq M, Wadood A, Khan KU, Rashid N, Atique MA, Bukhari FA: Characterization of four indigenous sheep breeds of Balochistan, Pakistan by random amplified polymorphic DNAs. *Afr J Biotechnol*, 11, 2581-2586, 2012. DOI: 10.5897/AJB11.3196

3. Celik S, Eyduran E, Karadas K, Tariq MM: Comparison of predictive performance of data mining algorithms in predicting body weight in Mengali rams of Pakistan. *Braz J Vet Res Anim Sci*, 46 (11): 863-872, 2017. DOI: 10.1590/S1806-92902017001100005

4. Zhang AL, Wu BP, Wuyun CT, Jiang DX, Xuan EC, Ma FY: Algorithm of sheep body dimension measurement and its applications based on image analysis. *Comput Electron Agric,* 153, 33-45, 2018. DOI: 10.1016/j.compag.2018.07.033

5. Sabbioni A, Beretti V, Superchi P, Ablondi M: Body weight estimation from body measures in Cornigliese sheep breed. *Ital J Anim Sci*, 19 (1): 25-30, 2020. DOI: 10.1080/1828051X.2019.1689189

6. Faraz A, Tirink C, Eyduran E, Waheed A, Tauqir NA, Nabeel MS, Tariq MM: Prediction of live body weight based on body measurements in Thalli sheep under tropical conditions of Pakistan using CART and MARS. *Trop Anim Health Prod*, 53 (2): 1-12, 2021. DOI: 10.1007/s11250-021-02748-6

7. Cam MA, Olfaz M, Soydan E: Body measurements reflect body weights and carcass yields in Karayaka sheep. *Asian J Anim Vet Adv*, 5 (2): 120-127, 2010. DOI: 10.3923/ajava.2010.120.127

8. Ghotbaldini H, Mohammadabadi M, Nezamabadi-Pour H, Babenko OI, Bushtruk MV, Tkachenko SV: Predicting breeding value of body weight at 6-month age using Artificial Neural Networks in Kermani sheep breed. *Acta Sci Anim Sci*, 41, 2019. DOI: 10.4025/actascianimsci.v41i1.45282

9. Olfaz M, Tirink C, Onder H: Use of CART and CHAID algorithms in Karayaka sheep breeding. *Kafkas Univ Vet Fak Derg*, 25 (1): 105-110, 2019. DOI: 10.9775/kvfd.2018.20388

10. Aytekin I, Eyduran E, Karadas K, Aksahan R, Keskin I: Prediction of fattening final live weight from some body measurements and fattening period in young bulls of crossbred and exotic breeds using MARS data mining algorithm. *Pak J Zool*, 50 (1): 189-195, 2018. DOI: 10.17582/journal.pjz/2018.50.1.189.195

11. Weber VAM, de Lima Weber F, da Silva Oliveira A, Astolfi G, Menezes GV, de Andrade Porto JV, Rezende FPC, de Moraes PH, Matsubara ET, Mateus RG, de Araújo TLAC, da Silva LOC, de Queiroz EQA, de Abreu UGP, da Costa Gomes R, Pistori H: Cattle weight estimation using active contour models and regression trees Bagging. *Comput Electron Agric*, 179, 105804, 2020. DOI: 10.1016/j.compag.2020.105804

12. Salawu EO, Abdulraheem M, Shoyombo A, Adepeju A, Davies S, Akinsola O, Nwagu B: Using artificial neural network to predict body weights of rabbits. *Open J Anim Sci*, 4, 182-186, 2014. DOI: 10.4236/ojas.2014.44023

13. Celik S, Yilmaz O: Prediction of body weight of Turkish tazi dogs using data mining Techniques: Classification and Regression Tree (CART) and multivariate adaptive regression splines (MARS), *Pak J Zool*, 50 (2): 575-583, 2018. DOI: 10.17582/journal.pjz/2018.50.2.575.583

14. Fatih A, Celik S, Eyduran E, Tirink C, Tariq MM, Sheikh IS, Faraz A, Waheed A: Use of MARS algorithm for predicting mature weight of different camel *(Camelus dromedarius)* breeds reared in Pakistan and morphological characterization via cluster analysis. *Trop Anim Health Prod*, 53 (1): 1-14, 2021. DOI: 10.1007/s11250-021-02633-2

15. Topal M, Macit M: Prediction of body weight from body measurements in morkaraman sheep. *J Appl Anim Res*, 25 (2): 97-100, 2004. DOI: 10.1080/09712119.2004.9706484

16. Huma ZE, Iqbal F: Predicting the body weight of Balochi sheep using a machine learning approach. *Turk J Vet Anim Sci*, 43 (4): 500-506, 2019. DOI: 10.3906/vet-1812-23

17. Hussain A, Akhtar P, Ali S, Younas M, Yaqoob M, Babar ME, Kaved K, Shakoor A: Factors influencing body weights at different ages in Thalli sheep. *J Anim Plant Sci*, 23 (1): 1-6, 2013.

18. Hussain A, Akhtar P, Ali S, Javed K, Younas M, Shakoor A, Waheed U: Genetic analysis of post-weaning growth traits of Thalli sheep under tropical conditions. *Trop Anim Health Prod*, 46, 1527-1531, 2014. DOI: 10.1007/s11250-014-0670-x

19. R Core Team: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2020. https://www.R-project.org/; *Accessed:* 11.03.2021.

20. Revelle W: Psych: Procedures for Personality and Psychological Research, Northwestern University, Evanston, Illinois, USA, 2020. https://CRAN.R-project.org/package=psych Version = 2.0.12; *Accessed:* 11.03.2021.

21. Wei T, Simko V: R package "corrplot": Visualization of a Correlation Matrix (Version 0.84), 2017. https://github.com/taiyun/corrplot; *Accessed:* 11.03.2021.

22. Kuhn M: Caret: Classification and Regression Training. R package version 6.0-86, 2020. https://CRAN.R-project.org/package=caret; *Accessed:* 11.03.2021.

23. Liaw A, Wiener M: Classification and regression by random forest. *R News*, 2 (3): 18-22, 2002.

24. Meyer D, Dimitriadou E, Hornik K, Weingessel A, Leisch F: e1071: Misc Functions of the Department of Statistics, Probability Theory Group (Formerly: E1071), TU Wien R package version 1.7-4, 2020. https://CRAN.R-project.org/package=e1071; *Accessed:* 11.03.2021.

25. Eyduran E: ehaGoF: Calculates Goodness of Fit Statistics. R package version 0.1.1., 2020. https://CRAN.R-project.org/package=ehaGoF; *Accessed:* 11.03.2021.

26. Pérez-Rodríguez P, Gianola D, Weigel KA, Rosa GJM, Crossa J: Technical Note: An R package for fitting Bayesian regularized neural networks with applications in animal breeding. *J Anim Sci*, 91 (8): 3522-3531, 2013. DOI: 10.2527/jas.2012-6162

27. Kayri M: Predictive abilities of bayesian regularization and Levenberg–Marquardt algorithms in artificial neural networks: A comparative empirical study on social data. *Math Comput Appl*, 21(2): 20, 2016. DOI: 10.3390/mca21020020

28. MacKay DJC: A practical Bayesian framework for backpropagation networks. *Neural Comput*, 4, 448-472, 1992. DOI: 10.1162/neco.1992.4.3.448

29. Foresee FD, Hagan MT: Gauss-Newton approximation to Bayesian learning. *Proceedings of International Conference on Neural Networks*, 3, 1930-1935, 1997. DOI: 10.1109/ICNN.1997.614194

30. Nguyen D, Widrow B: Improving the learning speed of 2-layer neural networks by choosing initial values of the adaptive weights. *1990 IJCNN International Joint Conference on Neural Network*, 3, 21-26, 1990. DOI: 10.1109/IJCNN.1990.137819

31. Dolezel P, Skrabane P, Gago L: Weight initialization possibilities for feedforward neural network with linear saturated activation functions. *IFAC-PapersOnLine*, 49 (25): 49-54, 2016. DOI: 10.1016/j.ifacol.2016.12.009

32. Linan MN, Gerardo B, Medina R: Modified weight initialization in the self-organizing map using Nguyen-Widrow initialization algorithm. *J Phys Conf Ser*, 1235:012055, 2019. DOI: 10.1088/1742-6596/1235/1/012055

33. Nguyen QT, Fouchereau R, Frénod E, Gerard C, Sincholle V: Comparison of forecast models of production of dairy cows combining animal and diet parameters. *Comput Electron Agric*, 170, 105258, 2020. DOI: 10.1016/j.compag.2020.105258

34. Smola AJ, Schölkopf B: A tutorial on support vector regression. *Stat Comput*, 14, 199-222, 2004. DOI: 10.1023/B:STCO.0000035301.49549.88

35. Kavaklioglu K: Modeling and prediction of Turkey's electricity consumption using Support Vector Regression. *Appl Energy*, 88, 368-375, 2011. DOI: 10.1016/j.apenergy.2010.07.021

36. Alonso J, Castañón ÁR, Bahamonde A: Support Vector Regression to predict carcass weight in beef cattle in advance of the slaughter. *Comput Electron Agric*, 91, 116-120, 2013. DOI: 10.1016/j.compag.2012.08.009

37. Patel AK, Chatterjee S, Gorai AK: Development of a machine vision system using the support vector machine regression (SVR) algorithm for the online prediction of iron ore grades. 2017 Fifteenth IAPR International Conference on Machine Vision Applications 12, 197-210, 2017. DOI: 10.23919/MVA.2017.7986823

38. Breiman L: Random forests. *Mach Learn*, 45 (1): 5-32, 2001. DOI: 10.1023/A:1010933404324

39. Rodriguez-Galiano V, Mendes MP, Garcia-Soldado MJ, Chica-Olmo M, Riberio L: Predictive modeling of groundwater nitrate pollution using Random Forest and multisource variables related to intrinsic and specific vulnerability: A case study in an agricultural setting (Southern Spain). *Sci Total Environ*, 476-477, 189-206, 2014. DOI: 10.1016/j.scitotenv.2014.01.001

40. Wang L, Zhou X, Zhu X, Dong Z, Guo W: Estimation of biomass in wheat using random forest regression algorithm and remote sensing data. *Crop J*, 4, 212-219, 2016. DOI: 10.1016/j.cj.2016.01.008

41. Akin M, Eyduran E, Niedz RP, Reed BM: Developing hazelnut tissue culture free of ion confounding. *Plant Cell Tissue Organ Cult*, 130 (3): 483-494, 2017. DOI: 10.1007/s11240-017-1238-z

42. Akin M, Eyduran E, Reed BM: Use of RSM and CHAID data mining algorithm for predicting mineral nutrition of hazelnut. *Plant Cell Tissue Organ Cult*, 128 (2): 303-316, 2017. DOI: 10.1007/s11240-016-1110-6

43. Kovalchuk IY, Mukhitdinova Z, Turdiyev T, Madiyeva G, Akin M, Eyduran E, Reed BM: Modeling some mineral nutrient requirements for micropropagated wild apricot shoot cultures. *Plant Cell Tissue Organ Cult*, 129, 325-335, 2017. DOI: 10.1007/s11240-017-1180-0

44. Friedman JH: Multivariate adaptive regression splines. *Ann Stat*, 19 (1): 1-67, 1991. DOI: 10.1214/aos/1176347963

45. Aksoy A, Erturk YE, Eyduran E, Tariq MM: Utility of MARS algorithm for describing non-genetic factors affecting pasture revenue of Morkaraman breed and Romanov x Morkaraman F1 crossbred sheep under semi intensive conditions. *Pak J Zool*, 51(1): 235-240, 2019. DOI: 10.17582/journal.pjz/2019.51.1.235.240

46. Akin M, Eyduran SP, Eyduran E, Reed BM: Analysis of macro nutrient related growth responses using multivariate adaptive regression splines. *Plant Cell Tissue Organ Cult*, 140, 661-670, 2020. DOI: 10.1007/s11240-019-01763-8

47. Arthur CK, Temeng VA, Ziggah YY: Multivariate Adaptive Regression Splines (MARS) approach to blast-induced ground vibration prediction. *Int J Min Reclam Environ*, 34 (3): 198-222, 2020. DOI: 10.1080/17480930.2019.1577940

48. Tatliyer A: The Effects of raising type on performances of some data mining algorithms in lambs. *KSU J Agric Nat*, 23(3), 772-780, 2020. DOI: 10.18016/ksutarimdoga.vi.651232

49. Ali M, Eyduran E, Tariq MM, Tirink C, Abbas F, Bajwa MA, Baloch MH, Nizamani AH, Waheed A, Awan MA, Shah SH, Ahmad Z, Jan S: Comparison of artificial neural network and decision tree algorithms used for predicting live weight at post weaning period from some biometrical characteristics in Harnai sheep. *Pak J Zool*, 47, 1579-1585, 2015.

50. Eyduran E, Zaborski D, Waheed A, Celik S, Karadas K, Grzesiak W: Comparison of the predictive capabilities of several data mining algorithms and multiple linear regression in the prediction of body weight by means of body measurements in the indigenous Beetal goat of Pakistan. *Pak J Zool*, 49 (1): 257-265, 2017.

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Research Article

Growth Performance, Ruminal Volatile Fatty Acids, Health Status and Profitability in Calves Fed with Milk Supplemented with Probiotics

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Abstract: This study aimed to determine the effects of milk supplemented with different amounts (10-15 mL/day) of probiotics (effective microorganism-EM) during the period until weaning (70 days) of the calves on the growth performance [live weight (LW), live weight gain (LWG), the feed conversion ratio (FCR), body measurements], rumen volatile fatty acids (VFA), health status and profitability. A total of 42 calves were divided into three groups as control and two treatment groups (EM10 and EM15) containing 14 calves in each with similar live weights (42±5 kg), ages (7±3 days), breeds (7 Holstein and 7 Simmental), and sex (7 female and 7 male). The control group had no supplement in the milk, whereas the calves in the treatment groups received 10 mL of EM per calf per day orally or 15 mL of EM with milk. According to the study results, using the 10 and 15 mL/day of EM in calves had no significant effect on the performance (LW, LWG, FC, body measurements), VFA, disease rates, and profitability (P>0.05). However, in the first 30 days of the study, the FCR of the EM10 group was positively affected compared to the control group (P<0.05). In conclusion, slightly better results were obtained in both treatment groups regarding body measurements, VFA, disease rates, treatment costs and profitability than the control group.

Keywords: *Calf feeding, Effective microorganism, Performance, Probiotic, Profitability*

Buzağı Beslemede Probiyotik Kullanımının Büyüme Performansı, Rumen Uçucu Yağ Asitleri, Sağlık Durumu ve Karlılığa Etkisi

Öz: Bu çalışmada sütten kesilene kadarki dönemde (70 gün) buzağılara farklı oranlarda (10-15 mL/gün) süte ilave edilen probiyotiğin (efektif mikroorganizma-EM) büyüme performansı [canlı ağırlık (CA), canlı ağırlık artışı (CAA), yemden yararlanma oranı (YYO), vücut ölçüleri], rumen uçucu yağ asitleri (UYA), sağlık durumu ve karlılık üzerine etkilerinin belirlenmesi amaçlanmıştır. Çalışmada toplam 42 buzağı, canlı ağırlıkları (42±5 kg), yaşları (7±3 günlük), ırkları (7 Holstayn, 7 Simental) ve cinsiyetleri (7 dişi, 7 erkek) benzer olacak şekilde bir kontrol ve iki deneme grubu (EM10 ve EM15) olmak üzere her grupta 14 buzağı olacak şekilde toplam 3 gruba ayrılmıştır. Deneme grubunda bulunan buzağıların sütlerine kontrol grubundan farklı olarak, EM10 grubunda buzağı başına günlük 10 mL EM ve EM15 grubunda ise 15 mL EM katılarak oral yolla içirilmiştir. Çalışma bulgularına göre, buzağılarda 10 ve 15 mL/gün EM kullanılması, performans (CA, CAA, YT, vücut ölçüleri), UYA, hastalık oranları ve karlılık değerlerini önemli oranda etkilememiştir (P>0.05). Ancak, çalışmanın 0-30. günleri arasında EM10 grubunda, YYO kontrol grubuna göre olumlu etkilenmiştir (P<0.05). Sonuç olarak, deneme gruplarında vücut ölçüleri, UYA, hastalık oranları, tedavi maliyetleri ve karlılık açısından kontrole göre nispeten daha iyi sonuçlar elde edilmiştir.

Anahtar sözcükler: *Buzağı besleme, Etkili mikroorganizma, Karlılık, Performans, Probiyotik*

Introduction

Healthily raising calves is very important for the economic sustainability of dairy cattle farms. One of the most critical problems of the dairy cattle industry globally and in our country is the high calf diseases and losses, especially in the pre-weaning period. The rate at which dairy calves die in farms is estimated to be over 10% in Turkey and

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5-10% in Europe [1-5]. The growth performance of young calves is strongly related to the type of consumed feed, the rearing system, and intestinal microbiota balance [6,7]. Nowadays, more intensive rearing is carried out due to the increasing scale of farms in animal husbandry, which brings hygiene, care-feeding, and management problems. Because of these problems, gastrointestinal infections, and diarrhea, which are seen in calves in the first months of life due to enteric bacteria imbalance, are the leading health problems that cause the deaths of calves, yield and economic losses. It has been claimed that the composition and individual variations of the intestinal microbiota of calves may play an essential role in the pathogenesis of gastrointestinal diseases and diarrhea and may be associated with susceptibility to enteric infections [8,9]. Therefore, developing a healthy intestinal microbiome is vital for the sustainability of animal production and its economic aspect [10]. Since the enteric infection causes growth retardation, increases the risk of diseases and death, and adversely effects on fertility and fertility parameters (delay in first calving age and first lactation) in the future, prevention of diarrhea and enteric diseases should be the primary goal in calves [11-16].

The use of antibiotics in calf nutrition, either directly or in whole milk or milk substitute formula, has been widely accepted as a strategy to reduce early diarrheal morbidity and mortality [17,18]. However, the possibility of the emergence of microbial resistance due to antimicrobials in animal production and the potential risks for human health and food safety have led to legal regulations regarding the use of antibiotics in animal husbandry. Therefore, new strategies are needed to minimize the susceptibility of calves to intestinal infections and diarrhea and improve intestinal health. Thus, studies on giving safer food additives instead of antibiotics to calves in the suckling period have increased. Probiotics have become a good option for manipulating the intestinal microbiome to improve calf health and development [19,20]. Despite the increasing interest in the use of probiotics to improve the performance and health of animals by balancing the gastrointestinal microbial ecosystem in recent years, the mechanism of action of probiotics is still not fully elucidated. Different mechanisms have been proposed to explain the effects of probiotics. Most common observed and hypothesized mechanisms include probiotics competing for nutrients, producing antibacterial compounds (e.g., organic acids, hydrogen peroxide, bacteriocins) in the intestinal lumen, production of biofilms by changing the bacteria population of the gastrointestinal tract, stimulation of fecal shedding of coliforms, invading certain areas of the intestinal mucosa, decrease concentration of stress hormones (cortisol), and activating the pre-existing immune system of calves [6,7,21]. Agazzi et al.[22] reported that the administration of probiotic to calves altered the microbiota balance and nutrient utilization in the GI tract and increased the growth performance.

It has been reported that the use of probiotics in calf nutrition reduces the weaning age, increases the number of rumen microorganisms and the digestion of feed, and thus contributes to the development of rumen flora and fauna earlier [23].

While probiotics were primarily used in monogastric animals, it has been observed that probiotics in ruminants, especially in preruminants, have become widespread in recent years. It has been reported that proper and enough probiotics can be added to milk or starter feeds in preruminant calves to improve intestinal health, promote early solid feed intake (FC) and improve growth [10]. In some of these studies, it was determined that probiotics significantly increased live weight (LW) [24-27], live weight gain (LWG) [24,25,27,28], feed consumption [28], feed efficiency [24] and significantly decreased the incidence/duration of diarrhea, and the fecal counts of coliforms [24,28], On the other hand, in some studies, probiotics did not affect the growth performance and the survival of calves [29,30]. The diversity of the results in the previous studies which do not fully support each other may be due to factors such as; the strain of the probiotic microorganism used, the dose, the quality of the feed consumed (nutrient and energy level), the amount of feed/milk consumed, the addition of the probiotic to the feed or milk, and the rearing conditions of the calves.

This study was performed to determine the effects of probiotics (effective microorganism-EM) added to the milk in different amounts (10-15 mL/day) during the pre-weaning period on the growth performance, rumen volatile fatty acids, health status, and profitability of calves.

Material and Methods

Ethical Statement

This study was approved by the Erciyes University Animal Experiments Local Ethics Committee (Approval date and number: 03.11.2021 and 21/235).

Commercial Probiotic Product

The probiotic additive used in the study (EM Agriton®, Okinova, Japan) contains *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus rhamnous*, *Lactobacillus casei,* and *Lactobacillus delbrueckii* lactic acid bacteria and *Saccharomyces cerevisiae* yeast. This commercial additive contains $1*10^7$ cfu/g microorganisms, and its pH value is 3-3.85.

Study Design and Calf Nutrition

In the study, 42 calves were divided into three groups

containing 14 calves with similar live weights (42±5 kg), ages (7±3 days), breeds (7 Holstein, 7 Simmental), and gender (7 female, 7 male) in each as a control group and two treatment groups (EM10 and EM15); Unlike the control group, the calves in the treatment groups were given either 10 mL of EM per calf per day or 15 mL of EM per calf per day via the oral route.

Calves of cows in 2nd and/or 3rd lactation were used in the study. All calves were fed from a bottle within the first 30 min after birth. In the study, all calves were given colostrum 8-10% of live weight (LW) in 3 meals. The calves were housed in individual compartments, and they all received pelleted calf starter feed (90%) and alfalfa hay (10%) mixed, and water *ad libitum* during the experiment. Calf starter feed (90%) and alfalfa hay (10%) mixed were given to the calves starting from the age of 10 days. A 5.8 L/day of full-fat milk (35°C) was given with a nursing bottle to each calf in all groups in two meals (at 8:00 and 18:00) during the 1st-30th days of the study, and during the 31st-70th days of the study, all the calves received 7.4 L/day of full-fat milk (35°C) in two meals.

All liquid and solid feeds given to the calves were weighed and recorded daily. Daily dry feed consumption was determined by collecting and weighing the remaining amounts of starter feed and alfalfa hay mix given to the calves every day. Total dry matter consumption was calculated from the sum of DM from milk and DM from dry feed (starter feed + alfalfa hay). The FCR was calculated by dividing the consumed total dry matter (DM) (milk DM + solid feed DM) to the total LWG of calves.

Health and Growth Records of Calves

The calves were individually weighed at the beginning (day 0), middle (day 30), and end (day 70) of the study on a scale with an accuracy of 0.1 kg, and their LWs were recorded. In the beginning and at the end of the study, the height at wither (WH), height at rump (RH), body depth (BD), chest circumference (CC), body length (BL), and rump width (RW) of all calves were measured individually before feeding using a tape measure and a measuring stick. The calves were observed for any disease symptoms (diarrhea, fever, etc.), and the treatment procedures and drugs used in the diseases were recorded during the study.

Determination of Volatile Fatty Acids in Rumen Fluid of Calves

At the end of the study, rumen fluids (approximately 2 hours after feeding) of 8 calves from each group were taken by a rumen tube. First 10 mL of rumen fluid discarded to minimize the saliva contamination and then collecting about 20 mL of rumen fluid (both solid and liquid fractions) for analysis. The rumen fluid was immediately brought to the laboratory in an aerobic environment and the thermos with the ice-bag into a falcon tube (50 mL volumetric) with screw cap to prevent volatile fatty acid loss. The concentrations (mmol/L) of volatile fatty acids (VFA's) (acetic, butyric, propionic, iso-butyric, valeric, hexanoic, iso-caproic, n-heptanoic, and iso-valeric acids) in the rumen fluids were identified in a GC-FID device (Thermo Trace 1300, Thermo Scientific, USA) with a polyethylene-glycol-based phase GC Column (Thermo ScientificTM, TRACE TR-WAX GC Column, USA) [31] using the Xcalibur™ software (Thermo Scientific™, USA).

Determination of Chemical Compositions of Starter Feed and Lucerne Hay and Milk

The feed samples were ground in a laboratory-type mill (IKA Werke, Germany) with a diameter of 1 mm. Dry matter (DM), diethyl ether extract (EE), crude protein (CP) (nitrogen x 6.25), and ash compositions of grounded samples were analyzed according to the AOAC [32]. The analyses of all these chemical compositions were carried out in triplicate. At the beginning of the experiment and regular intervals during the rest of the study (three-day intervals), analyses for nutrition and quality of the whole milk used in the experiment were carried out using a milk quality analyzer (Milkana® Superior Plus).

Economic Analyses

In the economic analysis, calf feeding (milk=$0.22/L; alfalfa hay=$0.11/kg; starter feed=$0.22/kg), treatment, and control expenditures were considered in the cost calculation. The calf price was assumed as $3.7/kg LW in the total income calculation (personal communication). Profitability was calculated by subtracting total cost from total income. A partial budget analysis applied for determining the effects of using EM in calf feeding. Partial budget analysis aimed to determine the positive or negative effects of change made in the production system. In the analysis, "Additional Income Increase" and "Decreased Costs" have a positive effect on the production system; "Decreased Income" and "Additional Costs" have a negative effect. The net income increase obtained as a result of the partial budget analysis was calculated with the help of the following formula;

Net Income = (Additional revenue increase + Reduced costs) – (Decreased revenue + Additional costs) [33].

Statistical Analyses

In the study, calf LWs, body sizes, solid and liquid DM amounts, rumen volatile fatty acid amounts, and the financial results were analyzed by using the One-Way ANOVA. Disease rates were evaluated with the chi-square test (SPSS, 22.0). Duncan's multiple range test was applied to determine the differences between the groups. Data were given in mean±standart error (X±Sx).

Results

The nutrient amounts of the milk, calf starter feed, and alfalfa hay consumed by the calves are given in *Table 1*.

The LW, LWG, FC, and FCR of the calves by the groups in the study are given in *Table 2*.

The use of 10 and 15 mL/day EM per animal in the study did not significantly affect the LW and LWG values on the 30th and 70th days (P>0.05). However, the highest LW and LWG values were found in the EM15 group. Although the DM consumption from liquid and solid feeds was highest in the EM15 group, no significant difference was determined (P>0.05). Compared to the control group, while the FCR was positively affected in the EM10 group (P<0.05), there was no difference in the EM15 group (P>0.05) for 0-30 days. On the other hand, there was no significant difference between the groups regarding FCR during days 31-70 and 0-70 (P>0.05; *Table 2*).

The body measurements (WH, RH, BL, BD, CC, RW) of the calves throughout the study are given in *Table 3*.

Supplementation of EM to calves in different amounts (10 mL-15 mL) did not significantly affect their body measurements (WH, RH, BL, BD, CC, RW) on the 70th day (P>0.05). However, it can be said that the changes in WH, BD, and BL (cm/day) were positively affected in the EM10 group *(Table 3)*.

The amounts and ratios of volatile fatty acids obtained from the rumen fluids of the calves at the end of the study are given in *Table 4*.

There was no statistical difference between the groups regarding the VFA rates in rumen fluids taken from calves at the end of the study (P>0.05). The highest ratios of acetic acid (51.0%) and propionic acid (30.8%) were found in the group given 10 mL/day of EM. The highest butyric acid ratio was found in the control group *(Table 4)*.

Disease rates in the calves throughout the study are given in *Table 5*.

Although there was no statistical difference between the groups regarding disease rates, the highest number of diseases was observed in the control group (P>0.05; *Table 5*).

Table 1. *Chemical compositions of starter feed and lucerne hay and milk*

% in DM	Starter Feed	Lucerne Hay	Milk
DM, % (feed basis)	91.35	92.86	12.76
CP	20.45	16.47	3.39
Ash	7.71	11.52	-
EE	4.08	3.40	-
CF	7.30	21.10	-
DM without fat	-	-	8.83
Fat	-	-	3.93

DM: *dry matter,* ***CP:*** *crude protein,* ***EE:*** *diethyl ether extract,* ***CF:*** *crude fiber*

Table 2. *Live weight, average daily gain, feed intake, feed efficiency of calves treated with or without probiotic (EM) during the first 70 days*

Parameter	Days	Control (X±Sx)	EM10 (X±Sx)	EM15 (X±Sx)	P
Total DM intake*g/day	0-30 days	319.4±42.8	222.4±23.2	332.6±42.7	0.081
	31-70 days	1126.7±114.5	1026.0±80.2	1226.1±104.0	0.391
	0-70 days	947.2±96.7	847.4±65.4	1027.5±88.9	0.339
LW, kg	0. day	41.8±1.4	42.7±1.1	42.3±1.8	0.912
	30. day	55.8±2.3	57.9±1.5	55.7±2.6	0.719
	70. day	93.5±4.8	94.2±2.7	97.1±4.3	0.801
LWG, g/calf/day	0-30 days	465.6±38.7	506.9±36.7	520.3±82.4	0.755
	31-70 days	944.0±65.9	908.6±48.7	1071.5±45.7	0.115
	0-70 days	738.9±51.5	736.4±36.9	835.2±47.7	0.251
FCR (g feed DM/g live weight gain)	0-30 days	0.69±0.06^{ab}	0.46±0.055^{a}	0.89±0.20^{b}	**0.044***
	31-70 days	1.17±0.07	1.15±0.09	1.16±0.10	0.983
	0-70 days	1.04±0.06	0.93±0.06	1.08±0.13	0.439

** Dry matter consumption from milk, g/calf/day + dry matter consumption from starter feed, g/calf/day + dry matter consumption from roughage, g/calf/day*

Table 3. *Body measurements of calves treated with or without probiotic (EM) during the first 70 days*

Body Measurements	Control (X±Sx)	EM10 (X±Sx)	EM15 (X±Sx)	P
Withers height on day 0, cm	76.6±0.8	77.2±0.7	75.4±1.5	0.443
Withers height on day 70, cm	89.9±1.2	92.1±1.0	88.6±1.3	0.080
Change of withers height (cm/day)	0.18±0.01	0.21±0.01	0.19±0.02	0.172
Rump height on day 0, cm	80.1±0.8	81.1±0.8	78.9±1.6	0.352
Rump height on day 70, cm	93.2±1.1	96.1±1.0	94.1±1.3	0.192
Change of rump height (cm/day)	0.19±0.04	0.21±0.01	0.22±0.02	0.213
Body depth at day 0, cm	31.7±0.5	31.4±0.3	31.8±0.5	0.855
Body depth at day 70, cm	42.5±0.6	42.9±0.5	42.0±0.5	0.519
Change of body depth (cm/day)	0.16±0.0	0.17±0.01	0.15±0.01	0.165
Chest circumference at day 0, cm	80.0±1.1	80.4±0.6	79.3±1.4	0.736
Chest circumference at day 70, cm	104.1±1.5	104.8±0.9	103.2±1.5	0.698
Change of chest circumference (cm/day)	0.35±0.01	0.35±0.01	0.34±0.01	0.918
Body length at day 0, cm	71.6±1.2	69.6±1.3	71.5±1.6	0.513
Body length at day 70, cm	89.6±1.1	88.4±0.9	89.8±1.6	0.689
Change of body length (cm/day)	0.26±0.01	0.27±0.02	0.26±0.02	0.912
Rump width on day 0, cm	23.6±0.4	23.2±0.4	23.5±0.5	0.768
Rump width on day 70, cm	26.9±0.4	27.6±0.3	27.4±0.6	0.463
Change of rump width (cm/day)	0.05±0.006	0.06±0.007	0.06±0.007	0.140

Table 4. *Rumen volatile fatty acids (VFA) amounts and ratios*

Volatile Fatty Acids	Control (X±Sx)	EM10 (X±Sx)	EM15 (X±Sx)	P
VFA, mmol/L	28.5±4.4	49.7±9.3	36.1±7.7	0.149
Individually volatile fatty acids as % in VFA				
Acetic acid	50.2±0.5	51.0±1.6	49.9±1.2	0.803
Propionic acid	30.0±1.4	30.8±1.8	30.3±1.4	0.938
Butyric acid	10.0±0.7	9.9±0.8	8.8±1.6	0.452
Valeric acid	3.8±0.4	3.3±0.3	4.0±0.4	0.407
iso-butyric acid	1.4±0.2	1.3±0.3	1.6±0.3	0.713
iso-valeric acid	1.5±0.2	1.4±0.4	2.0±0.3	0.359
Hexanoic acid	1.4±0.1	1.3±0.3	1.7±0.4	0.558
iso-caproic	0.78±0.2	0.26±0.1	0.53±0.3	0.199
n-heptanoic acid	0.92±0.1	0.73±0.2	1.07±0.3	0.565

VFA: *Total volatile fatty acids*

Table 5. *Disease rates in calves*

Groups	Preweaned Period			
	0-30 days		31-70 days	
	Positive	Negative	Positive	Negative
Control (n=14)	10 (%50)	4 (18.2%)	1 (33.3%)	13 (33.3%)
EM10 (n=14)	4 (%20)	10 (45.5%)	2 (66.7%)	12 (30.8%)
EM15 (n=14)	6 (%30)	8 (36.4%)	0 (0%)	14 (35.9%)
Total	20 (%100)	22 (100%)	3 (100%)	39 (100%)
Statistical values	N=42, χ^2 = 5.35, Sd=2, P =0.069		N=42, χ^2 = 2.15, Sd=2, P =0.341	

Table 6. *Nutrition, treatment and disease control costs of calves*

Cost Elements	Preweaned Period								
	0-30 days			31-70 days			Total (0-70 days)		
	Control	EM10	EM15	Control	EM10	EM15	Control	EM10	EM15
1. Nutrition	40.9	40.9	42.0	76.0	75.9	78.2	116.9	116.9	120.2
Milk	38.7	38.7	38.7	65.6	65.6	65.6	104.3	104.3	104.3
Feed	2.2	1.5	2.3	10.4	9.5	11.3	12.6	11.0	13.6
EM	-	0.7	1.0	-	0.9	1.3	-	1.6	2.3
2. Treatment	4.2	2.5	1.8	0.2	0.3	0.0	4.4	2.7	1.8
Drug	3.4	2.1	1.5	0.2	0.2	0.0	3.6	2.3	1.5
Labor	0.2	0.1	0.1	0.01	0.02	0.0	0.2	0.1	0.1
Veterinary	0.6	0.3	0.2	0.02	0.04	0.0	0.6	0.3	0.2
3. Control Expenditure	7.7	7.7	7.7	3.6	3.6	3.6	11.3	11.3	11.3
Total cost (1+2+3) (X±Sx)	52.8±1.4	51.1±1.2	51.5±1.0	79.8±1.0	79.8±0.7	81.8±0.8	132.6±1.8	130.9±1.4	133.3±1.5
P	0.576			0.162			0.547		

$=13.5 TRY

Table 7. *Economic reflection of use of EM in calf feeding*

Groups	Total Cost (X±Sx)	Total Income (X±Sx)	Profit (X±Sx)
Control	132.6±1.8	346.0±17.6	213.4±16.9
EM10	130.9±1.4	348.6±9.9	217.7±10.1
EM15	133.3±1.5	359.3±15.9	226.0±15.8
P	0.547	0.801	0.827

$=13.5 TRY

The costs of feeding, treatment, and disease control throughout the study are given in *Table 6*.

According to the study's findings, there was no statistically significant difference between the groups regarding total cost between days 0-30, 31-70, and 0-70 (P>0.05). In addition, when a comparison of feeding-related costs was made throughout the study, the highest cost ($120.2/calf) was that of the EM15 group. When the groups were compared in terms of treatment costs, the cost of treatment in the period covering 0-30 days, which had the highest disease rate, was higher than that of the cost of the period covering 31-70 days. In terms of the groups, the treatment cost was higher in the control group ($4.4/calf) than in the treatment groups. It was calculated that calf feeding cost alone constituted 88-90% of the total cost *(Table 6)*.

The results of the economic analysis (total cost, total income, and profit) of the study groups are given in *Table 7*.

According to *Table 7*, there was no statistical difference between the groups in terms of the total cost, total income, and profit (P>0.05). However, the total cost was calculated to be the lowest in the EM10 group ($130.9/calf) and the highest in the EM15 group ($133.3/calf), while the highest income ($359.3/calf) and profit ($226.0/calf) were seen in the EM15 group.

Discussion

In recent years, studies on the use of probiotics have increased for purposes such as; increasing calf feeding performance, reducing mortality, and improving intestinal health. In this study, although the LW and LWG increased numerically at the end of the study in calves given probiotics at different amounts (10-15 mL/day), they were not significantly affected. This finding was consistent with the results of the studies, which reported that probiotics positively but not significantly affected LW in calves [24,34-37]. However, it was determined that the daily use of 10 mL and 15 mL of probiotics in calf feeding increased profitability by 2% and 6%, respectively.

In contrast to the findings of this study, Gryazneva et al.[38] claimed that probiotic application consisting of *Lactobacillus* strains significantly increased the end-of-experiment LW in calves. Timmerman et al.[24] reported that veal calves, when fed with milk substitute feed with

probiotics, showed an increase in LW gain at one week old but showed limited beneficial effects during the first two weeks of life. The lack of the effect of the EM additive used in this study on LW and LWG or obtaining mixed results from some literature findings may support the view that the effects of probiotics are directly related to the type and dose of probiotic strain consumed by the calves, the feed consumed by the calves, the duration of the probiotics supplementation as well as the age and the rearing system of the calves.

A probiotic function may be associated with improved feed efficiency, especially in diets containing a high proportion of dry matter such as grain and forage [36], which positively affect ruminal development.

Similar to the results of the studies reporting that probiotics improve the FCR of calves [24,34,35,39], in this study, the FCR of calves receiving 10 mL/day EM for the first 30 days of their lives improved significantly. However, it was determined that EM consumption did not substantially affect FCR in the following periods (days 31-70). This finding supports the view that probiotics are most effective on calves in the neonatal period. In this study, compared to the control and EM10 groups, the total DM consumption from liquid and solid feeds was also numerically higher in the EM15 group, in which the highest LW and LWG values were observed. This study determined that feeding with EM did not significantly affect DM consumption in calves. This finding has supported the results of previous studies reporting no effects of probiotics on DM consumption [40-42]. On the other hand, Ruppert et al.[43] reported that probiotics increased the FC between the 2nd and 28th days.

Giving 10 and 15 mL/day of EM to calves up until their weaning slightly but not significantly increased their body measurements (withers height, rump height, body depth, chest circumference, body length, rump width) when compared to the control group. These findings have supported the results of studies reporting that probiotics do not significantly affect body measurements [36,44]. However, some studies reported that probiotics affected calves' CC [39,45], WH [37,45-47] and BL [45,47] developments positively.

Gastro-intestinal and respiratory diseases are the two main causes of calf mortality in early life. Gastrointestinal diseases, which are common in intensive breeding systems due to intestinal microbial imbalances, are among the most important factors affecting the growth and development of calves in the first few weeks of their lives, and thus the performance of calves in their later years and the financial status of the enterprises [36,48,49]. Producers are at great risk of sustaining significant direct and indirect economic losses due to negative effects on calf health and productivity and the investment in therapeutics [21]. Calves are particularly susceptible to intestinal infectious diseases in the first postpartum period and diarrhea, among other health problems, poses a significant risk. The use of probiotics in this period has been a frequently used tool in recent years to maintain the intestinal microbial balance and prevent the formation of opportunistic pathogenic bacterial populations [36,41].

It has been noted that probiotics reduce intestinal pH with the organic acids they secrete, stimulate the hydrogen peroxide and lactoperoxidase thiocyanate system, which have a bactericidal effect, thus preventing the increasing of *E. coli* that cannot grow in a neutral and acidic environment [50]. It has been reported that the use of probiotics prevents pathogen colonization in the digestive tract [51] or significantly reduces the prevalence of diarrhea in young calves [52].

Despite the lack of statistically significant differences between the groups regarding disease rates in the groups given EM, a slight decrease in diarrhea cases and tendency of improvements in the general health status of animals were observed in the groups receiving EM. In addition to this, it can be said that the use of EM reduces the treatment costs from $4.4 (control group) to $2.7 (EM10) and $1.8 (EM15). It is thought that this will also positively affect the future performances of the calves.

In some studies, similar to the current study, it has been reported that probiotics have a positive effect on intestinal health and at the same time reduce the severity, duration, and adverse effects of digestive system diseases such as diarrhea, which is a significant cause of mortality [25,36,42,53-56]. Isık et al.[35] reported in their study that diarrhea was not observed in the group given probiotics, but that it was observed in the control group. Diler and Aydın [46], in their study, detected a decrease in the rate of diarrhea in the treatment groups in comparison with the control group. Signorini et al.[53] also reported a significant reduction in gastrointestinal diseases with probiotic supplementation. On the other hand, studies report that probiotics are not effective on the disease rate in calves [36,41].

It is believed that supporting the growth of calves in this first period of their lives will significantly affect their fertility and fertility performance in the future. Thus, this improvement in the performance provided by probiotics will contribute to improving the production and economic indexes of the farms [11-16].

During the liquid feeding period of calves after birth, rumen fermentation is stimulated by providing concentrated feed (calf starter feed) with high starch and protein digestibility. It is aimed to provide rumen fermentation (feed digestion and microorganism flora) as in adult ruminants [57]. The effectiveness of rumen fluid VFA concentration in the pre-

weaning period on rumen development varies according to the diet consumed [58]. It is stated that the use of lactic acid producing *Streptococcus bovis* and *Lactobacillus* together with lactate-using *Probionibacterium acnes* or *Aspergillus oryzae* increases rumen papillae development and VFA production [50].

In this study, percentages of acetate, propionate, butyrate, iso-butyrate, iso-valerate, and valerate in VFA and molarity of VFA of rumen fluid level in calves fed with milk + dry feed before weaning was like the findings of previous studies [59]. In the present study, the fact that the addition of probiotics to milk in pre-weaned calf does not change the individual volatile fatty acids percentages in VFA during the milk feeding period may be due to the content of the consumed feed probiotic dose or environmental factors. Unlike the current study, in the study conducted by Windschitl [39], it was determined that probiotics increased the rate of VFA in the rumen. The present study determined that the molarity of VFA in the rumen fluid of calves consuming probiotics increased numerically. This result shows that probiotic supplementation may positively affect feed fermentation in the rumen. Adding probiotics to the milk of calves during the milk-drinking period (numerically; 28.5±4.4 vs. 49.7±9.3 and 36.1±7.7) positively impacts the molarity of volatile fatty acids in the rumen fluid taken at the time of weaning; commercial probiotics additive shows that the bacteria in its content will have the potential to increase rumen fermentation.

In conclusion, the results of this study have revealed that although the use of additional probiotics in the pre-weaning period does not affect some performance parameters (LW, LWG, FC, WH, RH, BL, BD, CC, RW) in calf feeding, it can be suggested that it has a potential to positively affect the molarity of volatile fatty acids in the calf rumen and in 0-30 days it has significantly improved FCR. Additionally, EM slightly decrease the disease rates and treatment costs.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author (S. Sarıözkan).

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

MK: Investigation, collected the data, analyzing, writing. VÖ: Designed the study material, collected the data. SS: designed the research, analyzing, supervision, reviewing and editing. BKG: Analyzing, supervision, reviewing and writing. KK: Analyzing, editing and writing.

References

1. Ayvazoğlu Demir P, Aydın E, Ayvazoğlu C: Estimation of the economic losses related to calf mortalities Kars Province, in Turkey. *Kafkas Univ Vet Fak Derg,* 25 (3): 283-290, 2019. DOI: 10.9775/kvfd.2018.20471

2. Aydoğdu M: Probiyotik kullanmanın süt emen simental buzağılarda performans ve sağlığı üzerine etkileri. *Yüksek Lisans Tezi,* Kırıkkale Üniv. Sağlık Bil Enst., 2017.

3. Akyüz E, Naseri A, Erkılıç EE, Makav M, Uzlu E, Kırmızıgül AH, Gökce G: Neonatal buzaği ishalleri ve sepsis. *KAUFBED*, 10 (2): 181-191, 2017.

4. Yüzbaşıoğlu R: Tokat ili merkez ilçesindeki buzağı yetiştiricilerinin sorunları. *GBAD*, 10 (3): 111-118, 2021.

5. Karakaş E: Bursa-Yenişehir ilçesinde yetiştirilen Holştayn buzağıların doğum ağırlığı, sütten kesim yaşı, süt tüketimleri ve yaşama güçleri. *Uludag Univ J Fac Vet Med*, 21, 77-81, 2002.

6. Verstegena MWA, Williamsa BA: Alternatives to the use of antibiotics as growth promoters for monogastric animals. *Anim Biotechnol,* 13 (1): 113-127, 2002. DOI: 10.1081/ABIO-120005774

7. Jouany JP, Morgavi DP: Use of 'natural' products as alternatives to antibiotic feed additives in ruminant production. *Animal,* 1 (10): 1443-1466, 2007. DOI: 10.1017/S1751731107000742

8. Oikonomou G, Teixeira AGV, Foditsch C, Bicalho ML, Machado VS, Bicalho RC: Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of *Faecalibacterium* species with health and growth. *Plos One,* 8 (4): e63157, 2013. DOI: 10.1371/journal.pone.0063157

9. Jiang X, Xu HJ, Cui ZQ, Zhang YG: Effects of supplementation with *Lactobacillus plantarum* 299v on the performance, blood metabolites, rumen fermentation and bacterial communities of preweaning calves. *Livest Sci,* 239, 104-120, 2020. DOI: 10.1016/j.livsci.2020.104120

10. Ewaschuk JB, Naylor JM, Chirino-Trejo M, Zello GA: *Lactobacillus rhamnosus* strain GG is a potential probiotic for calves. *Can J Vet Res*, 68 (4): 249, 2004.

11. Da Silva Abreu B, Pires LC, Dos Santos KR, Luz CSM, De Oliveira MRA, De Sousa Júnior SC: Occurrence of *Cryptosporidium* spp. and its association with ponderal development and diarrhea episodes in nellore mixed breed cattle. *Acta Vet Bras*, 13 (1): 24-29, 2019. DOI: 10.21708/avb.2019.13.1.7977

12. Gulliksen SM, Lie KI, Løken T, Østerås O: Calf mortality in Norwegian dairy herds. *J Dairy Sci*, 92 (6): 2782-2795, 2009. DOI: 10.3168/jds.2008-1807

13. Klein P, Kleinova T, Volek Z, Simunek J: Effect of *Cryptosporidium parvum* infection on the absorptive capacity and paracellular permeability of the small intestine in neonatal calves. *Vet Parasitol*, 152, 53-59, 2008. DOI: 10.1016/j.vetpar.2007.11.020

14. Pardon B, Hostens M, Duchateau L, Dewulf J, De Bleecker K, Deprez P: Impact of respiratory disease, diarrhea, otitis and arthritis on mortality and carcass traits in white veal calves. *BMC Vet Res,* 9:79, 2013. DOI: 10.1186/1746-6148-9-79

15. Soberon F, Raffrenato E, Everett RW, Van Amburgh ME: Preweaning milk replacer intake and effects on long-term productivity of dairy calves. *J Dairy Sci*, 95, 783-793, 2012. DOI: 10.3168/jds.2011-4391

16. Soberon, F, Van Amburgh ME: Lactation Biology Symposium: The effect of nutrient intake from milk or milk replacer of preweaned dairy calves on lactation milk yield as adults: A meta-analysis of current data. *J Anim Sci,* 91 (2): 706-712, 2013. DOI: 10.2527/jas.2012-5834.

17. Morrill JL, Dayton AD, Mickelsen R: Cultured milk and antibiotics for

young calves. *J Dairy Sci*, 60 (7): 1105-1109, 1976. DOI: 10.3168/jds.S0022-0302(77)83995-7

18. Donovan DC, Franklin ST, Chase CCL, Hippen AR: Growth and health of holstein calves fed milk replacers supplemented with antibiotics or enteroguard. *J Dairy Sci*, 85 (4): 947-950, 2002. DOI: 10.3168/jds.S0022-0302(02)74153-2

19. Cangiano LR, Yohe TT, Steele MA, Renaud DL: Invited review: Strategic use of microbial-based probiotics and prebiotics in dairy calf rearing. *Appl Anim Sci,* 36, 630-651, 2020. DOI: 10.15232/aas.2020-02049

20. FernándezCiganda S, Fraga M, Zunino P: Probiotic lactobacilli administration induces changes in the fecal microbiota of preweaned dairy calves. *Probiotics Antimicrob Proeinst*, 2021:2021. DOI: 10.1007/s12602-021-09834-z

21. Alawneh JI, Barreto MO, Moore RJ, Soust M, Al-Harbi H, James AS, Krishnan D, Olchowy TWJ: Systematic review of an intervention: The use of probiotics to improve health and productivity of calves. *Prev Vet Med,* 183:105147, 2020. DOI: 10.1016/j.prevetmed.2020.105147

22. Agazzi A, Tirloni E, Stella S, Maroccolo S, Ripamonti B, Bersani C, Caputo JM, Dell'Orto V, Rota N, Savoini G: Effects of species-specific probiotic addition to milk replacer on calf health and performance during the first month of life. *Ann Anim Sci,* 14 (1): 101-115, 2014. DOI: 10.2478/aoas-2013-0089

23. Burçak E, Yalçın S: Buzağı beslemede probiyotiklerin kullanımı. *Lalahan Hay Araşt Enst Derg,* 53 (2): 101-114, 2013.

24. Timmerman HM, Mulder L, Everts H, Van Espen DC, Van Der Wal E, Klaassen G, Rouwers SMG, Hartemink R, Rombouts FM, Beynen AC: Health and growth of veal calves fed milk replacers with or without probiotics. *J Dairy Sci*, 88 (6): 2154-2165, 2005. DOI: 10.3168/jds.S0022-0302(05)72891-5

25. Jatkauskas J, Vrotniakiene V: Effects of probiotic dietary supplementation on diarrhoea patterns, faecal microbiota and performance of early weaned calves. *Vet Med,* 55 (10): 494-503, 2010. DOI: 10.17221/2939-VETMED

26. Zábranský L, Poborská A, Malá G, Gálik B, Kantor M, Hanušovský O, Petrášková E, Kernerová N, Kučera J: Probiotic and prebiotic feed additives in calf nutrition. *J Cent Eur Agric,* 22 (1): 14-18, 2021. DOI: 10.5513/JCEA01/22.1.3057

27. Ülger İ: Effects of pre-weaning probiotic treatments on growth performance and biochemical blood parameters of Holstein calves. *Indian J Anim Res*, 53 (5): 644-647, 2019. DOI: 10.18805/ijar.B-816

28. Wu Y, Wang L, Luo R, Chen H, Nie C, Niu J, Chen C, Xu Y, Li X, Zhang W: Effect of a multispecies probiotic mixture on the growth and incidence of diarrhea, immune function, and fecal microbiota of pre-weaning dairy calves. *Front Microbiol,* 12:681014, 2021. DOI: 10.3389/fmicb.2021.681014

29. Jenny BF, Vandijk HJ, Collins JA: Performance and fecal flora of calves fed a *Bacillus subtilis* concentrate. *J Dairy Sci,* 74, 1968-1973, 1991. DOI: 10.3168/jds.S0022-0302(91)78364-1

30. Abu-Tarboush HM, Al-Saiady MY, Keir El-Din AH: Evaluation of diet containing Lactobacilli on performance, fecal coliform, and Lactobacilli of young milk calves. *Anim Feed Sci Technol,* 57, 39-49, 1996. DOI: 10.1016/0377-8401(95)00850-0

31. Ersahince AC, Kara K: Nutrient composition and *in vitro* digestion parameters of Jerusalem artichoke *(Helianthus tuberosus* L.) herbage at different maturity stages in horse and ruminant. *J Anim Feed Sci,* 26 (3): 213-225, 2017. DOI: 10.22358/jafs/76477/2017

32. AOAC: Official methods of analysis of the association of official analytical chemists. 15th Ed., Arlington, VA (USA), 1990.

33. Kara K, Sariözkan S, Konca Y, Güçlü BK: Bıldırcın *(Coturnix coturnix japonica)* karma yemlerine humat ilavesinin besi performansı ve gelire etkisi. *Vet Hekim Der Derg,* 83 (2): 17-24, 2012.

34. Zhang R, Zhou M, Tu Y, Zhang NF, Deng KD, Ma T, Diao QY: Effect of oral administration of probiotics on growth performance, apparent nutrient digestibility and stress-related indicators in Holstein calves. *J Anim Physiol Anim Nutr*, 100 (1): 33-38, 2016. DOI: 10.1111/jpn.12338

35. Işık M, Ekimler F, Özen N, Fırat MZ: Effects of using probiotics on the growth performance and health of dairy calves. *Turk J Vet Anim Sci*, 28 (1): 63-69, 2004.

36. Frizzo LS, Soto LP, Zbrun MV, Bertozzi E, Sequeira G, Armesto RR, Rosmini MR: Lactic acid bacteria to improve growth performance in young calves fed milk replacer and spray-dried whey powder. *Anim Feed Sci Technol,* 157 (3-4): 159-167, 2010. DOI: 10.1016/j.anifeedsci.2010.03.005

37. Bayatkouhsar J, Tahmasebi AM, Naserian AA, Mokarram RR, Valizadeh R: Effects of supplementation of lactic acid bacteria on growth performance, blood metabolites and fecal coliform and lactobacilli of young dairy calves. *Anim Feed Sci Technol,* 186 (1-2): 1-11, 2013. DOI: 10.1016/j.anifeedsci.2013.04.015

38. Gryazneva TN, Pavlova IB, Varonin ES: Biological methods of correction of microbial flora of ıntestines in calves. *Veterinariya-Moskva,* 7, 23-24, 1991.

39. Windschitl PM: Effect of probiotic supplementation on growth rate, rumen metabolism, and nutrient digestibility in holstein heifer calves. *Asian-Australas J Anim Sci,* 4 (4): 341-351, 1991. DOI: 10.5713/ajas.1991.341

40. Büyükkılıç Beyzi S: Buzağılarda yem katkı maddesi olarak probiyotiğin performans ve dışkı karakteristikleri üzerine etkileri. *Yüksek Lisans Tezi,* Ankara Üniv. Sağlık Bil. Enst., 2012.

41. Cruywagen CW, Jordaan I, Venter L: Effect of *Lactobacillus acidophilus* supplementation of milk replacer on preweaning performance of calves. *J Dairy Sci,* 79 (3): 483-486, 1996. DOI: 10.3168/jds.S0022-0302(96)76389-0

42. Görgülü M: Effect of probiotic on growing performance and health of calves. *Pak J Biol Sci,* 6 (7): 651-654, 2003.

43. Ruppert LD, McCoy GC, Bower NR, Hotjens MF: Probiotic suplemented calf diets. http//www.Traill.Uiuc.edu/dairynet; *Accessed:* 03.01.2022.

44. Alıç Ural D, Erdoğan S, Erdoğan H, Ural K: Effect of probiotic supplementation on some body measurement. *J Adv Vetbio Sci Tech,* 5 (2): 48-56, 2020. DOI: 10.31797/vetbio.696300

45. Nehru Arun P, Sunandhadevi S, Rama T, Muniyappan N: Effect of probiotic supplementation on growth performance of crossbred calves in an organized cattle farm. *J Anim Health Prod,* 5 (3): 89-91, 2017. DOI: 10.17582/journal.jahp/2017/5.3.89.91

46. Diler A, Aydın R: Rasyona probiyotik-enzim kombinasyonu ilavesinin İsviçre Esmeri ırkı buzağılarda büyüme performansı ve yemden yararlanma ve sağlık üzerine etkileri. *Hay Üret,* 50 (2): 22-28, 2009.

47. Noori M, Alikhani M, Jahanian R: Effect of partial substitution of milk with probiotic yogurt of different pH on performance, body conformation and blood biochemical parameters of Holstein calves. *J Appl Anim Res,* 44 (1): 221-229, 2016. DOI: 10.1080/09712119.2015.1031772

48. Murray CF, Fick LJ, Pajor EA, Barkema HW, Jelinski MD, Windeyer MC: Calf management practices and associations with herd-level morbidity and mortality on beef cow-calf operations. *Animal,* 10 (3): 468-477, 2016. DOI: 10.1017/S1751731115002062

49. Stanton AL, Kelton DF, LeBlanc SJ, Wormuth J, Leslie KE: The effect of respiratory disease and a preventative antibiotic treatment on growth, survival, age at first calving, and milk production of dairy heifers. *J Dairy Sci,* 95 (9): 4950-4960, 2012. DOI: 10.3168/jds.2011-5067

50. Kocaoğlu Güçlü B, Kara K: Ruminant beslemede alternatif yem katkı maddelerinin kullanımı: 1. Probiyotik, prebiyotik ve enzim. *Erciyes Üniv Vet Fak Derg,* 6 (1): 65-75, 2009.

51. Fuller R: Probiotics in man and animals. *J Appl Bacteriol,* 66, 365-378, 1989. DOI: 10.1111/j.1365-2672.1989.tb05105.x

52. Abe F, Ishibashi N, Shimamura S: Effect of administration of Bifidobacteria and lactic acid bacteria to newborn calves and piglets. *J Dairy Sci,* 78, 2838-2846, 1995. DOI: 10.3168/jds.S0022-0302(95)76914-4

53. Signorini ML, Soto LP, Zbrun MV, Sequeira GJ, Rosmini MR, Frizzo LS: Impact of probiotic administration on the health and fecal microbiota of young calves: A meta-analysis of randomized controlled trials of lactic acid bacteria. *Res Vet Sci,* 93 (1): 250-258, 2012. DOI: 10.1016/j.rvsc.2011.05.001

54. LeJeune JT, Wetzel AN: Preharvest control of *Escherichia coli* O157 in cattle. *J Anim Sci,* 85, 73-80, 2007. DOI: 10.2527/jas.2006-612

55. Morrison SJ, Dawson S, Carson AF: The effects of mannan oligosaccharide and *Streptococcus faecium* addition to milk replacer on calf health and performance. *Livest Sci,* 131 (2-3): 292-296, 2010. DOI: 10.1016/j.livsci.2010.04.002

56. Riddell JB, Gallegos AJ, Harmon DL, McLeod KR: Addition of a *Bacillus* based probiotic to the diet of preruminant calves: Influence on growth, health, and blood parameters. *Int J Appl Res Vet Med,* 8 (1): 78-85, 2010.

57. National Research Council (NRC): Nutrient Requirements of Dairy Cattle. *Seventh rev ed.,* Washington, USA, 2001.

58. Yohe TT, Schramm H, White RR, Hanigan MD, Parsons CLM, Tucker HLM, Enger BD, Hardy NR, Daniels KM: Form of calf diet and the rumen. II: Impact on volatile fatty acid absorption. *J Dairy Sci,* 102 (9): 8502-8512, 2019. DOI: 10.3168/jds.2019-16450

59. McCurdy DE, Wilkins KR, Hiltz RL, Moreland S, Klanderman K, Laarman AH: Effects of supplemental butyrate and weaning on rumen fermentation in Holstein calves. *J Dairy Sci,* 102 (10): 8874-8882, 2019. DOI: 10.3168/jds.2019-16652

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