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RESEARCH ARTICLE

Effects of Earthworm Antimicrobial Peptides and Probiotics on Intestinal Flora of Yellow-feathered Broilers

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Abstract: Earthworm antimicrobial peptides combined with probiotics were supplemented to the yellow-feathered broiler diet, and the cecal intestinal flora was subjected to 16S rDNA Qualcomm sequencing. The results showed that the total number of species in S1 (control group) was significantly higher than S2 (earthworm antimicrobial peptides) and S3 (Compound probiotic preparation), S4 (earthworm antibacterial peptide + composite probiotic preparation) (P<0.05), the colony structure of S1 is significantly different from S2, S3, and S4 (P<0.05). At the phylum level of each test group, the dominant bacterial groups were *Firmicutes*, *Bacteroides*, and *Proteobacteria*. Among them, S2, S3, and S4 were significantly higher than S1 in the relative abundance of *Firmicutes* and *Bacteroides* (P<0.05), the relative abundance of *Proteobacteria* was significantly lower than S1 (P<0.05). At the genus level, S2, S3, and S4 were significantly higher than S1 in the relative abundance of *Lactobacillus* (P<0.05), where S4 is higher than S3 (P>0.05). Earthworm antimicrobial peptides combined with compound probiotics can increase the number of beneficial bacteria and reduce the number of harmful bacteria to regulate intestinal flora, indicating that earthworm antimicrobial peptides combined with compound probiotics can be used as new green antibiotics in animal production.

Keywords: *Earthworm antibacterial peptides, Probiotic, 16S rDNA*

Topraksolucanı Antimikrobiyal Peptitleri ve Probiyotiklerinin Etlik Piliçlerin Bağırsak Florası Üzerine Etkileri

Öz: Etlik piliç rasyonlarına, topraksolucanı antimikrobiyal peptitleri ile birlikte probiyotikler ilave edildi ve sekum flora 16S rDNA Qualcomm sekanslamaya tabi tutuldu. Sonuçlar, S1'deki (kontrol grubu) toplam tür sayısının S2 (topraksolucanı antimikrobiyal peptit ilaveli), S3 (bileşik probiyotik preparatı ilaveli) ve S4'ten (topraksolucanı antibakteriyel peptid + kompozit probiyotik preparatı ilaveli) anlamlı derecede yüksek olduğunu (P<0.05) ve S1'in koloni yapısının S2, S3, S4'ten önemli ölçüde farklı olduğunu gösterdi (P<0.05). Her test grubunun filum seviyesinde, baskın bakteri grupları *Firmicutes*, *Bacteroides* ve *Proteobacteria* idi. Bunlar arasında, S2, S3 ve S4 gruplarında *Firmicutes* ve *Bacteroides*'in nispi yoğunlukları S1'den önemli ölçüde yüksekti (P<0.05), *Proteobacteria*'nın nispi yoğunluğu ise S1'den önemli ölçüde düşüktü (P<0.05). Cins düzeyinde, S2, S3 ve S4'ün nispi *Lactobacillus* yoğunluğu S1'den önemli ölçüde yüksekti (P<0.05), ancak S4'ün bu oranı S3'ten daha yüksekti (P>0.05). Farklı probiyotik türlerinin topraksolucanı antimikrobiyal peptitleri ile birlikte kullanımı, yararlı bakteri sayısını arttırabilir ve zararlı bakteri sayısını ise azaltarak bağırsak florasını düzenleyebilir. Bu da hayvansal üretimde, probiyotik karmaları ile topraksolucanı antimikrobiyal peptitlerinin bir arada yeni nesil antibiyotikler olarak kullanılabileceğinin göstergesidir.

Anahtar sözcükler: *Topraksolucanı antibakteriyel peptitleri, Probiyotik, 16S rDNA*

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Introduction

Since the discovery of penicillin in 1929 [1], antibiotics have become a protective shield for human health. However, with the widespread application of antibiotics in clinical practice, problems such as "superbacteria" and drug-resistant genes have emerged, especially the "abuse" of antibiotics in animal husbandry, which makes the problem of antibiotic resistance more and more serious. It poses a serious threat to human health and the development of animal husbandry, so it is urgent to seek alternative products.

Antimicrobial peptides refer to polypeptides with antibacterial activity in insects, which have strong alkaline, broad spectrum antibacterial activity and weak positive characteristics. Subsequently, antimicrobial peptides were also found in mammals such as pigs, cattle, sheep, and amphibians such as *Xenopus laevis*. Although different antimicrobial peptides from different sources have the same antibacterial effect, different antimicrobial peptides have different antibacterial effects, different bactericidal activities and different antibacterial spectrum, they all have research effects. In addition, from the known antibacterial peptides, antibacterial peptides with different protein structures have different antibacterial effects and mechanisms. Since this kind of active peptides have a wide spectrum and high bactericidal activity to bacteria, they are named "antibacterial peptides" [2-4].

Probiotics are active microorganisms beneficial to the host by colonizing the body and changing the composition of a specific part of the host flora. By regulating the immune function of the host mucosa and the system, or by regulating the balance of intestinal flora, promoting nutrient absorption to maintain intestinal health, thus producing single microorganisms or mixed microorganisms with precise composition in favor of health, also known as probiotics or compound microecological preparation [5,6].

In animal breeding, intestinal health plays an important role in the prevention and control of pathogenic microorganisms and the digestion and utilization of feed [7,8].Digestive system not only is a site for digestion and absorption of dietary nutrients, but also provides protection against pathogens and toxins and has a large microbiome and immune cells [9]. The microbiota in the gastrointestinal tract is associated with a broad range of functions within the host, including the fermentation of complex macronutrients, nutrient and vitamin production, cellulose fermentation, protection from pathogens, maintenance of the balance of the immune system, and physiological metabolism in distal organs or tissues [10-13]. When the body is affected by some abnormal factors, intestinal barrier damage, bacteria, and other pathogenic agents can enter, engraftment in the intestinal tract through the blood circulation to achieve internal organs organ enteritis causes a series of reactions and systemic infection, cause severe infection situation, bring irreparable economic benefits to farms. Intestinal tract is not only the main place for nutrient digestion and absorption, but also has a very important defense function. Intestinal environmental imbalance will lead to a series of intestinal diseases. Increased intestinal bacteria and toxins with the probability of intestinal infection, inflammation and other problems hinder the digestion and absorption of nutrients, resulting in reduced performance and even death of animals. In order to maintain animal health and improve the quality of products, digestion, absorption and synthesis of nutrients in poultry are often promoted by improving the dietary ratio. Antimicrobial peptides (AMP) are a family of peptides that exhibit a range of antimicrobial activities. Studies have found that through isolation of key growth nutrients, penetration of bacterial membranes, and other related mechanisms have been identified as key regulators of interactions between symbiotic microorganisms and host tissues [14]. Both antimicrobial peptides and probiotics could regulate the intestinal flora of animals. AMPs exhibit a broad spectrum of antimicrobial activity and inhibit microbial cells by interacting with their membranes or by other mechanisms, such as inhibition of cell-wall synthesis or suppression of nucleic acid or protein synthesis [15]. In prior analyses, we have found that cathelicidin-WA can enhance the barrier function of the intestinal epithelium, protecting hosts from enterohemorrhagic *E. coli* O157:H7 infection [16]. The results suggest that probiotics-feeding may enhance the immunodefense system mediated by AvBDs but not by cytokine, against infection by Gram-negative bacteria [17]. Further, the data showed that SGAMP could effectively enhance the contents of IEL, mast cells, and goblet cells in the intestine [16]. All the above studies showed that dietary antimicrobial peptides or probiotics can improve the changes of intestinal microbial environment of animals, 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 [18].

Therefore, this experiment intends to add earthworm antimicrobial peptides and probiotics to the yellow-feathered broiler's diet. Through 16S rDNA high-throughput sequencing of the contents of the cecum of chickens, the results of the sequencing are used to analyze the effects of earthworm antimicrobial peptides combined with probiotics on the cecum of yellow-feathered broilers. To explore whether the combined effect of the two is better than the effect of a single addition, and to provide a theoretical basis for the application of earthworm antimicrobial peptides in the poultry breeding industry, the current study was performed [19,20].

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.

Animal Feeding Experiment and Management

Two hundred and forty healthy 1-day-old yellow-feathered broilers with similar body weight were purchased from a local hatchery and randomly divided into 4 treatment groups with 6 replicates in each treatment and 10 broilers in each replicate. The treatments were basal diet group (S1 group), basal diet +100 mg/kg earthworm antimicrobial peptide group (S2 group), basal diet +200 mg/kg compound probiotics group (S3 group), basal diet +100 mg/kg earthworm antimicrobial peptide +200 mg/kg compound probiotics group (S4 group). Corn-soybean meal diet was used in the experiment, and the basal diet was prepared according to the NRC (1994) broiler nutritional standard, and its composition and nutritional level were listed in *Table 1*. The composition and nutrient level of the basal diet are shown in *Table 1*. Throughout the entire study, the indoor temperature for chickens was monitored at constant temperature. It was 32~35°C on the first day, then gradually decreased and remained at 22°C for the last two weeks. According to the commercial conditions, the implementation of the artificial lighting scheme of 23 h of all-day lighting. 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 64 days. The activity unit was 100 mg/kg, and the compound probiotic preparations (1000 mg/kg *yeast*, 200 mg/kg *Lactobacillus*, 500 mg/kg *Bacillus subtilis*) were purchased from Shaanxi Longzhou Biological Co., Ltd. (China).

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

Items	Content, %
Ingredients	
Corn	56.32
Soybean meal	34.80
Soybean oil	4.00
limestone	1.00
mountain flour	1.50
$CaHPO_4$	1.80
NaCL	0.32
L - lysine	0.10
DL - lysine	0.16
Total	100
Nutrient levels	
ME/(MJ/Kg)	12.88
CP	21.80
Ca	0.90
AP	0.44
Lys	1.14
Met	0.5

Composite premix: Cu: 8 mg, Fe: 100.0 mg, Mn: 120.0 mg, I: 0.7 mg, Se: 0.35 mg, Zn: 100 mg per kg; Multivitamins per kg of diet provide: Vit. A: 12.000 IU, Vit. D_3: 3.000 IU, Vit. E: 7.5 IU, Vit. K: 21.5 mg, Vit. B_1: 0.6 mg, Vit. B_2: 4.8 mg, Vit. B_6: 1.8 mg, Vit. B_{12}: 9 μg, Niacin 10.5 mg, D-pantothenic acid: 7.5 mg, Folic acid: 0.15 mg

The Sample Collection

On day 64, a total of 12 yellow-feathered broilers (male) were randomly selected for each replicate in each experimental group. After slaughter, the caecal contents of chickens were removed by opening the abdominal cavity and stored at -80°C.

Sample Testing

16S rDNA sequencing was commissioned by Shanghai Zhongke New Life Science Co., LTD. The samples were sent to Shanghai Zhongkexin Life Biotechnology Co., LTD for detection. The sequencing process was as follows: Firstly, the DNA of the samples was extracted by fecal genome DNA extraction kit, and the V3-V4 variable region was amplified and sequenced by Illumina Miseq sequencing platform after the detection was qualified by 1% agarose gel electrophoresis. There is a certain proportion of Dirty Data in the Raw Data obtained by sequencing. In order to make the results of information analysis more accurate and reliable, the original Data should be spliced, filtered and de-chimeric to obtain valid Data. OTUs (Operational Taxonomic Units) clustering and species classification analysis were then performed based on available data.

Results

In this experiment, 1265441 original Tags sequences were obtained, and the average value of each sample in Q20 and Q30 was in 98% and 94%, and the quality of the sequencing data was good, which could be used for further analysis of species abundance and diversity.

After sequencing results of Illumina MiSeq platform were obtained, all sequences were clustered with classifiable operating units according to 97% similarity. OTUs statistics were performed on the samples, as shown in *Fig. 1.* S1 obtained 1.904 OTUs numbers, 982 of which were unique; S2 obtained 1285 OTU numbers. S3 obtained 1202 OTU numbers, only 215; S4 got 1388 OTUs, 313 unique. Group S1 shared 702 OTUs with S2, S3 and S4. SI and S2 have 822 OTU numbers, S1 and S3 have 789 OTU numbers, S1 and S4 have 842 OTU numbers.

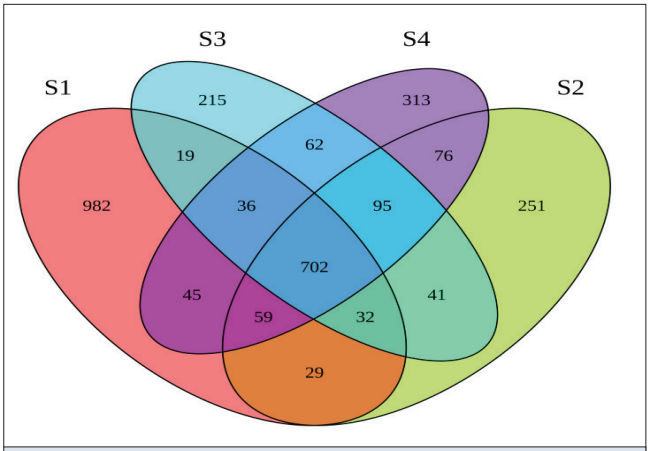

Fig 1. Venn plot of cecal intestinal flora of yellow-feathered broilers in different treatments

As shown in *Table 2*, there was no significant difference in Shannon and Simpson species richness among experimental groups (P>0.05). ACE and Chao 1 reflected the total number of species. The total number of species in S1 was significantly higher than S2, S3 (P<0.05), S4 (P>0.05), S2 was higher than S3 (P>0.05). Good coverage reaction sequencing depth, all test groups were 1, satisfying the sequencing depth.

As shown in *Fig. 2*, the Rarefaction Curve indicates that the predicted species richness of the tested samples is high. When the Curve tends to be flat, it indicates that the amount of sequencing data is reasonable. When Shannon index ranged from 3 to 5, the curve tended to be flat, indicating that the species discovered from the randomly selected sequencing number of each sample tended to be saturated, that is, nearly all OTUs were detected. The data are reliable and comprehensive.

Principal Component Analysis (PCA) is a method to simplify data Analysis and dimensionality reduction of multidimensional data, so as to extract the most important elements and structures in the data. Therefore, samples with high similarity in community structure tend to cluster together, while those with large difference in community structure tend to disperse. As shown in *Fig. 3*, S2, S3 and S4 are aggregated together with high similarity in colony structure, indicating that there is little difference in diversity of intestinal flora among S2, S3 and S4, and S2, S3 and S4 have high dispersion degree with S1. Based on Unweighted UnifracBeta distance, it can be obtained from *Fig. 4* the bacterial colony structure diversity of S1 was significantly different from S2, S3 and S4 (P<0.05).

As shown in *Table 3* and *Fig. 5*, *Bacteroidetes*, *Proteobacteria*, *Deiron-bacilli*, *Tautrophs*, *Epsilonbacteraeota*, *Verrucobacteriaceae*, *Actinomycetes*, *Cyanobacteria* and *Firmicutes* were the top 10 dominant flora in relative abundance. *Firmicutes*, *Bacteroidetes* and *Proteobacteria*

Table 2. *Effects of earthworm antimicrobial peptides and probiotics on cecal intestinal microflora α diversity of yellow-feathered broilers*

Parameter	S1	S2	S3	S4
Shannon	6.55±0.18	6.18±0.13	6.28±0.22	6.36±0.87
Simpson	0.96±0.005	0.95±0.003	0.95±0.01	0.95±0.04
ACE index	1140.68±117.1^{a}	941.91±67.04^{b}	883.29±38.11^{b}	1006.55±44.17^{ab}
Chao1 index	1146.84±110.34^{a}	902.86±56.75^{b}	849.35±39.48^{b}	961.67±34.12^{ab}
Good coverage	1	1	1	1

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

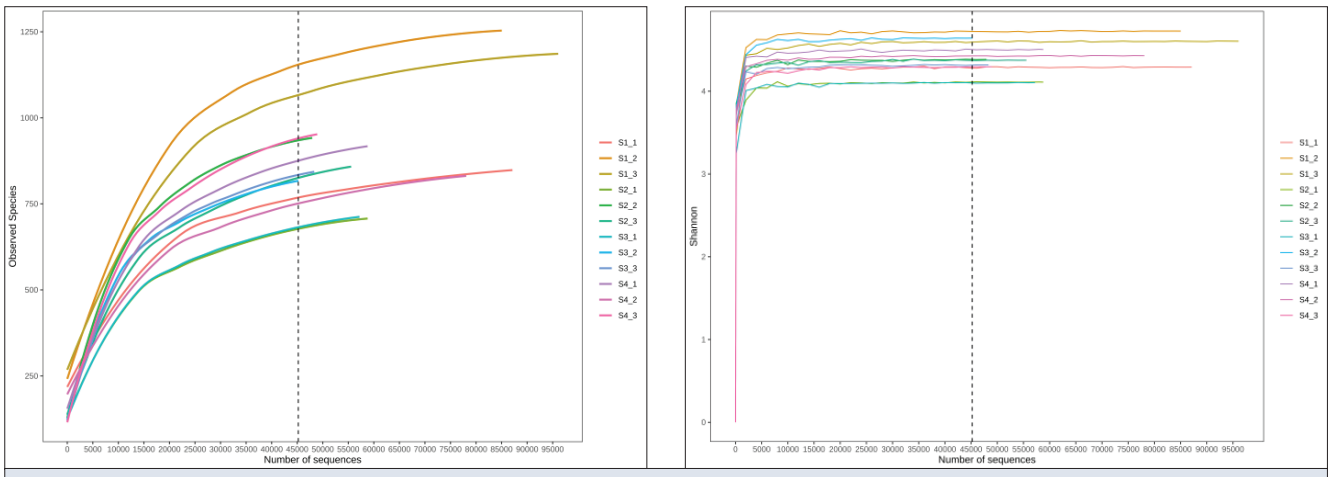

Fig 2. Sample Rarefaction Curve (A) Shannon Curve (B) (The abscissa are all randomly selected sequence numbers)

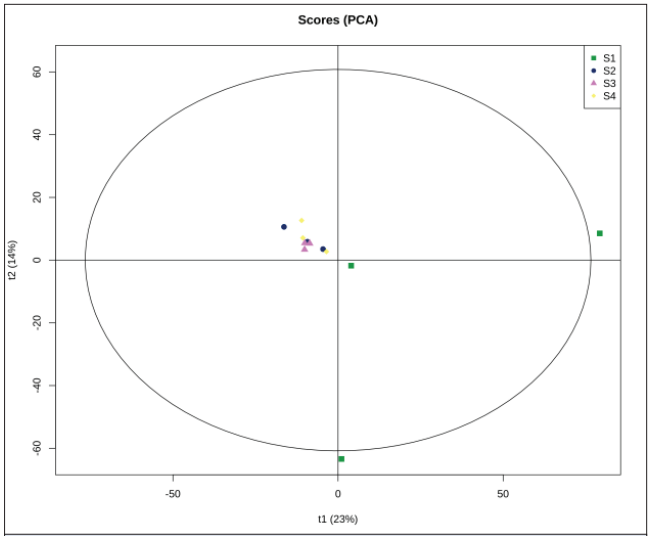

Fig 3. PCA of cecal intestinal flora of yellow-feathered broilers treated with different treatments

Table 3. Relative abundance of species in cecal intestinal microflora level of yellow-feathered broilers with different treatments

Parameter	S1	S2	S3	S4
Thick wall door	0.49	0.53	0.53	0.57
Bacteroidetes	0.21[a]	0.40[b]	0.34[b]	0.34[b]
Deformation of the fungus door	0.11[a]	0.03[b]	0.02[b]	0.03[b]
Deferrobacterium phylum	0.11[a]	0.00[b]	0.01[b]	0.01[b]
Put the door	0.01	0.02	0.03	0.01
Epsilonbacteraeota	0.03	0.00	0.00	0.01
Warts micro bacteria	0.01	0.01	0.03	0.01
Actinobacillus	0.01	0.00	0.01	0.00
Cyanophyta	0.01	0.00	0.00	0.00
Soft wall door	0.00	0.00	0.00	0.00
Other	0.01	0.00	0.00	0.00

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

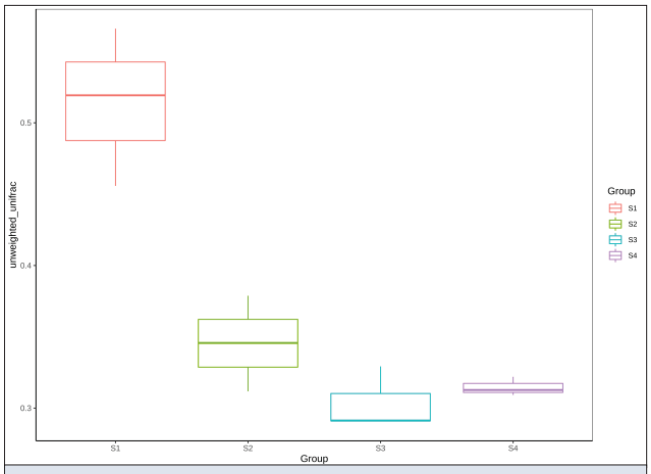

Fig 4. Weihted Box figure of cecal intestinal flora of yellow-feathered broilers treated with different treatments

were the top three dominant phyla in relative abundance of each experimental group. The relative abundance of *Firmicutes* was S4 > S3 > S2 > S1, and there was no significant difference among all groups (P>0.05). The relative abundance of S1 was significantly lower than S2, S3 and S4 (P<0.05). In relative abundance of *Proteobacteria*, S1 was significantly higher than S2, S3 and S4 (P<0.05), but there was no significant difference among S2, S3 and S4 (P>0.05). The relative abundance of S1 was significantly higher than S2, S3 and S4 (P<0.05). There was no significant difference in the relative abundance of *Microbacteria*, *Actinobacter*, *Cyanobacteria*, *Firmicutes*, *Epsilonbacteraeota* and *Actinobacter* among experimental groups (P>0.05).

As *Table 4* and *Fig. 4* shows, at the genus level, the top 10 dominant flora in relative abundance were *Bacteroidetes*, *Faecalis*, *Alistipes*, *Koala bacillus*, *Mucispirillum*, *Ruminococcus*

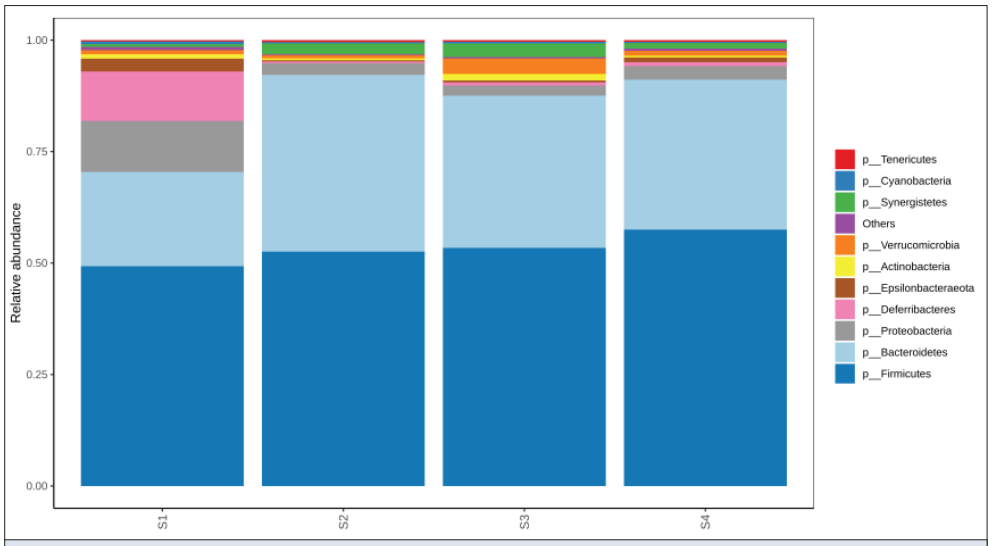

Fig 5. Histogram of relative abundance of species in cecal intestinal flora level of yellow-feathered broilers with different treatments

Table 4. *Species relative abundance of cecal intestinal flora of yellow-feathered broilers under different treatments*

Microorganism	S1	S2	S3	S4
Bacteroides	0.11^{c}	0.30^{a}	0.23^{b}	0.23^{b}
Fecal coli	0.10	0.06	0.05	0.09
Alistipes	0.04^{b}	0.08^{a}	0.09^{a}	0.07^{a}
Koala bacillus	0.02^{b}	0.07^{a}	0.06^{a}	0.04^{ab}
Mucispirillum	0.11^{a}	0.00^{b}	0.01^{b}	0.01^{b}
Ruminococcaceae UCG-014	0.04	0.05	0.04	0.05
Ruminococcus torques group	0.04	0.03	0.03	0.04
Unclassified genus of bacteria	0.02	0.03	0.05	0.02
Lactobacillus	0.01^{b}	0.04^{a}	0.02^{b}	0.03^{b}
Rumen fungi NK4A214	0.01	0.02	0.02	0.02
Other	0.51	0.34	0.41	0.40

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

UCG-014 and *Ruminococcus* torques Group, unclassified bacteria, *Lactobacillus,* rumen bacteria family NK4A214. The dominant flora in S1 were *Bacteroides*, *Faecalis*, and *Mucispirillum*; in S2 and S3 were *Bacteroides*, *Faecalis*, and *Phascolarctobacterium*; in S4, *Bacteroides*, *Faecalis*, and *Alistipes*. In the relative abundance of *Bacteroides*, S1 was significantly lower than S2, S3 and S4 (P<0.05), and S2 was the highest with no significant difference from S3 and S4 (P>0.05). In relative abundance of *Faecalis*, S1 was higher than S4, S2 and S3 (P>0.05); In the relative abundance of *Alistipes*, S2, S3 and S4 were significantly higher than S1 (P<0.05), and S3 had the highest relative abundance with no significant difference with S2 and S4 (P>0.05). In the relative abundance of *Koala bacillus*, S2 and S3 were significantly higher than S1 (P<0.05), and S4 was higher than S1 (P>0.05). In relative abundance of *Mucispirillum*, S1 was significantly lower than S2, S3 and S4 (P<0.05). In the relative abundance of *Lactobacillus*, S2, S3 and S4 were significantly higher than S1 (P<0.05), and S2 was the highest. There was no significant difference in the relative abundance of *UCG-014*, *Ruminococcus torques group* and NK4A214 of *Ruminococcus* among all groups (P>0.05).

LefSe (LDA Effect Size) analysis can be used to find with significant differences in abundance between groups through comparative analysis between and within groups. The differences are expressed by LDA Score, and the larger THE LDA Score value is, the greater the impact of species is. A total of 47 species, including 6 S2, 8 S3 and 33 S4, showed significant differences in abundance between groups. As shown in *Fig. 4*, *Fig. 5*, compared with other groups, S2 has significant differences in species including *Bacteria*, *Negativicutesc*, *Selenomonadales*, *Rikenellaceae* and *Alistipes*. The LDA Score of Bacteria was greater than 4, followed by the LDA Score of *Negativicutesc*, *Selenomonadales* and *Rikenellaceae* was greater than 3.5. S3 Compared with other groups, the species with significant differences are *ultradClostridiabacterium*, *Bacteroidales*, *Bacteroidetes*, *Bacteroidaceaec*, *Bacteroidia*, *Bacteroides*, *Phascolarctobacterium*, *Acidaminococcaceae*, *Itured-Firmicutesbacterium*, the largest affect is the *ulturedClostridiabacterium* and order, phylum, family, class, and genus of Bacteroides, LDA Score were greater than 3.5; Compared with other groups, the species of S4 group were *Betaproteobacteriales*, *Rhizobiales*, *Burkholderiaceae*, *Rhizobiaceae,* *Ochrobactrum*, Other, *Magnetospirillaceae*, *Chitinophagaceae*, Other, *Chitinophagales*, *Asticcacaulis*, *Caulobacter*, Other, *Rhodobacteraceae*, Other, *Acidobacteria*, *Rhodobacterales*, *Diplorickettsiles*, *Planctomycetes*, *Oxyphotobacteria*, *Chloroplast*, *Ralstonia*, *Pararhizobium_Rhizobium*, *Unclassified*, *Planococcaceae*, *Blastocatellia - Subgroup4*, *Altererythrobacter*, *Moraxellaceae*,

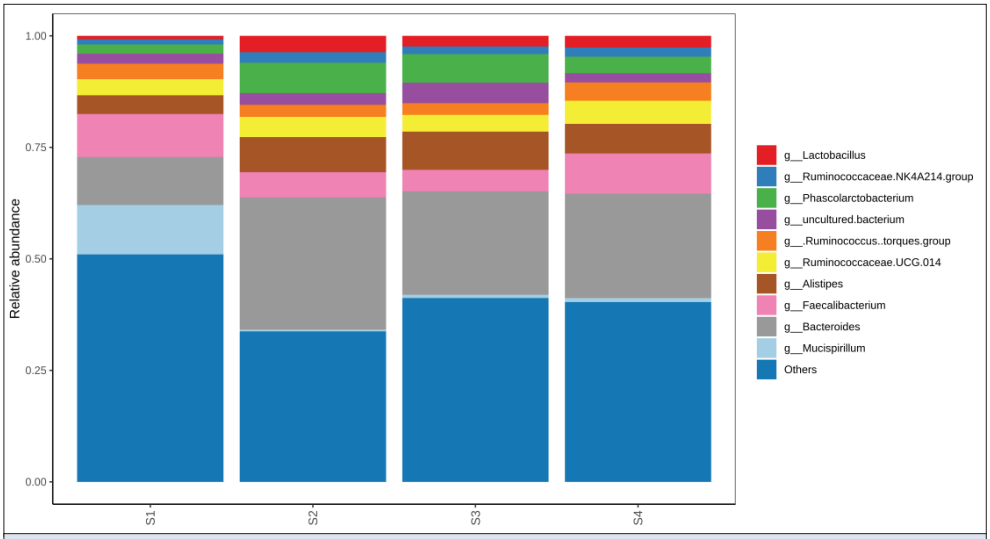

Fig 6. Column chart of species relative abundance in cecal intestinal flora of yellow-feathered broilers treated with different treatments

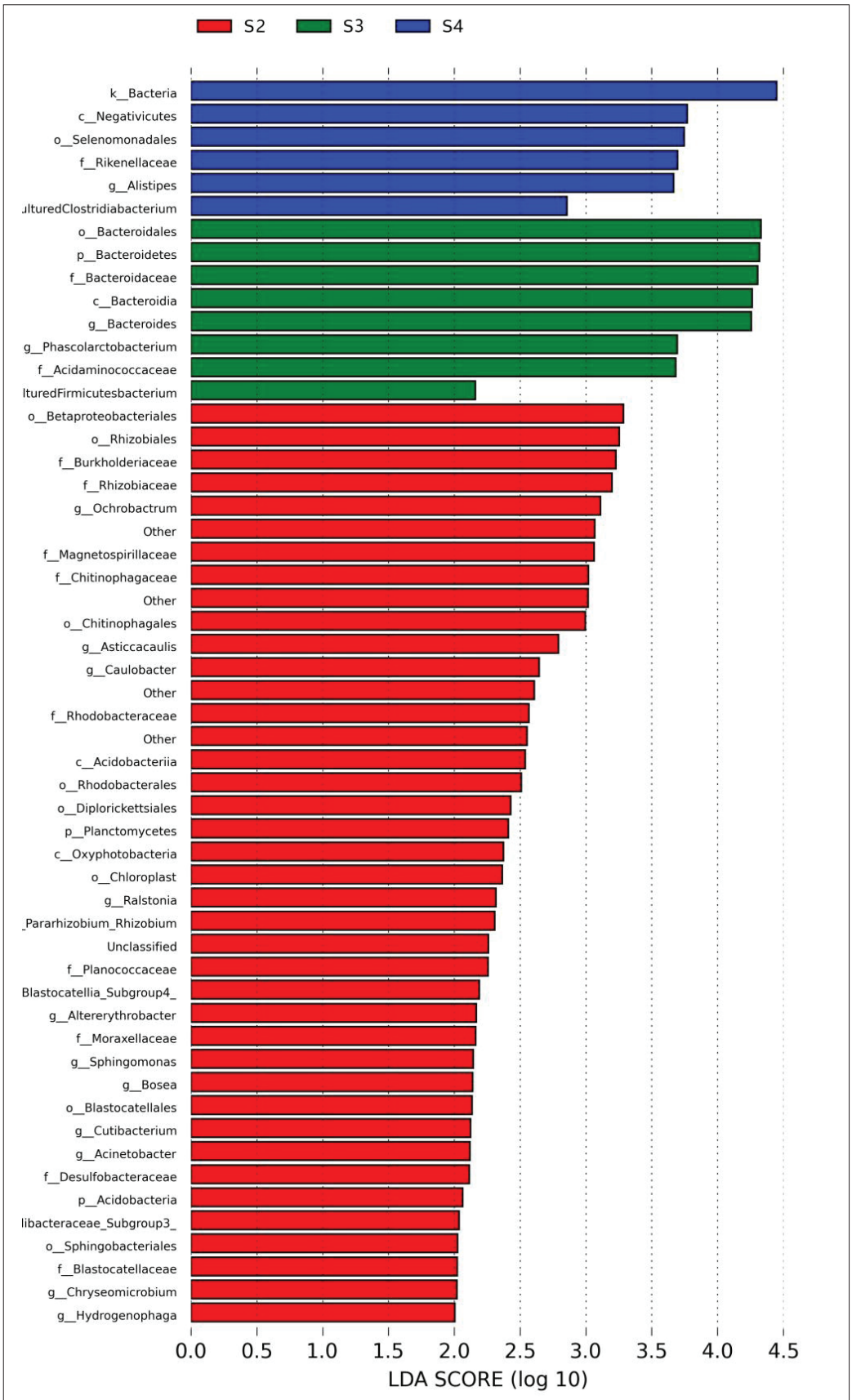

Fig 7. Column chart of cecal intestinal flora LAD of yellow-feathered broilers treated with different treatments. The length of the histogram represents the size of the impact of significantly different species, and different colors represent different samples

Sphingomonas, *Bosea*, *Blastocatelales*, *Cutibacterium*, *Acinetobacter*, *Desulfobacteraceae*, *Acidobacteria*, *Ibacteraceae _Subgroup3*, *Sphingobacteriales*, *Blastocatellaceae*, *Chryseomicrobium*, and *Hydrogenophaga*, Among them, *Betaproteobacteriales*, *Rhizobiales* and *Burkholderiaceae* had the greatest influence with LDA Score greater than 3, *Rhizobiaceae*, *Ochrobactrum,* Other, *Magnetospirillaceae*, *Chitinophagaceae*, Other, *Chitinophagales*. Species with no significant difference between S1 and other groups (*Fig. 6*, *Fig. 7*).

DISCUSSION

AMP has good potential as a suitable alternative to conventional antibiotics used in the pig and poultry industry [21]. AMP has been reported to benefit growth performance, reduce incidence of diarrhea and increase the rate of weaned pigs [22]. Daneshmand et al.[23] reportedly showed that AMPs can protect broiler chickens from challenging *E. coli* in vivo. The digestive tract microecosystem is an important component of livestock and poultry body weight and has an effect on the metabolism of macromolecules. AMPs has been reported to have beneficial effects on growth performance, intestinal microflora and morphology, immune function and nutrient digestibility of chickens [24,25]. The chicken gastrointestinal (GI) tract is home to a complex microbial community that underlines the links between diet and health. The GI tract is rich in microbial biodiversity, playing home to ≥500 phylotypes or 1 million bacterial genes, which equates to 40-50 times the number in the chicken genome [26]. *In vitro* culture is traditionally used in the study of intestinal microorganisms, but most of them are difficult to be screened and isolated by traditional methods. Therefore, molecular biology is an important direction to explore the composition of intestinal microbiota in the future. At present, intestinal microbes are mostly studied from microbial 16S rRNA sequence. Studies on the cloning and sequencing of 16S rRNA sequence show that more and more intestinal bacteria that cannot be isolated and cultured *in vitro* have been found. The study explored the bacterial community present in water, sediment and intestine samples from an aquaculture site using high-throughput sequencing [27]. This finding indicated that sediment and water are major sources of intestinal microbes. Changes in the qualitative and quantitative composition of the caecal microbiota were less pronounced than in the crop [28]. Daneshmand et al.[23]. used 16S rRNA gene mapping technology to study the diversity of intestinal microbes and found that the diversity and distribution of intestinal microbes were relatively stable. The diversity of intestinal microorganisms of laying hens was investigated by PCR-DOGE test. It was found that the cecum was the most suitable organ for the study of microorganisms in the digestive tract of laying hens. Dietary AMP has been reported to improve intestinal tissue structure and promote growth [29-31].

The results of this experiment showed that the total number of species in S1 was significantly higher than S2, S3 and S4 and the colony structure of S1 was significantly different from S2, S3 and S4. At the phylum level of each experimental group, the dominant flora were *Firmicutes*, *Bacteroides* and *Proteobacteria*. The relative abundance of S2, S3 and S4 in *Firmicutes* and *Bacteroides* was significantly higher than S1, and the relative abundance of Proteobacteria was significantly lower than S1. *Proteobacteria* was the largest phylum in bacteria, including many pathogenic bacteria. Such as *Escherichia coli*, *Salmonella*, *Vibrio Cholera*, *Helicobacter pylori* and other harmful bacteria. On the genus level, S2, S3, and S4 in the *Phascolarctobacterium*, significantly higher than in the relative abundance of the genus lactobacillus S1. the *Phascolarctobacterium* with *Clostridium* difficile bacteria for succinic acid salt to inhibit the growth of clostridium difficile bacteria, lactic acid bacillus genus flora is beneficial to the body, there are few pathogenic, and can improve the body resistance, formany spoilage organisms, and pathogenic bacteria have inhibition. Chicken intestinal microflora plays an important role in immune regulation and disease control. Intestinal microorganisms can be divided into intestinal symbiotic bacteria, conditioned pathogenic bacteria and enterohost pathogenic bacteria [32]. Intestinal opportunistic pathogens, such as facultative anaerobe *Escherichia coli*, are present in low concentrations, but when intestinal homeostasis is disrupted, for example when the body is infected with a virus, *Escherichia coli* proliferates rapidly and leads to intestinal disturbances [33]. According to the report, diacetyl by gram-negative bacteria binding protein reaction of arginine, which interfere with the use of arginine, inhibit the growth of gram negative bacteria , in Newcastle disease virus can be isolated from the dead broilers was added in the diet of earthworm antibacterial peptide and composite probiotic preparations can reduce the relative abundance of harmful bacteria, increase the relative abundance of the beneficial bacteria, thereby regulate the intestinal flora and improve immunity.

LefSe analysis of cecal intestinal flora of yellow-feathered broilers showed that there were 47 species with significant differences in abundance between groups, including 6 species in S2, 8 species in S3 and 33 species in S4. The phylum actinomycetes is a group of prokaryotes and Gram-positive bacteria, once thought to be a cross between bacteria and molds because of their morphology, are prokaryotes without nuclei. Antimicrobial peptides have antibacterial activity against most gram-positive bacteria, gram-negative bacteria, mycoplasma and some

viruses, but have no toxicity to fungi and prokaryotes, so earthworm antimicrobial peptides have no effect on actinomycetes, but compound probiotics can reduce their relative abundance and complement antibacterial peptides. It provides a theoretical basis for earthworm antimicrobial peptides and compound probiotics to maintain the balance of intestinal flora and improve the immune performance of poultry.

After adding earthworm antibacterial peptides and compound probiotics to the diets of yellow-feather broilers, 16S rDNA sequencing analysis of cecum intestinal flora showed that the total number of species in S1 was significantly higher than that in S2, S3 and S4, and the colony structure of S1 was significantly different from S2, S3 and S4 ($P<0.05$). At the phylum level, the dominant bacteria groups of the experimental groups were *Firmicutes*, *Bacteroidetes* and *Proteobacteria*, among which the relative abundance of S2, S3 and S4 in *Firmicutes* and *Bacteroidetes* was significantly higher than that of S1 ($P<0.05$), and the relative abundance of *Proteobacteria* was significantly lower than that of S1 ($P<0.05$). The relative abundance of S2, S3 and S4 of *Phascolarctobacterium* and *Lactobacillus* were significantly higher than that of S1 ($P<0.05$). Earthworm antimicrobial peptides and compound probiotics could increase the relative abundance of beneficial bacteria, reduce the relative abundance of harmful bacteria, and regulate the cecal intestinal flora of broilers. Combined use had complementary effects.

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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.

Ethical Statement

The study was approved by the Animal Experimentation Ethics Committee of the School of Animal Science and Technology, Shihezi University.

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. JY completed the main experimental content. ZW and SG collected and analyzed data. LD and XZ wrote the first draft of manuscript. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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RESEARCH ARTICLE

Content of 17β-Estradiol in Raw Milk in Ukraine

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Abstract: The main source of estrogen supply of steroid origin in the organism of consumers is milk and dairy products, which consume 60 to 80% of these hormones. Excess estrogen, including 17β-estradiol in the serum, has been linked to female and male genital cancers. The purpose of the search was to determine the amount of 17β-estradiol in milk on various farms in Ukraine and during its processing at the dairy plant. The determination of 17β-estradiol in cow's milk samples was performed by enzyme-linked immunosorbent assay. It was found that the average value of 17β-estradiol content in raw milk obtained during the day on one farm does not depend on the time of milk production. At the same time, the content of 17β-estradiol in whole milk from one herd changes significantly during the year of its receipt. Mixing whole milk from different farms at the processing plant does not lead to a significant difference in 17β-estradiol content compared to milk produced on a particular farm in the region. It was found that whole milk with a higher mass fraction of milk fat contains more 17β-estradiol. Separating milk influences the distribution of 17β-estradiol between cream and skim milk. The amount of 17*ß*-estradiol in skim milk was almost 5-7 times less than in cream. Therefore, to possibly reduce the daily intake of 17β-estradiol and dairy products during consumption, it is necessary to reduce the mass fraction of fat in them.

Keywords: *17β-estradiol, Whole milk, Skim milk, Cream*

Ukrayna'da Çiğ Sütte 17β-Östradiol İçeriği

Öz: Tüketicilerin bünyesindeki östrojen orijinli steroidin temel kaynağını süt ve süt ürünleri oluşturur ve bu hormonlar %60-80 oranında alınmaktadır. Serumdaki 17β-östradiol de dahil olmak üzere aşırı östrojen, kadın ve erkek genital kanserleriyle ilişkilendirilmiştir. Bu çalışmanın amacı, Ukrayna'daki çeşitli çiftliklerde ve süt fabrikasında işlenme esnasındaki sütte 17β-östradiol miktarının belirlenmesiydi. İnek süt örneklerinde 17β-östradiol miktarı, enzyme-linked immunosorbent assay ile analiz edildi. Bir çiftlikte gün boyunca elde edilen çiğ sütteki ortalama 17β-östradiol miktarının süt sağım zamanına bağlı olmadığı tespit edildi. Aynı zamanda, bir sürüden alınan tam yağlı sütteki 17β-östradiol içeriği, alındığı yıl boyunca önemli ölçüde değişmekteydi. Farklı çiftliklerden gelen tam yağlı süt karışımının 17β-estradiol içeriği, bölgedeki belirli bir çiftlikte üretilen sütteki içerik ile karşılaştırıldığında önemli bir farklılık saptanmadı. Yüksek kütle oranına sahip tam yağlı sütün daha fazla 17β-östradiol içerdiği saptandı. Sütün, krema ve yağsız süt olarak ayıklanması 17β-östradiol dağılımını etkilemekteydi. Yağsız sütteki 17β-östradiol miktarı kremadakinden neredeyse 5-7 kat daha azdı. Bu nedenle, tüketim sırasında 17β-östradiolün günlük alımını azaltmak için, süt ürünlerinin içeriklerindeki yağın kütle fraksiyonunu azaltmak gerekmektedir.

Anahtar sözcükler: *17β-östradiol, Tam yağlı süt, Yağsız süt, Kaymak*

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Introduction

It is well known that milk and dairy products are considered essential sources of nutrients and are the basis of the ration for most of the population [1,2]. Milk contains the optimal ratio of calcium and phosphorus for their assimilation, milk proteins are complete, and raw milk is the basis for producing many dairy products [3-5]. However, the usefulness of milk and dairy products can be only if they meet the quality and safety indicators. Contaminants of milk can be various xenobiotics, including antibiotics and detergents and disinfectants [6,7], heavy metal salts [8], pesticides, hormones, etc. [9,10]. Recently our research has shown that beef containing the synthetic hormone zeranol, which is forbidden for use as a stimulant of animal weight gain, has been processed [11]. Therefore, the research of products of animal origin, in particular milk, for the presence of steroid hormones, which are considered stimulants to increase production, is constantly relevant, despite the fact that the use of hormones is officially prohibited.

Milk contains a group of estrogenic hormones of natural origin (17β-estradiol, 17α-estradiol, estriol, and estrone), which have an influence on several vital functions in the organism (protein synthesis, signaling transmission between receptors, etc.), including the regulation of reproduction [12]. Estrogen steroid hormones of natural origin are present in milk as a result of secretion of internal glands, respectively, a certain amount of them will always be present in raw milk [13]. Hormones of artificial origin are present in milk in the treatment of the reproductive system of animals, as well as in intended use to increase production [14,15]. However, modern methods for determining 17β-estradiol in milk and dairy products cannot identify its origin. Therefore, the research aims to establish a safe content of 17β-estradiol in milk and dairy products, namely the amount of hormone synthesized by animals under normal physiological conditions [12]. However, there are cases when animals are given 17β-estradiol to increase milk production [15,16], in which case the amount of this hormone rises significantly in milk. High levels of 17β-estradiol in milk and dairy products are a concern among scientists, as the use of these products leads to an increase in estrogen levels in the blood of consumers, which is considered dangerous because these hormones are responsible for various cancers (breast, uterine, uterine, ovaries, testes, prostate) [17-20]. In addition, the intake of significant amounts of estrogen with food in the organism of prepubertal children causes disorders of the development of genitourinary and central nervous system [21,22].

It is reported that the content of 17β-estradiol in whole milk of healthy cows depends on many factors related to the physiological state of the organism (gestation period, estrus), from the composition of feed ration, breed, and age of animals [23]. The researchers found in drinking milk which was selected from different regions of Iran from 75.5 pg/mL to 922.3 pg/mL of 17β-estradiol, with an average of 330 pg/mL [24]. Another search [25] reported an amount of 17β-estradiol in pasteurized and raw milk in the range of 5.6-51 pg/mL. In the research of samples of pasteurized and sterilized skim milk, it was found that the average level of 17β-estradiol was 8.2±0.59 pg/mL [26]. According to Malekinejad and Rezabakhsh [12] and Janowski et al.[27], the content of 17β-estradiol in drinking milk averaged 20 mg/mL.

According to the requirements of the Codex Alimentarius Commission, the maximum amount of external (synthetic) estradiol entering the organism together with food should not exceed 50.000 pg/kg/day [28]. The analysis of Ukrainian normative documents revealed that the control of raw milk coming for processing on the content of 17β-estradiol is not provided.

Due to the significant influence of high estrogen concentrations on the organism of consumers to date, the safe quantitative levels of 17β-estradiol in milk and dairy products are not entirely justified, given the intensive technology of milk production. Therefore, conducting systematic research, including the influence of the maximum number of factors (fat content, period of lactation, seasonality, feeding, breed) on the level of 17β-estradiol in raw milk, is promising and relevant.

The research aimed to determine the amount of 17β-estradiol in milk on various farms in Ukraine and during its processing at the dairy plant.

Material and Methods

A milk sampling for research was conducted on three dairy farms in Ukraine in the Western and Central regions and on one milk processing plant. Milk is got on these farms from cows of the Ukrainian Black-Spotted breed. Milk samples were taken and delivered to the laboratory in a refrigerated state at a temperature of +2±1°C. The research was conducted in State Scientific and Research Institute for Laboratory Diagnostics and Veterinary and Sanitary Expertise (Kyiv).

Quantitative determination of 17β-estradiol in cow's milk samples was performed by enzyme-linked immunosorbent assay using a test system RIDASCREEN®17β-őstradiol Art. No. R2301 (manufactured by firm Art-Biopharm / R-Biopharm, Darmstadt, Germany). Before use, the test system lasted 20-30 min. at a temperature of 20-25°C, the reagents were prepared according to the manufacturer's protocol. Standard solutions of 17β-estradiol with concentrations of 0; 50; 200; 800; 3200; 12800 pg/mL were used to construct the calibration curve.

Before the research, the milk samples were heated in a thermostat to a temperature of 20-25°C and homogenized using an IKA homogenizer (T 18 Basic) with nozzles (S 18 N-10 G), to ensure homogeneity. 20 µL of standard solutions, test samples, 50 µL of a diluted antibody preparation, and 17β-estradiol conjugate were added to the wells of the microtiter plate sensitized to antibodies to 17β-estradiol. The tablet was incubated for 2 h at a temperature of 20-25°C. Then the device for washing tablets (BIORAD PW 40) washed the wells of the tablet with distilled water. 50 µL of substrate and chromogen solution was added to each well and incubated for 30 minutes at 20-25°C. After incubation, 100 µL of stop reagent was added to the wells. The optical density was measured on an enzyme-linked immunosorbent reader, Sunrise (Austria), at a wavelength of 450 nm. Specialized RIDA®Soft software was used for computer processing of measurement results.

Statistical analysis was performed using disperse analysis with Fisher's criteria (ANOVA). The data are presented as x ± SD (mean ± standard error). Significance of the obtained data was evaluated according to F-criterion with the significance levels of $P<0.05$, $P<0.01$, $P<0.001$ (taking into account Bonferroni correction) by SAS (Version 9.2, 2009).

Results

At the first stage of the research, the amount of 17β-estradiol in the milk of different milking (morning, lunch, evening) was determined on three dairy farms. Milk samples were taken after milking from the cooler tank (common milking) three times a day to get milk for morning, lunch, and evening milking. The experiment was conducted for two months in February-March. The research results are given in *Table 1*.

The searches show in *Table 1* that a significant difference between the average content of 17β-estradiol in raw milk obtained at different periods of the day was not found, both within one farm and on three other farms. The amount of hormone ranged from 439.8±41.8 pg/mL to 585.5±61.2 pg/mL in morning milk, from 497.6±46.5 pg/mL to 605.7±71.0 pg/mL in lunch milk, and from 543.3±53.2 pg/mL to 641.3±62.8 pg/mL in evening milk. At the same time, significant fluctuations of the content of 17β-estradiol in raw milk were found during milking, both at one and in different periods of the day. In particular, on farm number №1, the difference between the minimum and maximum amount of hormone was from 3.0 to 3.7 times ($P<0.01$); on the second farm, this difference was the smallest from 2.3 to 2.5 times ($P<0.01$) and the largest difference was found on the third farm between the minimum and maximum amount of hormone from 3.4 to 4.5 times ($P<0.01$).

Therefore, the difference between the maximum and minimum amount of 17β-estradiol in collected raw milk obtained during the day on one farm fluctuates significantly, but the average value does not depend on the milk production time.

In order to determine the influence of various factors, particularly the year, on the change in the content of 17β-estradiol in raw milk, the amount of this hormone was determined during the year. Milk samples were taken at these three farms twice a month. The results are given in *Table 2*.

Table 1. *Content of 17β-estradiol in raw milk on three farms in Ukraine, pg/mL, (x ± SD; n = 90)*

Farm Number	Milk Sampling Time	Number of Tested Samples	Minimum Quantity	Maximum Number	Average Number, x ± SE
№1	1	n=10	257.3	851.6*	514.9±66.5
	2	n=10	242.5	918.3*	605.7±71.0
	3	n=10	268.2	797.4*	583.2±55.3
№2	1	n=10	324.7	819.9*	439.8±41.81
	2	n=10	348.5	833.7*	497.6±46.5
	3	n=10	371.7	875.1*	543.3±53.2
№3	1	n=10	198.6	903,4*	585.5±61.2
	2	n=10	206.5	747.1*	604.8±59.4
	3	n=10	238.8	825.5*	641.3±62.8

1- milk of morning milking; 2 - milk of lunch milking; 3 - milk of evening milking; P<0.01 - compared to the minimal content of 17β-estradiol*

Table 2. *The amount of 17β-estradiol in raw milk during the year, pg/mL, (x±SD; n=48)*

Months of Research	Number of Tested Samples	Minimum Quantity	Maximum Number	The Average Number of x ± SE
January – February	12	582.3	1.243	836.9±79.2*
April – May	12	247.7	739.5	404.5±40.6
July – August	12	358.4	912.1	512.4±47.6
October – November	12	436.5	875.8	571.7±54.3

** P<0.05 - compared with the content of 17β-estradiol in milk taken in other months*

As can be seen from *Table 2*, we see that the highest amount of 17β-estradiol was found in milk samples taken on farms in January and February - 836.9±79.2 pg/mL. The amount of 17β-estradiol in this period was 2.0 times higher (P<0.05) compared with the content in milk selected in April and 1.6-1.5 times (P<0.05) higher than in July-August and September-October, respectively. The increase in the amount of 17β-estradiol in milk in January-February can be explained by the fact that probably in this period, the most significant number of cows in the third trimester of pregnancy physiologically produce milk with high levels of this hormone [29,30]. The lowest number of pregnant cows giving milk was in April and May.

Thus, searches indicate that the content of 17β-estradiol in whole milk from one herd undergoes significant changes during the year of its receipt.

Due to the fact that whole milk after delivery to the processing plant can be mixed with milk from other farms and reserved in large tanks for processing, we determined the amount of 17β-estradiol in whole milk before pasteurization. The search was performed in October and November *(Table 3)*.

Table 3 shows that the content of 17β-estradiol in whole milk collected at the processing plant generally reflects the amount of the hormone in milk on farms. The average amount of 17β-estradiol in October ranged from 423.4±40.3 pg/mL to 518.7±52.5 pg/mL, i.e., fluctuations within100 pg/mL. In November, the content of 17β-estradiol increased to an average of 578.7±52.8 pg/mL.

Therefore, the researches show that mixing processing plant of whole milk from different farms does not lead to a significant difference in 17β-estradiol content compared to milk produced on a particular farm in a given region.

The next step was to determine and compare the content of 17β-estradiol in the whole milk, depending on the content of mass fraction of fat. It is known that the steroid hormone 17β-estradiol belongs to the lipophilic ones, which are more concentrated in the milk fraction. The results of the research are given in *Table 4*.

As can be seen from *Table 4* we can see that a significant difference between the content of 17β-estradiol in raw milk with a mass fraction of fat from 2.8% to 3.5% is not observed. However, in whole milk with a fat content of 3.6% to 4.0%, the amount of 17β-estradiol was, on average, 20% higher than in milk with a fat content of 2.8% - 3.0%. In milk with a fat content of 4.1% to 4.5%, the amount of 17β-estradiol was 704.8±58.6 pg/mL, which is 27.8% (P<0.05) more compared to milk with the lowest milk fat content.

Therefore, the data indicate that whole milk with a higher mass fraction of milk fat contains more 17β-estradiol.

Considering that the level of steroid hormones in milk depends on the concentration of milk fat, the influence of skimming on the distribution of 17β-estradiol by fractions (cream, skim milk) was studied. Milk with different initial content of 17β-estradiol was taken into the experiment *(Fig. 1)*.

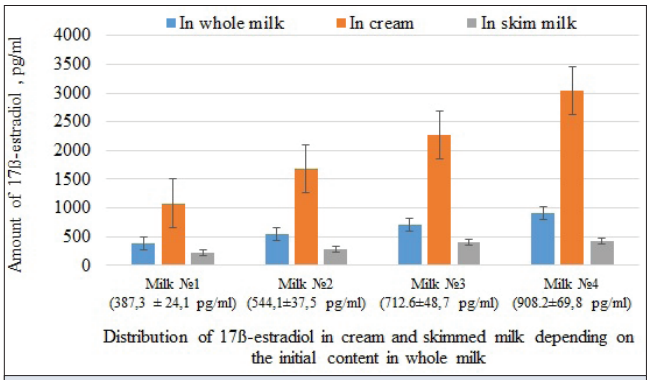

Fig 1. The influence of skimming whole milk on 17β-estradiol content

Table 3. *Content of 17ß-estradiol in raw milk at the dairy processing plant of Ukraine, pg/mL, (x±SD; n = 30)*

Months of Research	Research Days	Investigated Samples	Minimum Quantity	Maximum Number	The Average Number of x ± SE
October	1	n=5	302.5	627.2	423.4±40.3
	15	n=5	323.2	716.4	518.7±52.5
	30	n=5	281.6	519.3	437.5±42.7
November	40	n=5	311.8	775.1	514.2±45.1
	50	n=5	353.2	807.9	564.1±50.3
	60	n=5	430.7	756.4	578.7±52.8

Table 4. *The amount of 17ß-estradiol in raw milk, depending on the mass fraction of fat, (x±SD; n = 40)*

Milk with Fat Content, %	Number of Tested Samples	Minimum Quantity	Maximum Number	The Average Number of x ± SE
2.8 – 3.0	n=10	327.1	689.3	551.4±51.7
3.1 – 3.5	n=10	373.3	707.6	565.7±54.5
3.6 – 4.0	n=10	395.2	819.5	663.5±57.3
4.1 – 4.5	n=10	398.8	925.6	704.8±58.6*

** P<0.05 - compared with the content of 17ß-estradiol in milk with a fat content of 2.8-3.0%*

The results of *Fig. 1* show that the milk separation process influences the distribution of 17β-estradiol between cream and skim milk. In particular, the highest level of 17β-estradiol was found in the cream. The content of this hormone was 2.8-3.3 times (P<0.01) higher in cream than in whole milk. At the same time, skimmed milk contained 1.7 to 2.1 times (P<0.05) less 17β-estradiol than whole milk. The results of this investigation indicate that fat-containing dairy products will be a much greater source of intake of steroid hormone 17β-estradiol than low-fat. As the amount of 17β-estradiol in skim milk was practically 5-7 times (P<0.001) less than in cream, it is evident that reducing the daily intake of 17β-estradiol and dairy products during consumption is necessary to reduce the mass fraction of fat in them.

Discussion

It is believed that the main source of estrogen of steroid origin in the organism of consumers is milk and dairy products, which consume 60% to 80% of these hormones [25,26]. The presence of excessive amounts of estrogen, in particular, 17β-estradiol, in the serum has been associated with the development of female and male genital cancer [17-20]. Therefore, the importance of milk and dairy products in the development of these pathologies today is considered twofold. Some authors confirm the connection between the consumption of dairy products and the emergence of oncology [31-33], while others point to the ambiguity of this issue, and the correlation is probable [34-36]. Therefore, our research aimed at determining the amount of 17β-estradiol in milk on various farms in Ukraine and processing it at the dairy plant. After all, no study has been conducted to determine this hormone in Ukraine, and available foreign data indicate that the amount of 17β-estradiol in milk is very variable and depends on many factors.

When determining 17β-estradiol on three farms, we found that the amount of hormone ranged from 439.8±41.8 pg/mL to 585.5±61.2 pg/mL in morning milking, from 497.6±46.5 to 605.7±71.0 pg/mL in lunch milking and from 543.3±53.2 pg/mL to 641.3±62.8 pg/mL in evening milking. At the same time, significant fluctuations of the content of 17β-estradiol in raw milk got during milking were found, both at one and in different periods of the day. In particular, the difference between the minimum and maximum amount of the hormone ranged from 3.0 to 4.5 times (P<0.01). Thus, our data are consistent with the results of researches by Iranian scientists on fluctuations in the content of 17β-estradiol from 75.5 pg/mL to 922.3 pg/mL of milk [24]. However, they differ from other researchers, who found significantly lower amounts of 17β-estradiol in whole cow's milk - from 5 pg/mL to 51 pg/mL [25-27]. We also found that the highest amount of 17β-estradiol was found in milk samples taken from farms in January and February - 836.9±79.2 pg/mL. The amount of 17β-estradiol in this period was 2.0 times higher (P<0.05), compared with the content in milk taken in April and May and 1.6-1.5 times (P<0.05) higher than in July-August and September-October, respectively. Seasonal increase in the amount of 17β-estradiol in raw milk, we explain the presence on the farm in this period a significant number of cows in the second and third trimesters of pregnancy, which according to scientists [29,30] in this period of pregnancy produce milk with 17β- estradiol is 26-33 times more than non-pregnant cows [25,29,37].

Research of raw milk at a milk processing plant revealed that mixing milk from different farms did not lead to a significant difference in 17β-estradiol content compared to milk obtained on a particular farm in the region. This gives reason to believe that the range of 17β-estradiol in whole milk from one herd undergoes significant changes during production during the year.

In a research of the content of 17β-estradiol, depending on the mass fraction of fat in raw milk, it was found that a significant difference between the content of the hormone in milk with a mass fraction of fat from 2.8% to 3.5% is not observed. Changes were detected in milk with a fat content of 3.6% to 4.0%, in particular, the amount of 17β-estradiol was, on average, 20% higher than in milk with a fat content of 2.8% - 3.0%. In milk with a fat content of 4.1% - 4.5%, the amount of 17β-estradiol was 27.8% (P<0.05) higher compared with milk with a fat content of up to 3.0%. The obtained results confirm researchers' data [26] on the lipophilicity of 17β-estradiol, as a result, milk with a higher mass fraction of fat contained a higher content of this hormone. It was reported [38] that in pasteurized milk with a fat content of 1.0% and 2.0% fat, the amount of 17β-estradiol was 1.8 and 1.3 times (P<0.05) less than in whole milk.

Our results indicate the lipophilic nature and redistribution of 17β-estradiol by separation technology. Thus, it was found that the process of separation of milk influences the distribution of 17β-estradiol between cream and skim milk. In particular, the amount of 17β-estradiol in cream was 2.8-3.3 times (P<0.01) higher than in whole milk. At the same time, skimmed milk contained 1.7 to 2.1 times (P<0.05) less 17β-estradiol than whole milk. In searches [38], the hormone content in cream was 3.2 time (P<0.01) higher than in whole milk and 15 times higher than in skim milk. Therefore, we believe that foods fortified with milk fat are also a significant source of 17β-estradiol for consumers.

So, summarizing the study, we can note the following. Significant fluctuations in 17β-estradiol in cows' milk may be mainly due to the presence of different levels of fat

in the herd of cows at different stages of pregnancy, milk production with different fat content, or dietary influence, as phytoestrogens of the green feed in summer, may increase steroids hormones in the blood [23,39]. All these factors must be investigated in a specific experiment. Also, it is likely that significant differences in the content of 17β-estradiol in milk may be due to the use of different methods for its determination (enzyme-linked immunosorbent assay, radioimmunoassay, chromatographic). In addition, considering that 17β-estradiol is a lipophilic hormone whose solubility is better in fats, it is advisable to indicate the mass fraction of milk fat when comparing its content in dairy products. This will allow a more objective assessment of the level of 17β-estradiol in milk and the calculation of possible daily consumption.

It was found that the average value of 17β-estradiol in raw milk obtained during the day on one farm does not depend on the time of milk production. At the same time, the content of 17β-estradiol in whole milk from one herd changes significantly during the year of its receipt. Mixing whole milk from different farms at the processing plant does not lead to a significant difference in 17β-estradiol content compared to milk produced on a particular farm in the region.

It was found that whole milk with a higher mass fraction of milk fat contains more 17β-estradiol. The process of separating milk affects the distribution of 17β-estradiol between cream and skim milk.

The amount of 17β-estradiol in skim milk was almost 5 - 7 times less than in cream. Therefore, to possibly reduce the daily intake of 17β-estradiol and dairy products during consumption, it is necessary to reduce the mass fraction of fat in them.

Availability of Data and Materials

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

Financial Support

No funding was received.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Statement

Ethics committee approval is not required for this study because of performing *in vitro* in the laboratory.

Author Contributions

MK, VS and GK conceived and executed the idea, designed experiments, analyzed results and a deep revision of the manuscript. ZM, KM, YH and OP collected samples, performed experiments, contributed to tand implementation of the research. All authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication.

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Research Article

The Effect of Concentration and Storage Time on Short-Term Storage of Ram Sperm

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Abstract: The present study was conducted to observe the effect of different dilution rates on ram sperm motion characters (motility, progressivity, velocity and average of speed), head diameter, mitochondrial membrane potential (MMP) and live intact acrosome rate during liquid storage for 96 h at 5°C. Normospermic semen samples that obtained from 4 adult rams were pooled. Pooled semen was split into two aliquots; Low Dilution Rate (LDR) (400 million) and High Dilution Rate (HDR) (20 million) motile spermatozoa per insemination dose/0.25 mL straw and were diluted with a skim milk-egg yolk-based extender. It was observed that the Total Motility (TM) and total progressivity of the LDR group decreased more rapidly than HDR group over time, in general. From the 12th h to the 96th h; the rapid progressivity ($P<0.01$), MMP ($P<0.05$), live sperm rates with intact acrosome ($P<0.01$) and straightness (STR) ($P<0.001$) of the HDR group results were statistically better than LDR. It was concluded that the spermatological properties examined during storage were damaged at different times and were affected by sperm concentration and also storage time.

Keywords: *Acrosome, Concentration, Kinematics, MMP, Motility, Ram, Semen, Extender, Storage*

Koç Spermasının Kısa Süreli Saklanmasına Konsantrasyonun ve Muhafaza Süresinin Etkisi

Öz: Bu çalışma, koç spermasının 5°C'de 96 saat süre ile saklanmasında farklı sulandırma oranlarının sperm hareketleri ile ilişkili (motilite, progressivite ve ortalama hız) parametreleri, baş çapı, mitokondriyal membran potansiyeli ve canlı sağlam akrozoma sahip spermatozoa oranlarına etkilerinin ortaya konulması amacıyla gerçekleştirildi. Dört baş erişkin koçtan elde edilen normospermik özelliğe sahip ejekulatlar birleştirildi. Birleştirilen ejekulatlar ikiye bölünerek; Düşük Dilüsyon Oranı (LDR) (400 milyon) ve Yüksek Dilüsyon Oranı (20 milyon), motil spermatozoa tohumlama dozunda/0.25 mL payet olacak şekilde yağsız süt tozu yumurta sarısı bazlı sulandırıcı ile sulandırıldı. Genel olarak Düşük sulandırma oranı (LDR) grubunda TM ve progressivitenin Yüksek sulandırma oranı (HDR) grubuna göre daha hızlı azaldığı gözlemlendi. On ikinci saatten 96. saate kadar ki saklama süresinde, HDR grubunda elde edilen hızlı progresif motilitenin ($P<0.01$), mitokondriyal membran potansiyelinin ($P<0.05$) ve sağlam akrozoma sahip canlı spermatozoa oranının ($P<0.01$), doğrusallığın (STR) ($P<0.001$) LDR grubuna göre istatistiksel olarak daha yüksek olduğu gözlemlendi. Saklama süresince incelenen spermatolojik özelliklerin farklı zamanlarda zarar gördüğü, ayrıca sperm konsantrasyonu ve saklama süresinden de etkilendiği sonucuna varıldı.

Anahtar sözcükler: *Akrozom, Koç, Konsantrasyon, Kinematikler, MMP, Motilite, Saklama, Sperma, Sulandırıcı*

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Introduction

Artificial insemination (AI) is an assisted reproductive technique performed by transplanting a certain amount of fresh, diluted, cooled, or frozen sperm into the female reproductive channels. In livestock, AI is a helpful technique for genetic improvement and management of reproduction [1]. With the application of sperm storage (short-term or long-term) techniques, it is possible to benefit more from the genetically superior males in breeding programs [2].

The damaging effects of cryopreservation on ram sperm morphology, and function, and the high cost of intrauterine insemination; makes chilled, liquid preservation the most realistic current option for semen storage and cervical AI. Compared to frozen-thawed semen, liquid-stored semen possesses the advantages of convenient easier handling, higher fertility results, and economy [3]. Moreover, cooled semen insemination doses require fewer spermatozoa per dose, which means more AI doses per ejaculate. The main disadvantage of diluted, chilled, or cooled semen is its short fertile life compared to frozen semen, which makes it unfeasible for long storage periods or transportation over long distances [4,5]. For that reason, fresh, diluted or cooled ram semen AI is only an alternative to frozen semen when the insemination is done within a short period of time after collection [6-8].

There are many studies about liquid storage of mammalian semen published recently [9-16]. Lowering mammalian semen temperatures (5-12°C) results in a reduction of cellular metabolism and reactive oxygen species, thus prolonging sperm survival [17]. Furthermore, hypothermia destabilizes the activity of the sodium-potassium pump by causing intracellular sodium levels to rise to cytotoxic levels. Accordingly, it causes membrane damage [17,18].

During the dilution, cooling, storage and freeze-thawing process, the semen extenders protect spermatozoa metabolism in many ways, including stabilizing the plasma membrane and maintaining intracellular and intramembrane ionic concentrations, thus reducing cold shock damage and osmotic shock. The control of pH of the medium and bacterial growth provides extra support to reduce oxidative damages [17,19-22]. Sperm dilution rate affects the buffering capacity of the extender and its ability to remove metabolic residues [23]. Reducing the sperm concentration could facilitate not only the increased usage of selected sires but also the more prolonged storage period of liquid semen [24]. Lipid peroxidation is a metabolic process where reactive oxygen species (ROS) are formed by the oxidative degeneration of poly-unsaturated fatty acids [25]. Increasing sperm concentrations would increase ROS levels, also the presence of nonviable or poor-quality spermatozoa and cell debris in the milieu escalate ROS level. ROS overproduction negatively affects sperm function and morphology. The reduction of sperm concentration and lowering temperature decrease both the ROS production and acidification of the extender through the accumulation of lactic acid and CO_2 from glycolysis and oxidative phosphorylation, respectively [9,22,25,26]. Although high sperm concentration supports fertility, it can also shorten overall lifespan of spermatozoa by exhausting energy resources [21].

The sperm concentration and days of storage were associated with the deterioration in the structural, functional and sperm motility parameters [10,27-30]. A reduction in the quality of stored semen when the sperm dilution rate decreased was reported in ram [29,31], stallion [32], alpaca [30], and bulls [24,33,34]. There are relatively few studies on the effect of semen concentration on ram semen quality during liquid storage for 96 h at 5°C [29,35]. For intra-uterine insemination, the minimum effective dose of both fresh and frozen semen is $20x10^6$ motile spermatozoa [36-39] and for intravaginal insemination it is 400 x 10^6 motile spermatozoa [8,36,39]. Therefore, the objective of this study was to determine time-related changes in motility and kinetics parameters, MMP and acrosome integrity of ram semen stored at two different concentrations (High dilution rates (HDR): 20 x 10^6 and Low dilution rates (LDR): 400 x 10^6 motile spermatozoa/insemination dose) at 5°C for 96 h in skim milk-egg yolk-based extender.

Material and Methods

Ethical Statement

Semen collection procedures of the present study were approved by the local ethics committee of Sheep Breeding Research Institute, Ethics Committee on Animal Research (2021/048).

Animal Management and Semen Collection

This study was carried out during the breeding season, using 2-3 elderly fertility proven 2 Kivircik (Local Breed) and 2 Ramlic (Rambouillet*Daglic (Local Breed)) rams, which were raised in the Sheep Breeding Research Institute, which is located at 40.32 latitude, 27.91 longitude coordinates. During the study, the lowest and the highest temperatures were 17-26°C and relative humidity averaged 70%. During the study, rams and sheep were kept closed; 1 kg of cut feed (Barley, SSM, Salt and Marble powder) was given to the rams. In addition, 100 grams of raisins and 1 egg per day were given to the rams as supplementary nutrients.

Ram semen was collected by an artificial vagina and then quickly transferred to a 30°C water bath. Ejaculates, which have normospermic properties; (volume >0.9 mL, concentrations >1800 x 10^6/mL, mass movement >4 (1-

5) and total motility ≥70%) were used [23]. Initially neat sperm motility and mass activity were examined using a microscope (Olympus Corporation, CX31, Japan). Sperm concentration was determined using a sperm density meter (IMV Technologies, Ovine Accucell®, France). The experiment was repeated until 8 replicas were completed.

Semen Processing

Ejaculates which have normospermic characteristics were pooled to avoid the individual effect. Each pooled ejaculates were divided into 2 groups (High Dilution Rate (HDR) and Low Dilution Rate (LDR). The HDR group and LDR groups were diluted at 30°C with skim milk-based extender (10 g Skim milk powder (Sigma® 70166), 1 g of glucose (Sigma® G7021), 5% egg yolk) [16] at a final concentration 80 x 10^6 spermatozoa/mL and 1600 x 10^6 spermatozoa/mL, respectively. The extender osmolality was 380 mOsm.

Diluted semen temperatures were gradually reduced from 30°C to 4°C (at -0.3°C/min) within 2 h and stored for 4 days (until 96 h after collection) for further evaluation. Spermatological examinations; motility and kinematic parameters, live/intact acrosome, MMP were evaluated after dilution at 30°C (0^{th} h), after cooling to the 4°C (2^{nd} h), and at 12^{th}, 24^{th}, 48^{th}, 72^{nd} and 96^{th} hours during the storage period.

Semen Evaluation

- Sperm Motion Characteristic

A computer-assisted sperm analyzer (CASA) (SCA®, Microoptics, Spain) was used for the sperm motion characteristic [40]. For each evaluation semen was re-diluted with Tris-based egg yolk free extender at 16 x 10^6 spermatozoa/ml concentration to avoid over concentration failure. The 3 µL of re-diluted semen were placed in a specific slide (Leja®, Ref. 025107, IMV Technologies, France) for CASA. The CASA system provides progressive motility and total motility values with curvilinear velocity (VCL), rectilinear velocity (VSL), average path velocity (VAP), linearity (LIN) and straightness (STR), also; rapid, medium, slow sperm were examined. When evaluating spermatozoon motion properties, field settings; at least-up to 15-70 µm², speed settings; static <10 µm/s, slow to medium >45 µm/s, fast >75 µm/s, progressive (STR>80). Additionally, sperm head area was also analyzed. The analysis continued until at least 7 areas or at least 500 spermatozoa were analyzed.

- Flow Cytometry Analyses

All flow cytometer analyzes were performed on the Guava easyCyte® microcapillary flow cytometry device, which includes the CytoSoft program. (Guava Technologies Inc., Hayward, CA, USA; distributed by IMV Technologies). The device has a single blue laser (488 nm), two photodiodes (forward scatter, side scatter), three photo multiplicators (green: 525/30 nm, yellow: 583/26 nm, and red: 655/50 nm) and appropriate optical filters and brackets. Each analysis was carried out until 5000 sperm cells with scatter and fluorescent properties were counted. The performance of the device is checked daily with the Guava Check kit (Guava Technologies®, Inc., Millipore, Billerica, MA, USA).

- Plasma and Acrosome Integrity

Plasma and acrosome integrity of dead and living spermatozoa were analyzed by Easykit 5 kit (ref. 025293; IMV Technologies) that measures the integrity of the acrosome and cytoplasmic membrane simultaneously according to the manufacturer's protocol. For this purpose, 200 µL EasyBuffer B (Ref. 023862; IMV Technologies) and 5 µL of tris diluted semen (8000 spermatozoa/µL) were added to ready to use 96 well plate. The sperm was incubated at 37°C for 45 min. It was kept in a dark environment until the analysis was carried out. The analysis continued until 5000 sperm and the ratio of live/intact acrosome sperm were calculated by the program (EasySoft, ref. 024842; IMV Technologies) [41].

- Mitochondrial Membrane Potential (MMP)

Sperm MMP, (polarized/ depolarized) were analyzed by EasyKit 2 (ref. 024864; IMV Technologies) according to the manufacturer's protocol. Sperm with high concentrations of fluorochromes match as polarized mitochondria (with Δψm high) whereas mitochondria accumulating lower concentrations of fluorochromes were calculated as depolarized (and have a low Δψm). For this purpose, 10 µL of pure ethanol has been added to ready to use 96 well plate for the purpose of dissolving fluorochrome. Then 50000 sperm in total with 190 µL Easybuffer B and 5 µL of Tris diluted semen (8000 spermatozoa/µL) were added to the well plate. The sperm was incubated at 37°C for 30 min and kept in the dark until the analysis was performed. The analysis continued until 5000 sperm and mitochondrial potential density/ratio was calculated in the program (EasySoft, ref. 024842; IMV Technologies) [41].

Statistical Analysis

Obtained semen parameters were expressed as Mean ± Standard Error. Homogeneity of spermatological and kinematic parameters was controlled by Levene's test, one-way variance (One Way ANOVA) in comparing the difference between the groups; GLM procedure and Bonferroni multiple comparison test was applied in comparison of data obtained at different times in groups. All statistical analyses were performed using the SPSS package program (IBM® SPSS Statistics for Windows, Version 23).

Results

The changes of cooled sperm motilities (total, progressive, rapid, medium, slow and static), MMP and live sperm with intact acrosome in the function of concentration and storage period for 96 h were given in *(Fig. 1, Fig. 2, Fig. 3, Fig. 4, Fig. 5, Fig. 6, Fig. 7)*. The dilution rates had no effect on the semen characteristic except rapid and medium progressivity, Head diameters and STR at the dilution stage.

For the HDR group, the sperm total motility (P<0.001), progressive motility (P<0.001), rapid progressive motility (P<0.05), medium progressive motility (P>0.05) and MMP (P<0.05) were significantly decreased as storage time increased. For the velocity parameters; while sperm with rapid motion rate was decreased (P<0.001); the medium (P>0.05), slow (P<0.01) and static (P<0.001) sperm rates were increased. Sperm speed-related parameters VCL, VAP, VSL, ALH, and BCF decreased over time (P<0.001). For the LDR group, total motility (P<0.001), progressivity (total (P<0.001), rapid progressive motility (P<0.01) and medium progressive motility (P<0.001), MMP (P<0.01) and live sperm with intact acrosome (P<0.001) were significantly decreased as storage time increased. For the velocity parameters; while sperm with rapid motion rate was decreased (P<0.001); the medium (P>0.05), slow (P<0.001) and static (P<0.001) sperm rates were increased. The speed-related values VSL (P<0.001), STR (P<0.001), LIN (P<0.001), and BCF (P<0.01) were decreased with increased duration of storage (P<0.001).

After cooling at 5°C within 2nd h, while the sperm samples with HDR revealed higher progressive motility (P<0.01),

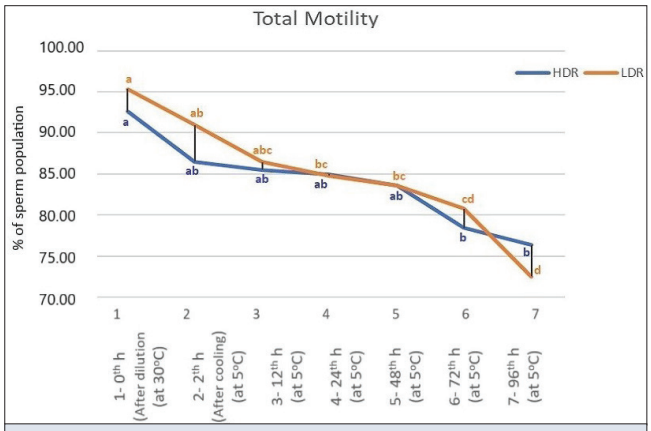

Fig 1. Total motility parameters in HDR and LDR groups for 96 h storage.

HDR: High Dilution Rate, LDR: Low Dilution Rate, **a,d:** The difference between the different letters during the storage is statistically significant in HDR and LDR groups (P<0.05)

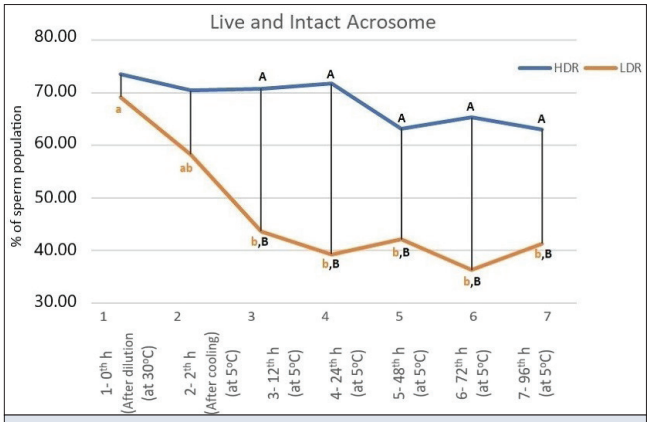

Fig 3. The sperm with live and intact acrosome parameters in HDR and LDR groups for 96 h storage

HDR: High Dilution Rate, LDR: Low Dilution Rate, **a,b:** The difference between the different letters during the storage is statistically significant in HDR and LDR groups (P<0.05), **A,B:** The difference between the different letter between the HDR, LDR groups is statistically significant at the same time zone (P<0.05)

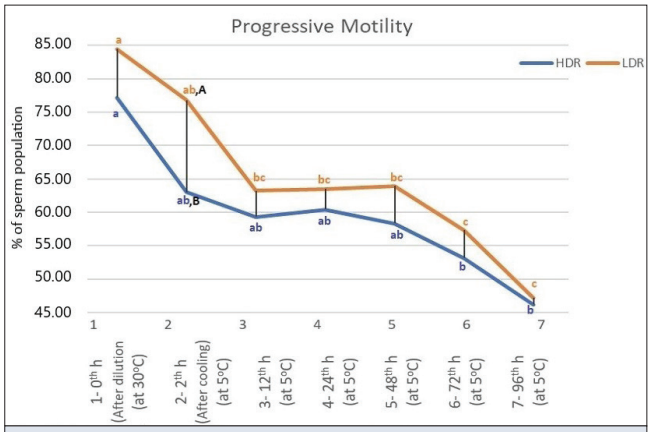

Fig 2. Progressive motility parameters in HDR and LDR groups for 96 h storage.

HDR: High Dilution Rate, LDR: Low Dilution Rate, **a,c:** The difference between the different letters during the storage is statistically significant in HDR and LDR groups (P<0.05), **A,B:** The difference between the different letter between the HDR, LDR groups is statistically significant at the same time zone (P<0.05)

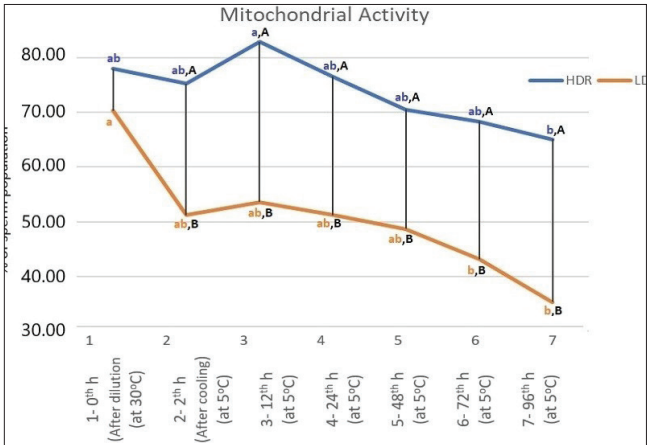

Fig 4. MMP parameters in HDR and LDR groups for 96 h storage.

HDR: High Dilution Rate, LDR: Low Dilution Rate, **a,b:** The difference between the different letters during the storage is statistically significant in HDR and LDR groups (P<0.05), **A,B:** The difference between the different letter between the HDR, LDR groups is statistically significant at the same time zone (P<0.05)

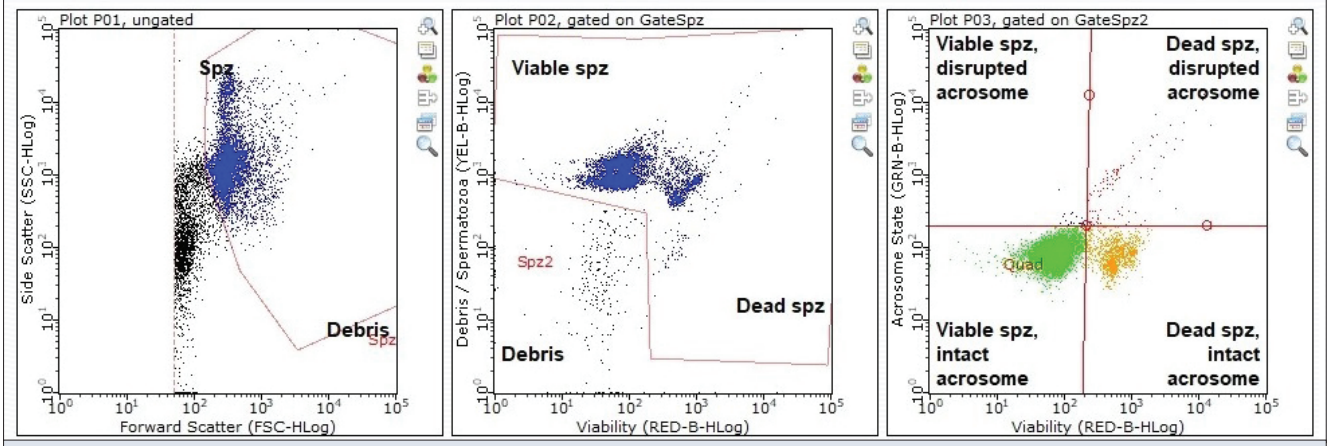

Fig 5. The obtained dotplots from flow cytometer analysis viability and acrosome ıntegrity at the rightest histogram. Green population: Viable and intact acrosome, Orange population: Dead and intact acrosome, Red points: Dead and damaged acrosome, Blue points: Viable but damage acrosome

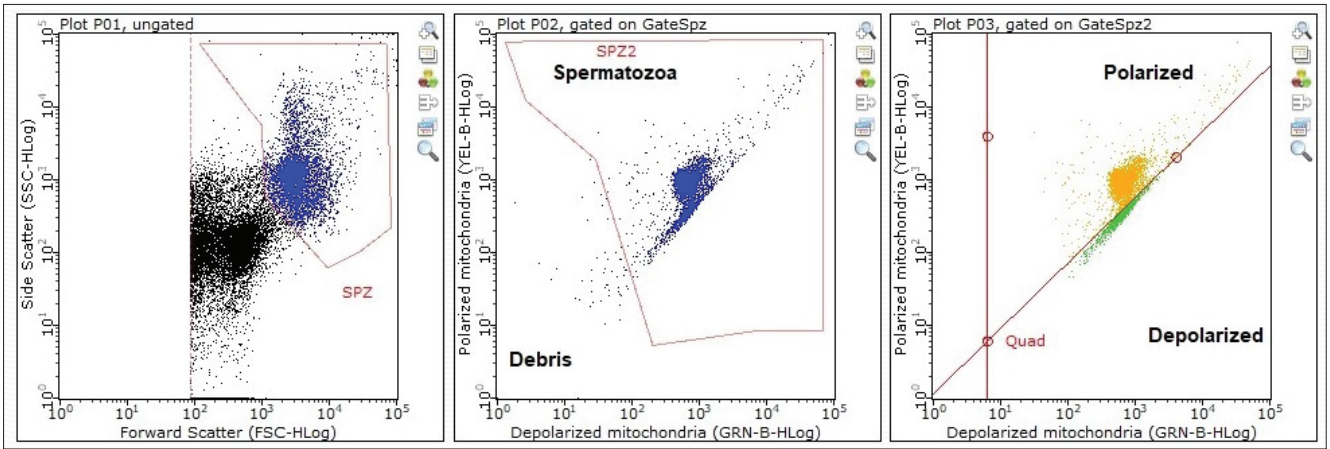

Fig 6. The obtained dotplots from flow cytometer analysis (Mitochondrial membrane potential)

At the rightest histogram (Orange population; Polarized mitochondria/High Mitochondrial Potential), (Green population; Depolarized mitochondria/Low Mitochondrial Potential)

rapid progressive motility (P<0.01), MMP (P<0.001) and slow sperm ratio (P<0.01), the sperm samples with LDR revealed higher progressive motility (P<0.05), medium (P<0.001) progressive motility and head diameters (P<0.05). From 12th h to 96th h; the rapid progressivity (P<0.01), MMP (P<0.05), live sperm rates with intact acrosome (P<0.01) and STR (P<0.001) of the HDR group results were better than LDR. The LIN (P<0.01) was better until 72nd h. The LDR group medium progressivity (P<0.01) and VAP (P<0.05) were better than HDR from the 12th to the 72nd h.

It was observed that the TM and PM of the LDR group decreased more rapidly than HDR group over time. Statistically, while both TM and PM of the HDR group decreased at the 72th h, TM and PM in the LDR group decreased at the 24th h and 12th h; respectively (P<0.05). The rapid progressive motility rates of the HDR were close to LDR during storage. While the medium progressive motility rate of the HDR group was similar to the diluted semen until 96th h, the LDR group differed at 72nd h of the storage. The MMP of HDR group was similar to the diluted semen until 72nd h but in the LDR group it was similar until 48th h (P<0.05). For the sperm rates with intact acrosome, in the HDR group, the increasing dilution rates protected sperm rates with intact acrosome until 96th h, but in the LDR group this protection was only until 2nd h (P<0.05). The velocity parameters were similar except rapid and slow spermatozoa rates at 2nd h in general.

However, in the both groups time-related head diameter differences at the different time zones were similar, the LDR group head diameter was slightly higher than HDR group in general.

The kinematic parameters in HDR and LDR groups for 96th h were given in *Table 1*. While VCL, VAP and ALH of LDR group were similar to the diluted semen until 96th

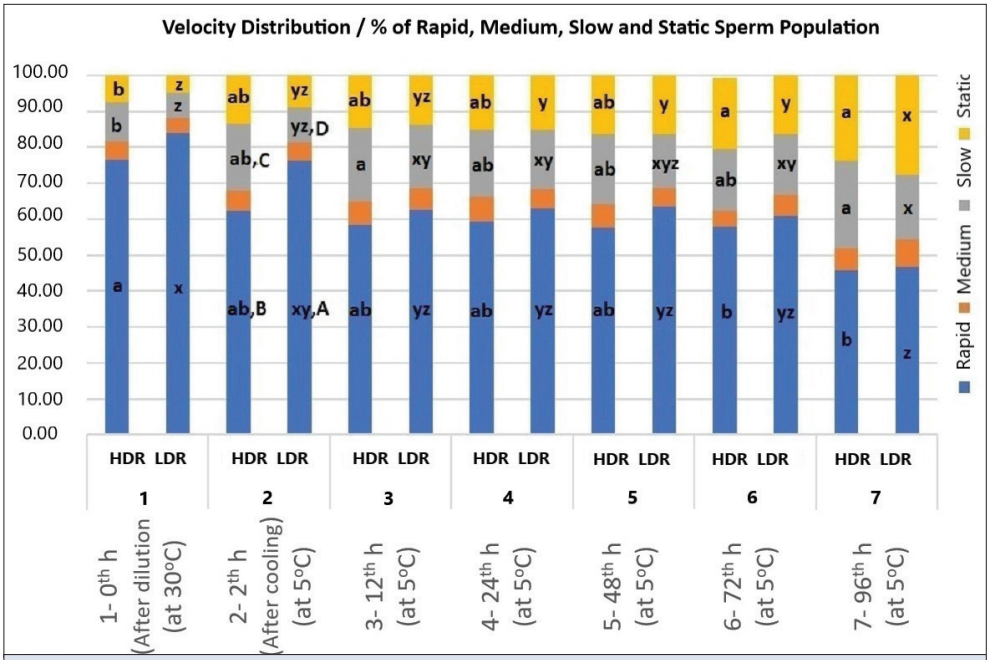

Fig 7. Velocity releated sperm ratios in HDR and LDR groups for 96 h storage.

HDR: High Dilution Rate (Laparoscopic insemination dose 20x10^6 sp/straw), **LDR:** Low Dilution Rate (Cervical insemination dose 400x10^6 sp/straw), **a,b:** The difference between different letters for the same characteristic (rapid, medium, slow or static) is statistically significant in HDR group. (P<0.05), **x,y,z:** The difference among different letters for the same characteristic (rapid, medium, slow or static) is statistically significant in LDR group (P<0.05), **A,B:** The difference between different letters for the same characteristic (rapid) is statistically significant in the same time zone (P<0.05), **C,D:** The difference between different letters for the same characteristic (slow) is statistically significant in the same time zone (P<0.05)

h, these parameters in the HDR group were until 2nd h, 2nd h, and 12nd h, respectively (P<0.05). The VSL of both groups were similar to the diluted semen until 72nd h. The cooling, storage period, and dilution rates did not affect the LIN and STR of the HDR group until 96th h but in the LDR group this similarity was until 24th h. In addition, the WOB and BCF of both groups were similar to the diluted semen until 96th h.

Discussion

In this study, we evaluated the effects of different dilution rates on ram sperm motion related (motility, progressivity, velocity and average of speed) parameters, head diameter, MMP and live intact acrosome rate during liquid storage for 96 h at 5°C. Fresh ram semen volume, concentration and motility ranges were 0.5 to 2.0 mL, 3.5 x 10^9 to 6.0 x 10^9 spermatozoa/mL and greater than 85%, respectively. The characteristics of fresh semen were in agreement with the data on ram semen reported in the literature [42-45].

Semen dilution removes adsorbed proteins, natural antioxidants, and other beneficial components in seminal plasma that are required for the maintenance of the membrane integrity and function of spermatozoa [33]. There have been considerable studies on the changes that occur when semen is diluted with different media which is known as dilution effect [33]. Dilution effect may be minimized by appropriate dilution rate and by extender with balanced salt, energy and buffering capacity [33]. The higher volume of seminal plasma and its contents may be one of the reasons for slightly better preservation of functional parameters at the low diluted group at 0th h. The increasing dilution rates increased rapid progressive motility and STR but not medium progressive motility and head diameters (P<0.05). The loss of intracellular water affects protein structure and function, which alter cell enzyme activity [46]. In this study, sperm head diameters of the LDR group were bigger than the HDR group (P>0.05).

Mainly, liquid sperm preservation involves slowing metabolic and biochemical function of spermatozoa by declining temperatures to 0-15°C [23,32,35]. Sperm cooling to +5°C has a controversial effect on semen parameters [47]. It was observed that cooling to +5°C within 2 h negatively affected observed semen parameters. Reducing sperm temperatures for cold storage to 5°C inflicts sub-lethal damages to spermatozoa [21].

Storage temperature and duration, concentration, and extender ingredients can influence sperm quality and survivability during liquid preservation [21]. At the 2nd h of the storage, the HDR revealed higher rapid progressivity (P<0.01), MMP (P<0.001), and slow velocity (P<0.01), and lower total (P<0.05) and medium (P<0.001) progressivity, medium velocity and head diameters (P<0.05), compared to LDR group, in the study.

Table 1. *The average values of kinematic parameters and head areas in HDR and LDR groups for 96 h storage*

Kinematic Parameters		Group	n	0th h	2nd h	12th h	24th h	48th h	72nd h	96th h	*P Value*
				Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	
Average Values of Speed	Head Area (μm²)	HDR	8	42.06±0.34^{B}	41.58±0.45^{B}	41.68±0.26^{B}	41.87±0.41	41.73±0.42^{B}	40.93±0.20^{B}	40.94±0.34	0.204
		LDR	8	43.08±0.22^{A}	43.13±0.36^{A}	42.87±0.32^{A}	42.57±0.46	42.97±0.36^{A}	42.57±0.31^{A}	42.11±0.64	0.573
			P Value	0.025	0.019	0.014	0.281	0.043	0.001	0.136	
	VCL (μm/s)	HDR	8	250.80±11.50^{a}	211.50±14.80^{ab}	198.98±8.08$^{bc.B}$	191.19±8.37$^{bc.B}$	189.30±8.29^{bc}	194.14±9.79^{bc}	157.86±9.35^{c}	0.001
		LDR	8	256.40±29.60	237.50±28.50	242.60±16.50^{A}	263.20±19.80^{A}	245.70±27.60	230.50±16.20	187.70±23.80	0.390
			P Value	0.862	0.430	0.033	0.005	0.070	0.075	0.263	
	VAP (μm/s)	HDR	8	152.14±7.55^{a}	125.96±8.44^{ab}	116.47±5.72$^{bc.B}$	114.99±4.98$^{bc.B}$	113.71±5.40$^{bc.B}$	116.29±6.18$^{bc.B}$	91.84±6.47^{c}	0.001
		LDR	8	159.10±15.50	147.30±14.50	145.07±8.95^{A}	158.50±11.00^{A}	146.30±13.40^{A}	145.00±8.49^{A}	118.40±12.00	0.313
			P Value	0.692	0.223	0.018	0.003	0.040	0.016	0.072	
	VSL (μm/s)	HDR	8	95.01±6.08^{a}	76.35±7.35^{ab}	75.68±4.17^{ab}	74.50±4.14^{ab}	70.12±5.78^{b}	71.96±4.29$^{ab.A}$	55.01±4.83^{b}	0.001
		LDR	8	85.55±8.37^{a}	80.47±7.98^{a}	75.74±5.59^{a}	77.95±5.91^{a}	66.74±5.34^{ab}	59.94±3.33$^{ab.B}$	47.66±4.58^{b}	0.001
			P Value	0.376	0.710	0.993	0.639	0.674	0.044	0.288	
	STR (%)	HDR	8	59.40±2.42^{A}	56.39±2.74	60.49±1.42^{A}	60.00±2.00^{A}	56.89±2.54^{A}	56.87±1.20^{A}	52.68±2.02^{A}	0.157
		LDR	8	52.37±1.10$^{a.B}$	52.80±1.38^{a}	50.65±1.49$^{a.B}$	48.80±1.65$^{ab.B}$	44.34±1.24$^{bc.B}$	40.85±1.62$^{c.B}$	39.68±0.78$^{c.B}$	0.001
			P Value	0.019	0.260	0.001	0.001	0.001	0.001	0.001	
	LIN (%)	HDR	8	35.31±1.37	32.41±1.47	34.32±1.32^{A}	34.97±1.34^{A}	33.16±2.06^{A}	33.36±1.25^{A}	28.72±1.83	0.075
		LDR	8	33.37±1.31^{a}	33.36±1.21^{a}	29.37±0.61$^{ab.B}$	28.71±0.84$^{abc.B}$	25.81±0.67$^{bc.B}$	24.74±1.15$^{bc.B}$	24.27±1.30^{c}	0.001
			P Value	0.326	0.626	0.004	0.001	0.004	0.001	0.067	
	WOB (%)	HDR	8	60.15±1.89	58.38±1.38	56.85±1.83	58.20±0.84	57.81±1.37	57.96±1.24	53.99±1.83	0.192
		LDR	8	64.11±2.45	63.60±2.02	59.13±1.00	59.35±0.81	58.04±1.81	60.34±0.72	60.33±2.36	0.129
			P Value	0.221	0.051	0.292	0.344	0.921	0.119	0.052	
	ALH (μm)	HDR	8	2.20±0.09^{a}	1.92±0.12^{ab}	1.84±0.06^{abc}	1.77±0.07$^{bc.B}$	1.75±0.06^{bc}	1.79±0.07^{bc}	1.54±0.07^{c}	0.001
		LDR	8	2.21±0.23	2.07±0.22	2.12±0.13	2.27±0.15^{A}	2.14±0.23	1.98±0.13	1.68±0.19	0.407
			P Value	0.969	0.562	0.093	0.013	0.125	0.258	0.501	
	BCF (Hz)	HDR	8	30.27±1.85^{a}	24.57±1.96^{ab}	23.90±1.34^{ab}	24.26±1.29^{ab}	23.27±1.98^{ab}	23.50±1.51^{ab}	18.03±1.36^{b}	0.001
		LDR	8	26.58±1.71^{a}	24.84±1.44^{ab}	21.94±1.51^{ab}	21.61±1.40^{ab}	22.07±1.57^{ab}	20.40±0.85^{ab}	18.73±1.13^{b}	0.005
			P Value	0.165	0.913	0.348	0.189	0.644	0.097	0.699	

HDR: *High Dilution Rate (Laparoscopic insemination dose. 20x10⁶ sp/straw);* ***LDR:*** *Low Dilution Rate (Cervical insemination dose. 400x10⁶ sp/straw);* ^{a,d} *The difference between different letters in the same row is statistically significant (P<0.05)*
0th h *(After dilution. at +30°C);* ***2th h*** *(After cooling. at +5°C. -0.3°C/min);* ***12th h, 24th h, 48th h, 72th h, 96th h*** *(at +5°C)*
^{A,B} *The difference between different letters in the same column is statistically significant (P<0.05)*

Ram sperm concentration significantly affect the sperm structural and functional parameters during the storage period at 4°C [29]. Both sperm concentrations and storage duration significantly affected the motility, morphology and membrane integrity [35]. For the HDR and LDR group; the sperm total motility (P<0.001 and P<0.001), progressive motility (P<0.001 and P<0.001), rapid progressive motility (P<0.05 and P<0.01), medium progressive motility (P>0.05 and P<0.001), MMP (P>0.05 and P<0.01) and live sperm with intact acrosome (P>0.05 and P<0.001) were significantly decreased as storage time increased, respectively. The declines in these sperm parameters during storage period may be originated from the metabolic activity, metabolic products, and pH changes [19,21]. For the velocity-related parameters of both HDR and LDR groups, it was observed that while there was a time-dependent decrease with sperm rapid motion (P<0.001); there was an increase with medium (P>0.05), slow (P<0.01) and static (P<0.001) motion. For the HDR group the VCL, VAP, VSL, ALH (P<0.001) and BCF and for the LDR group the VSL, STR

and LIN (P<0.001), and BCF (P<0.01) values of speed were decreased with increased duration time.

The decrease of MMP over time may occur due to the metabolic activities at 4°C and the changes in the pH due to metabolic products [29]. At low sperm concentrations, reduced ROS production persists throughout the storage period [24]. The MMP of HDR group was similar to the diluted semen until 72nd h but the LDR group was similar until 48th h (P<0.05). The lower numbers of sperm consume less glucose from media than with higher concentrations so that more energy source remains in the environment. The group with lower sperm numbers retained higher viability from 6 h to 5 d compared with treatments with higher concentrations [24]. Also as expected; the rapid progressivity (P<0.01), MMP (P<0.05), live sperm rates with intact acrosome (P<0.01), STR (P<0.001) and LIN (P<0.01) in the HDR group retained better than that of in LDR group; from the 12th h to the 96th h, in our study.

It was observed that different spermatological motion features are changed at different times and it was affected by the sperm concentration. The possible physiological reasons for lower motility in LDR group may be due to the effects of endogenous free radical production.

The semen extenders reduce the protective effects of the beneficial components of seminal plasma that are required for the maintenance of membrane integrity and functions of spermatozoa [48]. The VCL, VAP and ALH were similar to the diluted semen until 96th h in LDR group but until 2nd h, 2nd h, and 12nd h in the HDR group were, respectively (P<0.05). The VSL of both groups was similar to the diluted semen until 72nd h. The higher volume of seminal plasma and its contents in LDR group may be one of the reasons for better preservation of functional parameters.

The sperm diluted to 10 million spermatozoa/mL and stored at ambient temperature retained higher viability, and lower osmotic and ROS stress compared with the sperm stored at 60 million spermatozoa/mL [24,34]. A decline in acrosome integrity of alpaca sperm stored at 4°C is not observed until 72 h after the onset of liquid storage [30]. In the HDR group, the increasing dilution rates protected spermatozoa acrosomes until 96th h, but in LDR group it was until only 2nd h (P<0.05). This difference could be explained by differences of the osmotic stress and ROS level of both groups.

A low sperm number in a high osmotic environment causes more water to be released into the environment compared to the high number group. The LDR group head diameter was slightly higher than HDR in general. However, time-related head diameter differences of both groups were similar. The larger mean values of sperm head area are associated with larger mean values of ALH, a parameter which is related to sperm hyperactivation [49]. The ALH was slightly higher in LDR group that have larger head diameters.

In summary, we conclude that different spermatological motion features, MMP and live/intact acrosome rates changed at different times and it was affected by the sperm concentration and storage time. Following storage for 96 h, better sperm characteristics were achieved in the HDR group. While important sperm characteristics for fertility (such as; total motility, progressive motility, rapid progressive motility, medium progressive motility, MMP, live and intact acrosome, VSL, STR and LIN) did not change dramatically until 72th h in HDR group, however, these semen characteristics of the LDR group changed statistically earlier (at the 2nd h).

Availability of Data and Materials

The authors declare that the data that support the findings of this study are available from the corresponding author (C. Dayanıklı), upon reasonable request.

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Conflict of Interest

None of the authors have any conflict of interest to declare.

Ethical Statement

Semen collection procedures of the present study were approved by the local ethics committee of Sheep Breeding Research Institute, Ethics Committee on Animal Research (2021/048).

Author Contributions

CD: experimental design, methodology and investigation, semen collection, spermatological analysis, formal analysis, wrote manuscript. ES: semen collection, spermatological analysis. BB: contribution to experimental design, methodology and investigation, formal analysis, editing manuscript. BU: editing manuscript. ZN: experimental design, writing manuscript & editing, visualization, supervision. All authors read, revised, and approved the final manuscript.

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Research Article

Determination of Macro and Trace Element Levels of Serum, Tears, Saliva, and Hair Samples in Kilis Goats with ICP-MS

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Abstract: This study is focused on the evaluation of macro and trace elements concentration including sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), copper (Cu), zinc (Zn), selenium (Se), nickel (Ni), titanium (Ti), manganese (Mn), cobalt (Co), and chromium (Cr) of the serum, saliva, tears, and hair samples in Kilis goats. The study involved 33 goats without clinical signs of disease. Individual serum, saliva, tears, and hair samples from each goat were collected for element quantifications through the inductively coupled plasma mass spectrometry (ICP-MS) method after acid digestion in a microwave system. Element concentrations varied and depended on the sample type. The most predominant element in serum and saliva samples was Na (3265±53.6 and 3559±55.2 mg/L, respectively), followed by K>Ca>Mg. Potassium was the most abundant element analyzed in tears and hair samples (3506±305 and 4664±100.5 mg/L, respectively). The major trace element was Fe in all sample types except hair samples. Nickel was detected only in serum samples (83.9±6.35 µg/L) and Mn was detected only in hair samples (12903±3142 µg/L). In all samples, Co was below the detection limit. There was a significant correlation of some elements between serum and hair (Na, K, Mg, Cu), tears and hair (K, Mg, Fe), serum and tears (K) samples. Trace elements concentration did not pose a risk for deficiency or excess. It was concluded that tears and saliva are potential alternative materials for element analysis and simultaneous tears, saliva, and hair along with serum could be useful in predicting the inorganic metabolic status of goats.

Keywords: *Element, Goat, Hair, ICP-MS, Saliva, Tears*

Kilis Keçilerinde Serum, Gözyaşı, Tükürük ve Kıl Örneklerinin Makro ve Eser Element Düzeylerinin ICP-MS ile Belirlenmesi

Öz: Bu çalışma, Kilis keçilerinde serum, tükürük, gözyaşı ve kıl örneklerinde makro ve eser elementlerden sodyum (Na), potasyum (K), kalsiyum (Ca), magnezyum (Mg), demir (Fe), bakır (Cu), çinko (Zn), selenyum (Se), nikel (Ni), titanyum (Ti), manganez (Mn), kobalt (Co) ve krom (Cr) konsantrasyonlarını belirlemeye odaklanmıştır. Klinik hastalık belirtisi göstermeyen 33 Kilis keçisinin herbirinden, mikrodalga sisteminde asitle parçalamanın ardından indüktif eşleşmiş plazma-kütle spektrometresi (ICP-MS) yöntemiyle element miktar tayini için serum, tükürük, gözyaşı ve kıl örnekleri toplandı. Element konsantrasyonları değişkendi ve örnek tipine bağlıydı. Serum ve tükürük örneklerinde en çok bulunan element Na (sırasıyla 3265±53.6 ve 3559±55.2 mg/L) ve ardından K>Ca>Mg'du. Potasyum, gözyaşı ve kıl örneklerinde analiz edilen en bol elementti (sırasıyla 3506±305 ve 4664±100.5 mg/L). Kıl örnekleri hariç tüm örnek tiplerinde ana eser element Fe idi. Nikel sadece serum örneklerinde (83.9±6.35 µg/L) ve Mn sadece kıl örneklerinde (12903±3142 µg/L) tespit edildi. Tüm örneklerde Co, tespit limitinin altındaydı. Serum-kıl (Na, K, Mg, Cu), gözyaşı-kıl (K, Mg, Fe) ve serum-gözyaşı (K) örnekleri arasında bazı elementlerde önemli korelasyonlar vardı. Eser element konsantrasyonları eksiklik veya fazlalık için bir risk oluşturmamaktaydı. Gözyaşı ve tükürüğün element analizi için potansiyel alternatif materyaller olduğu ve eş zamanlı gözyaşı, tükürük ve kılın serum ile birlikte keçilerin inorganik metabolik durumunu tahmin etmede faydalı olabileceği sonucuna varıldı.

Anahtar sözcükler: *Element, Gözyaşı, ICP-MS, Keçi, Kıl, Tükürük*

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Introduction

Goat breeding is a global activity and Turkey makes a significant contribution to this activity. Kilis goats, consist of nearly half a million of Turkey's goat population and are mostly spread in the South-eastern region of Anatolia, draw attention to their high milk capacity and breeding performance under comprehensive farming systems [1].

Elements are vital for the metabolism of all mammals as they are essential in biological processes [2]. Essential elements are classified as macro and trace elements, depending on the concentration needed in diet and animal tissues. The macro elements exist in all tissues and fluids, and they act a crucial part in nerve transmission, muscle contraction, a healthy immune system, and acid-base homeostasis [3]. Trace elements are vital components of wide biological functions such as oxygen transport, vitamin and hormone synthesis, cell metabolism, collagen synthesis, energy production, and enzyme activity [4]. An increase or decrease in trace element concentrations is generally associated with abnormalities in metabolic, reproductive, immunological, and hormonal functions. This imbalance could be of varying severity and occasionally causes characteristic clinical signs [5,6]. Element deficiencies that cause reproductive disorders, reduced growth and milk production, and high mortality in small ruminants may not always be accompanied by clinical symptoms. Therefore, reliable biological material research involving different tissue, body fluids and hair comes into prominence in the diagnosis of inorganic metabolism imbalances in veterinary medicine [7].

The tear is essential for the health and homeostasis of the ocular surface [8]. Information on the elemental concentration of the tear fluid is scarce. The balance of the components of the tear fluid is disturbed in many systemic diseases. Therefore, it has the potential to be a reliable source of information on many disease states. There are current studies on the use of tears in the diagnosis of many diseases, including systemic sclerosis, cancer, thyroid disorders, diabetes mellitus, diabetic peripheral neuropathy, diabetic retinopathy, Alzheimer's disease, Parkinson's disease, multiple sclerosis (MS), migraine, and cystic fibrosis in humans [9].

Saliva reflects serum biomarkers, as serum components could reach the entire saliva by passive diffusion or gingival crevicular fluid [10]. In recent years, various studies on different animal species have reported that the analysis of health status markers in saliva samples is more sensitive than those performed in serum samples. At the same time, more economical, easy, fast, non-invasive sampling with minimal equipment makes saliva a good choice as an alternative diagnostic fluid. Saliva is more than a reflection of animal serum biomarkers [11,12].

Hair has a very active metabolism during its growth and is affected by health and nutrition status. Due to its structure, elements that cannot be detected in the blood at the same time can be found in the hair at a significant concentration, which has led to the evaluation of the hair as an important biological material in element analysis [13]. The analysis of animal hair is considered one of the fast and most effective ways to obtain sufficient information to assess inorganic metabolism and health status [14,15].

The below-average production and reproductive performance of ruminants require assessment of metabolic status. In the evaluation of the inorganic metabolic state, which is an important part of the general metabolic state, studies that include the determination of reliable biological materials and methods are valuable. The studies on the element concentrations in the various body fluids and tissue of goats are quite limited. The purposes of this study were to (i) determine inorganic metabolism status by simultaneously detecting the macro and trace element concentrations of serum, saliva, tears, and hair samples in Kilis goats, (ii) state the suitability of different biological materials for the ICP-MS method, and (iii) indication the correlation between the element concentrations of serum, tears, saliva, and hair samples.

Material and Methods

Ethical Statement

The study was conducted with the permission of Harran University Animal Experiments Local Ethics Committee (HRUHADYEK) with the number 2021/009/02.

Animals and Sampling

One-three years old 33 without clinical signs of disease Kilis goats lived in mid-lactation period were included in the present study. All animals were fed on pasture in Şanlıurfa (37°10'N: 38°47'E) at an altitude of 518 m above sea level. Fresh water was available *ad libitum*. The serum, tears, saliva, and hair samples were collected once from each goat in April-May.

Blood samples were collected from V. jugularis into vacuum serum tubes, 10 mL tubes without anticoagulant with a negative pressure system for serum. A capillary tube was used to collect tears samples. The standing animals had their heads tilted slightly and their eyelids opened gently. Tears accumulated in the lateral canthus were transferred to eppendorf tubes by capillary tubes [16]. For saliva samples, the sponge was placed on the cheek of the goat for an average of 1 minute with the help of forceps. Sponges were placed in tubes and transported to the laboratory [12]. All blood, tears, and saliva samples were separated by centrifugation (3000 rpm for 10 min) and stored at -20°C until analysis.

The hair samples were collected from the shoulder area using ethanol-precleaned stainless steel scissors and before being analyzed were washed in acetone in 10-15 minutes and then rinsed thrice in ultrapure water [17].

Chemicals and Standard Solutions

The chemicals, 37% (v/v) HCl, 65% (v/v) HNO_3, 30% (v/v) H_2O_2, were purchased from Merck (Germany). Stock standard solutions of elements were provided by Agilent Japan: Lot Number: 10-160YPY2. Standart certified reference material (NIST SRM 8435) was obtained from Nova Chimica (Milano, Italy). Argon gas (99.9990%) was provided by Linde Gases (Linde Group, Turkey).

Sample Preparation and Microwave Acid Digestion

For the purpose of eliminating possible contamination of elements, the whole equipment was previously kept for one night in 10% HNO_3 and then washed with ultrapure water. A closed microwave-assisted digestion procedure was used for all samples mineralization. Each serum (1.0 mL), saliva (100 µL) and tears (100 µL) samples were taken in tetrafluoroethylene vessels was mixed with 0.5 mL HNO_3, 1.5 mL HCl, and 0.25 mL H_2O_2. Hair samples (100 mg) taken into tubes were mixed with 4 mL HNO_3 and 2 mL H_2O_2. After digestion was complete tetrafluoroethylene vessels were cooled to room temperature. To optimize the pH for analysis by ICP-MS, the samples were transferred into a 50 mL polyethylene volumetric flask and diluted to 50 mL with ultrapure water (MES MP Mini pure, Türkiye). They were stored at +4°C until analyzed. The microwave digestion procedure of blanks was the same as the samples.

ICP-MS Procedure and Quality Assurance

Element analyses were determined on an Agilent 7500ce with an octopole reaction system inductively coupled plasma-mass spectrometer with an autosampler (Cetac ASX-520) and a nebulizer (Agilent, Japan). All samples were analyzed in three times. These isotopes were preferred to minimize interferences and maximize sensitivity. Recovery of 13 elements in all samples was between 96.8-102.0%.

Statistical Analyses

Statistical calculations were carried out using SPSS 22.0 software (SPSS Inc., Chicago, USA). Element concentrations of serum, tears, saliva and hair were compared by analysis of variance with repeated measures. A paired sample t-test was used for multiple comparisons. Relationships among numeric variables were developed using the Spearman rank correlation coefficient. The P-value lower than 0.05 was considered statistically significant. The graphics were created using the R "Hmisc" and "corrplot" packages [18,19]. The correlations between the elements of serum, tears, saliva, and hair were calculated while the significance value was evaluated.

Results

The concentration and comparison of macro and trace elements in serum, tears, saliva, and hair samples are presented in *Table 1*. There were statistical differences between the Na, K, Ca, Mg, Fe, Cu, Zn, Se, Ti, and Cr concentrations of different biological material samples ($P<0.05$). Nickel was detected only in serum samples and Mn was detected only in hair samples. In all samples, Co was below the detection limit.

Sodium was the most abundant macro element in serum and saliva samples (3265±53.6 and 3559±55.2 mg/L, respectively), followed by K>Ca>Mg. The major trace element found in serum samples was Fe (3.33 mg/L), followed by Zn>Ti>Cu>Cr>Se. Manganese and Co levels were below the detection limit in serum samples. In saliva samples, the most abundant trace element was Fe (1.79 mg/L), followed by Cr>Zn>Cu>Se>Ti. In saliva samples, Ni, Mn, and Co levels were below the limit of detection.

The most abundant macroelement in tears samples was K (3506 mg/L), followed by Na>Ca>Mg. The major trace element found in tears samples was Fe (5.02 mg/L), followed by Cr>Zn>Cu. Selenium, Ni, Ti, Mn, and Co levels were below the limit of detection in tears samples.

According to our study results, in hair samples the most predominant macroelement was K (4664 mg/kg), followed by Ca>Mg>Na. As for the trace elements, Zn (118.0 mg/kg) was the most notable in the hair samples, followed by Fe>Ti>Se>Mn>Cu. The levels of Ni, Co, and Cr were below the detection limit in hair samples.

The correlation of elements in serum, tears, saliva, and hair samples are presented in *Table 2*. Most of the significant correlations among elements were found in the hair samples. In all samples, the elements most correlated with other elements were Na, K, and Fe. *Fig.1* shows a correlation heatmap between serum, tears, saliva, and hair elements.

Table 3 represents correlations for pairs of elements between serum, tear, saliva, and hair samples. These correlations were verified, taking into concideration only those significant coefficient ($P<0.05$). A very strong correlation was detected between the serum and hair Na concentrations (rho: 0.703) ($P<0.01$). In terms of K values, there was a strong correlation between serum and tears (rho: -0.530), serum and hair (rho: -0.383), and tears and hair (rho: 0.492). In addition, a significant correlation was detected in Fe concentrations between the tears and hair samples (rho: -0.475) and in Cu concentrations between serum and hair samples (rho: 0.400).

Table 1. *Element concentrations and comparison in serum, tears, saliva, and hair samples*

Element	Serum		Tears		Saliva		Hair		P Value
	Min-Max	X ± Sx (n)	Min-Max	X ± Sx (n)	Min-Max	X ± Sx (n)	Min-Max	X ± Sx (n)	
Na (mg/L)	2477-4031	3265±53.6[a] (33)	448-3111	1989±159[b] (33)	3088-4120	3559±55.2[c] (33)	382-2709	1534±88.6[1,b] (33)	***
K (mg/L)	148.8-362.1	232.3±10.1[a] (33)	1410-5011	3506±305[b] (33)	166.1-491.1	243.6±17.6[a] (33)	3464-5684	4664±100.5[1,c] (33)	***
Ca (mg/L)	61.4-112.7	89.8±2.05[a] (33)	16.5-64.7	37.2±3.0[b] (33)	5.34-23.24	14.18±1.17[c] (33)	454.5-4678.5	2731±215.1[1,d] (33)	***
Mg (mg/L)	14.4-38.4	24.9±0.76[a] (33)	9.2-48.4	20.8±1.96[b] (33)	5.57-36.8	10.7±1.6[c] (33)	15.9-882.7	437.3±39.6[1,d] (33)	***
Fe (mg/L)	1.1-19.1	3.33±0.52[a] (33)	0.84-7.87	5.02±2.35[b] (33)	1.15-3.09	1.79±0.1[c] (33)	44.9-593.4	114.8±19.7[1,d] (33)	***
Cu (µg/L)	271.7-1212.5	435.4±34.7[a] (33)	53.8-315.6	193.4±21.9[b] (33)	177.7-395.1	285.7±14.9[c] (33)	8440-11725	10007±192.4[2,d] (33)	***
Zn (mg/L)	0.194-2.684	1.392±0.574[a] (33)	0.591-3.117	1.669±0.168[a] (33)	0.057-1.334	0.370±0.107[b] (12)	87.56-186.6	118.0±4.56[2,c] (33)	***
Se (µg/L)	169-537	231.2±25.2[a] (30)	-	<LOD	133-382	136±13.8[b] (33)	5812-20037	13802±655[2,c] (33)	***
Ni (µg/L)	53.3-131.1	83.9±6.35 (12)	-	<LOD	-	<LOD	-	<LOD	N.A.
Ti (µg/L)	355.2-606.9	497.2±10.7[a] (33)	-	<LOD	90.2-179.8	125.3±5.58[b] (33)	11780-86820	18699±931[2,c] (33)	***
Mn (µg/L)	-	<LOD	-	<LOD	-	<LOD	632-70050	12903±3142[2] (33)	N.A.
Co (µg/L)	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-
Cr (µg/L)	73-845	427.7±43.6[a] (23)	1474-1898	1733.4±62.2[b] (6)	93-1283	821±93.4[c] (33)	-	<LOD	***

[1] Concentration is expressed as mg/kg, [2] concentration is expressed as µg/kg, limit of detection is expressed as LOD, [a-d] Different letters in the same row indicate significant differences (P<0.05)

Table 2. *The Spearman's rank significant correlation of macro and trace elements in serum, tears, saliva and hair samples*

Serum		Tears		Saliva		Hair	
Element	rho	Element	rho	Element	rho	Element	rho
Zn-Na	-0.584**	Na-K	0.627**	K-Cu	0.750**	Fe-Na	0.634**
Zn-K	-0.449**	Na-Fe	0.766**	K-Fe	0.602**	Fe-Mg	0.548**
Zn-Ca	0.647**	Na-Cu	0.775**	Ti-Mg	0.680**	Fe-Ca	0.625**
Zn-Ti	0.459**	K-Fe	0.810**	Ti-Ca	0.677**	Fe-Ti	0.754**
Zn-Fe	0.613**			Cu-Cr	0.658**	Fe-Mn	0.696**
Cu-Na	0.481**					Cu-Na	0.927**
Cu-K	0.566**					Cu-K	-0.544**
Ti- Mg	0.526**					Cu-Fe	0.600**
Ti-Ca	0.915**					Mn-Mg	0.860**
Na-K	0.688**					Mn-Ca	0.866**
Ca-Mg	0.576**					Se-Na	-0.497**
						Se-K	0.651**
						Ti-Mg	0.880**
						Ti-Ca	0.944**
						K-Na	-0.693**
						Ca-Mg	0.950**

*** P<0.01*

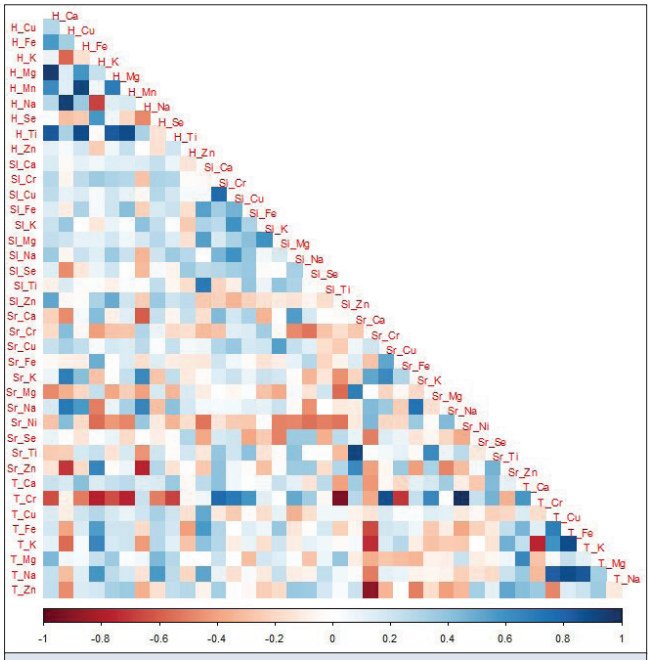

Fig 1. The correlation heatmeap of serum, tears, saliva, and hair samples. H: Hair, Sl: Saliva, Sr: Serum, T: Tear

Table 3. *Significant Spearman's rank correlations for macro and trace elements between serum, tears, and hair samples*

Element	Serum-Tears	Serum-Hair	Tears-Hair
Na-Na		0.703**	
K-K	-0.530*	-0.383*	0.492*
Mg-Mg		-0.389*	0.527*
Fe-Fe			-0.475*
Cu-Cu		0.400*	

** P<0.05, ** P<0.01*

Discussion

This study tries to determine the various macro and trace elements in the serum, saliva, tears, and hair samples of Kilis goats. The assessment of the inorganic metabolic status of livestock is a valuable tool in herd health management of animals, both individually and on a herd basis. Therefore, below-average production and reproductive performance in ruminants is a condition that should be evaluated for inorganic metabolism status.

In this study, the macro elements measured in samples were Na, K, Ca, and Mg, and their mean serum concentrations were 3265±53.6, 232.3±10.1, 89.8±2.05, and 24.9±0.76 mg/L, respectively. Overall, all the concentrations for serum macroelements were within physiological ranges in goats [20].

The serum Na concentrations observed in this study were mostly in agreement with studies with other goat breeds [21-24]. In our study serum Na levels in Kilis goats were higher than those in Guizhou black [25] and Halep goats [26], and it was lower than Kilis [27] and Angora goats [26].

The mean serum K concentration was parallel to those reported by Schweinzer et al.[2]. Previous studies have reported K concentration of different goat breeds and Kilis goat serum ranging from 119-199 mg/L [21-28].

Na is the main element responsible for maintaining the osmotic pressure and volume of the plasma, while K is responsible for maintaining the intracellular osmotic pressure. Therefore, the similarity between our results and the literature, as well as the results being within physiological ranges, suggests that these elements are less affected by external factors.

Calcium plays a significant role in maintaining the homeostasis of animals, including coagulation, mineralization of bones and teeth, hormone secretion, and neural excitability. In our study, the mean serum Ca concentration was 89.8±2.05 mg/L and it was higher than Honamlı goats [21] and Kilis goats in late gestation and parturition period [29]. It was lower than Kilis goats before and during, gestation, mid-gestation, and after the parturition period [29] and also lower than the findings of Schweinzer et al.[2], and Lima et al.[3]. Our value was similar to those previously reported in goats from China [25], South Italy [22], North-Eastern, Algeria [23], and Turkey [26,28]. Although serum concentrations of Ca vary in physiological conditions such as pregnancy and lactation, they are tried to be maintained within certain limits by homeostatic mechanisms.

Magnesium is the fourth most common cation in the body. As an intracellular cation, it functions as a catalyst or activator of hundreds of enzymes and active in all major metabolic processes. Previous studies have reported Mg concentration of goat serum ranging from 18.3-39.3 mg/L [2,21,22,25,26,28]. The mean serum Mg concentration of this study (24.9±0.76 mg/L) was agreed with those reported within the literature.

The results of our study documented that all macro elements were measured in serum, tears, saliva, and hair samples, although they differed in distribution. The macro elements exist in all tissues and fluids, and they are vital. Indeed, when compared with previous study results, although they show differences due to changes in different races, geography, physiological conditions, etc., and also the similarities of serum macro element levels and keeping them within physiological limits prove that macro element concentrations are under strict homeostatic control.

In the present study, the trace elements investigated in samples were Fe, Cu, Zn, Se, Ni, Ti, Mn, Co, and Cr. The mean Fe concentration of serum samples was 3.33±0.52 mg/L. Serum Fe concentrations in small ruminants may

vary within 1.93-2.09 mg/L [5,30] thus, the results of this study demonstrate high serum concentrations of this element. Several studies have reported values between 0.779-2.93 mg/L [2,3,21,31-33]. However, the serum Fe concentration was lower than the results of the study conducted with Omani [4], Boer [34], and Guizhou black goats [25].

The physiological range of serum Cu concentrations in small ruminants was notified between 0.43-1.39 mg/L, and a concentration of less than 0.08 mg/L has been reported to cause deficiency symptoms [28,35]. The mean serum Cu concentration of this study was 435.4±34.7 µg/L, which was mostly lower than the values reported in previous studies with goats [2-4,24,25,31,33,35-38]. However, it was higher than the results of Erdogan's study (0.33 mg/L) [28].

In our study, the concentration of Zn in serum samples was 1.392±0.574 mg/L. It was lower than those reported by Zhou et al.[34], Yun and Mei [25], Shawaf et al.[4], and Kachuee et al.[31], while lower concentrations were found those reported in previous studies (0.210-0.967 mg/L) [2,3,28,33,36,37]. When we compare the Zn content of serum samples in our study the literature showed that the results were in agreement with those reported in literature by Nazifi et al.[32].

Selenium is an essential trace element in animal nutrition and has many actions related to animal production, fertility, and disease prevention In the present study, the mean serum Se concentration was 231.2±25.2 µg/L. Harris reported a normal serum Se concentration of 100 µg/L for goats [39]. In previous studies conducted in different geographical regions and with different methods, serum Se concentrations of goats have been reported between 11-377 µg/L [2,4,25,28,31,32,36,37,40].

Nickel has gained much attention in recent years because it is related to the activity of the urease enzyme and is also a cofactor of enzymes involved in nitrogen metabolism [41]. Although Ni imbalance causes many serious problems in animals, data on small ruminants are insufficient. In this study, Ni has been detected in only serum samples and its mean value was 83.9±6.35 µg/L. Our value was lower than reported by Bashir et al.[41], Yazar et al.[37], and the permissible limits (0.4 mg/L) determined by the National Research Council [42]. Nickel remains below the detection limit in samples other than serum samples could be explained by the serum Ni level does not exceed the threshold level for its transition to the analyzed biological materials.

Titanium plays a role in the growth of mammals and is considered a growth factor [43]. Therefore, it is an essential element in animal nutrition. However, under normal farm conditions, its insufficiency is not expected [7]. In our study the concentration of Ti in serum and saliva samples were 497.2±10.7 and 125.3±5.58 µg/L, respectively. The Ti concentration of tears samples was below the detection limit. The possible reason for this was thought to be the existence of a mechanism that prevents the transition of Ti to tears.

Cobalt concentration was below the detection limit in all samples. Studies on Co deficiency in goats are scarce, however, these results indicate that there could be a lack of Co in the ration of Kilis goats and a need for Co supplementation in the ration.

Chromium is an essential element that supports mammals using carbohydrates, lipids, and proteins as an insulin activator. The tolerable concentration of Cr was reported as 1.0 mg/L in the blood [44]. In this study, the mean Cr concentration in serum samples of Kilis goats was 427.7±43.6 µg/L and below the tolerable limit. The Cr content of goats was lower in our study than in Khan's [44] and Yazar's study [37].

The bioavailability and requirement of the trace elements is affected by many factors including species, breed, sex, and age, as well as physiological factors such as the stage of pregnancy and lactation, inflammation, antagonists, concentrations of forage (soil type, age of plant, forage species, etc.) season, and climate [2,45]. For instance, Fe requirement of the animal body is a factor that greatly affects Fe absorption. Animals with low Fe status or fed an Fe-deficient diet will have greater Fe absorption and retention for physiological needs. Similarly, Cu and Zn absorption is regulated by their dietary concentrations, related to the animal's requirements, and the physiological conditions of the organism [45]. Another factor affecting trace element bioavailability in ruminants is the interactions between the elements. Differences in the sulfur content of the feeds or the presence of cyanogenetic glycosides, which are anatagonists for Se, may cause differences in the bioavailability of Se. It is also reported that high dietary Ca may reduce Mn absorption [46]. The fact that trace element concentrations are affected by many different factors makes it difficult to determine the reference values and causes great differences between these values. For this reason, serum trace element values reported in the literature selected from studies with goats and the levels compared in this study showed variable differences.

According to the results of our study, Na, K, Ca, Mg, Fe, Cu, Zn, and Cr were detected in tear and saliva samples, although their concentrations differed. In addition, unlike tears, Se and Ti were also measured in saliva samples. The measurement of all macro elements and some trace elements in serum, tears, and saliva samples simultaneously suggests that there are different mechanisms in the transition from serum, which acts as an element transport pool, to other body fluids.

In this study, the predominant macro element in hair samples was K, and the lowest concentrations of Na were also measured in hair samples. At the same time, a significant negative correlation was detected between Na and K (rho:-0.693) in the hair samples. This result is thought to be related to the tendency of Na and K to disperse in biological systems. Sodium is an extracellular element and is closely related to water. Therefore, in water-poor tissues, K is expected to be more than Na.

The complete reference ranges of macro and trace elements of hair are not available for goats, only partial information can be found for some elements. According to the results of a study conducted by Battini et al.[47] on the hair of dairy goats, the Na, K, Ca, Mg, Fe, Cu, and Zn concentrations of normal hair were reported as 1575.99±194.22, 3310.06±229.1, 896.93±42.34, 339.65±11.42, 161.58±24.84, 4.44±0.42, 103.49±2.54 ppm, respectively. Our results for Na were similar to normal goat hair, while other element concentrations were found to be higher except Fe. The highest concentration of Fe was detected in hair samples (114.8±19.7 mg/L) in our study and it was lower than reported by Battini et al.[47] and Yun and Mai [25]. However, it was higher than Boer goats [34]. The mean hair Zn concentration was 118.0±4.56 mg/kg in this study, which was higher than the reported by Pavlata et al.[36]. According to the present study, both the mean hair Zn and Se concentrations in Kilis goat were lower than the reported for the Boer and Guizhou black goat [25,34]. Studies with various animal species have shown that hair is a good indicator of Cu [7]. In our study, there was a significant correlation between serum and hair Cu concentrations (rho: 0.400). The mean hair Cu concentration was lower than the reported value for the Boer goats [34] and it also was higher than the value reported by Yun and Mei [25].

The obtained data demonstrate that the highest values of K, Ca, Mg, Fe, Cu, Zn, Se, Ti, and Mn were measured in the hair samples. Combs et al [48] concluded that elements are accumulated in hair in higher concentrations than in blood. Our results of element concentrations of hair samples also indicate that these elements tend to accumulate in the hair.

Knowledge about Mn metabolism especially in goats is scarce. It was reported that the Mn deficient nutrition had no effect on the Mn concentrations in the serum of goats, but decreased the Mn content in the hair, so the hair was a reliable indicator of Mn in animals [49]. The fact that Mn could only be measured in hair samples in the present study, supports this knowledge. The mean Mn concentration of hair samples was measured as 12903±3142 µg/kg. This value was consistent with the reported for normal goats [7].

The conclusions of this study can be listed as follows; *(i)* Saliva, tears, and hair samples could be used as alternative biological material in macro and trace element analysis by ICP-MS method. (*ii*) In goats, it may be useful for diagnosis and follow-up through the correlation of some elements between serum-hair (Na, K, Mg, Cu), tears-hair (K, Mg, Fe), and serum-tears (K). (*iii)* Hair Mn concentrations can be considered as a reliable parameter for investigating Mn metabolism in goats. *(iv)* In particular, trace element concentrations are affected by a wide variety of factors and reference values sometimes show great differences. Thus, the determination of especially the trace element concentrations with more comprehensive studies including the factors affecting element concentrations with small ruminants in the region will be beneficial as it may prevent possible economic losses in terms of yield and reproduction.

Availability of Data and Materials

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

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Conflıct of Interest

The authors declared that there is no conflict of interest.

Ethical Statement

The study was conducted with the permission of Harran University Animal Experiments Local Ethics Committee (HRUHADYEK) with the number 2021/009/02.

Author's Contribution

NP designed and supervised the study. NP, EEÖ, ÜY, and Mİ collected the samples. NP and EEÖ performed laboratory analysis. FB analyzed the data. The first draft of the manuscript was written by NP and Mİ. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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RESEARCH ARTICLE

The Method Evaluation of Culturing DF-1 to Proliferate Canine Distemper Virus in Mink with Cephodex Microcarrier

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Abstract: As an acute and highly lethal infectious disease, there is no specific therapeutic drug for canine distemper (CD). Although the process of large-scale production of canine distemper virus (CDV) vaccine of mink has been greatly improved, there are still many deficiencies to be perfected. As one of the most promising technologies for large-scale vaccine production, microcarrier suspension culture technology needs to be further improved. In this study, the application effect of the new Cephodex microcarrier in CDV culture was evaluated to establish a set of technical process for DF-1 cell high-density growth and CDV efficient proliferation. To perfect the large-scale CDV production process, Cephodex was used to suspension culture DF-1 cells for proliferating CDV. In a shake flasks culture system, the optimal culture conditions were established by optimizing culture temperature, virus inoculation and harvest time. Therefore, mink CD vaccine high-efficiency production was laid on the preliminarily established technology of CDV microcarrier suspension culture. The cell density could reach over 3×10^6 cells/mL after 72 h cultured with Cephodex microcarrier at 37°C. Proliferated at 35°C, the CDV titer after 72 h was about $10^{0.5}$ $TCID_{50}$/0.1mL higher than that at 33°C and 37°C. These results show that the Cephodex microcarrier could be used for large-scale culture of DF-1 cells and efficient proliferation of CDV.

Keywords: *Canine distemper virus, DF-1 cells, Microcarrier, Suspension culture, Vaccine*

Minklerde Canine Distemper Virüsünün Cephodex Mikro Taşıyıcı İle Çoğaltılması İçin DF-1 Kültür Yönteminin Değerlendirmesi

Öz: Akut ve oldukça ölümcül bir enfeksiyöz hastalık olan köpek distemperi (CD) için spesifik bir terapötik ilaç yoktur. Minklerde canine distemper virüsü (CDV) aşısının kitlesel üretim süreci büyük ölçüde geliştirilmiş olsa da, halen iyileştirilmesi gereken birçok eksiklikleri vardır. Büyük ölçekli aşı üretimi için en umut verici teknolojilerden biri olan mikro taşıyıcı sıvı kültür teknolojisinin daha da geliştirilmesi gerekmektedir. Bu çalışmada, yüksek yoğunluklu DF-1 hücre eldesi ve CDV'nin daha verimli üremesi için bir dizi teknik süreç oluşturmak amacıyla yeni Cephodex mikro taşıyıcısının etkinliği değerlendirildi. Büyük ölçekli CDV üretim sürecinin iyileştirilmesi amacıyla, CDV'yi üretmede kullanılan DF-1 hücre kültürünü sıvı hale getirmek için Cephodex kullanıldı. Çalkalamalı kültür şişeleri sisteminde, kültür sıcaklığı, virüs inokülasyonu ve toplama zamanı optimize edilerek optimal kültür koşulları oluşturuldu. Böylelikle, önceden kurulmuş CDV mikro taşıyıcı sıvı kültürü teknolojisinin üzerine yüksek verimlilikte mink CD aşısı üretimi gerçekleştirildi. Hücre yoğunluğu, Cephodex mikro taşıyıcı ile 37°C' de kültüre edildikten sonra 72 saat sonunda 3 x 10^6 hücre/mL' nin üzerine çıktı. 35°C'de üretilen CDV titresi, 72 saat sonra 33°C ve 37°C' dekinden yaklaşık $10^{0.5}$ $TCID_{50}$/0.1 mL daha yüksekti. Bu sonuçlar, Cephodex mikro taşıyıcısının, büyük ölçekli DF-1 hücre kültürü ve CDV'nin verimli bir şekilde üretilmesi için kullanılabileceğini göstermektedir.

Anahtar sözcükler: *Canine distemper virus, DF-1 hücresi, Mikro taşıyıcı, Sıvı kültür, Aşı*

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Introduction

Canine distemper (CD) is an acute and highly lethal infectious disease of canines, which causes great losses to the global fur economic animal breeding every year [1]. China is a large fur animal breeding country, which is also greatly affected by this disease [2]. Since the first report on the prevalence of mink CD in China, it has spread to Chinese major fur animal breeding areas after half a century, such as Hebei, Shandong, Heilongjiang. Especially in recent years, fur animals have developed rapidly with the breeding concentrated & huge scale and the fur animal active market, which have created favorable conditions for the prevalence of this disease [3]. At present, there is no specific therapeutic drug for the disease, and immunization is still an effective mean of prevention and control [4,5]. Therefore, high-quality vaccine products are particularly important. Usually, virus antigen is the core factor affecting the quality of vaccine. The proliferation level of canine distemper virus (CDV) on cells is generally not high, which affects the immunogenicity of antigen in vaccine and leads to clinical immune failure [6-8].

For a long time, the traditional two dimensional (2D) cell culture technology was mostly used to proliferate CDV, but the efficiency was low with low viral titer. So it was difficult to have an ideal application effect in large-scale production. Afterwards, some methods were performed such as screening high-quality cell lines and optimizing the culture process to improve the CDV proliferation efficiency and the viral titer, and some results had been achieved certainly [9,10]. Yang et al.[11] constructed a Vero/dSLAM cell line with the gene expressing dog signal lymphocyte activating molecule (dSLAM), and successfully cultured CDV (CD1901 strain) in the cells. The results showed that the highest viral titer of CD1901 could reach $10^{5.5}$ 50% tissue culture infectious dose ($TCID_{50}$)/mL in the Vero/dSLAM cells at the 4 day post inoculation. Because microcarrier culture technology has the characteristics of the three dimensional (3D) cell culture technology, it can effectively improve cell density and production efficiency. In recent years, this technology has been widely used in large-scale animal cell culture and industrialized vaccine production [12]. Saykally et al.[13] used microcarriers to culture ARPE-19 cells for human cytomegalovirus (HCMV) proliferation in bioreactor. The virus yield was 100 times that of the traditional culture plate method. At the same time, the treatment time was shortened by 90% compared with the traditional culture method, which greatly improved the work efficiency. Liu et al.[14] discovered that Cephodex microcarrier was very suitable for large-scale culture of CIK cells and the grass carp reovirus (GCRV) titer could reach the $10^{8.5}$ $TCID_{50}$/ml in this way, thus a solid basis could be established for the large-scale preparation of vaccine against the grass carp hemorrhage. In this study, the screened DF-1 cell line was cultured in high density with a new Cephodex microcarrier, and the technical conditions such as culture temperature were optimized to achieve the purpose of CDV efficient proliferation.

Material and Methods

Ethical Approval

The study was approved by the local Ethics Committee of Shandong Binzhou Animal Science and Veterinary Medicine Academy, Binzhou, China (Approval no: SDBZASVM-2020-002).

Reagents

Cell growth medium was DMEM (10-203, Wuxi Meidi biological products company) solution with 6% (v/v) FBS (11011-8611, Zhejiang Tianhang Biotechnology Co., Ltd), and cell maintenance medium was DMEM solution with 2% (v/v) FBS.

Cell Resuscitation and Subculture

One cryopreservation tube containing DF-1 seed cells (ATCC CRL-12203, Shandong Binzhou Animal Science and Veterinary Medicine Academy) was put into 37°C water bath, and these cells in which were thawed quickly and centrifuged at 1000 rpm at room temperature for 5 min, and then the supernatant was discard. After the cells were resuspended with cell growth medium, them were transfered into T75 flask aseptically. The flask was added with an appropriate amount of growth medium and put into incubator with 37°C and 5% CO_2. At 72 h, DF-1 cells grew into intact monolayer were digested by trypsin and dispersed by blowing, and then passaged with the expanded ratio of 1:3.

Microcarrier Preparation and Suspension Culture

Six 0.1 g Cephodex microcarriers (MC10001, Binzhou Biocarrier Biotechnology Co., Ltd) were put into six 50 mL conical shake flasks respectively, and 10ml PBS was added to each flask, and then the microcarrier was stirred and suspended with a glass rod. Six shake flasks were sterilized at 121°C for 30 min. After sterilization, the microcarrier was suspended when the temperature decreased to about 80°C to prevent microcarrier agglomerating. Replaced the sterile PBS, the shake flasks were injected 1~2 mL of cell growth solution, and then were put into incubate at 37°C for 24~72 h for sterility test.

Passing the sterility test, the supernatant was discarded and the microcarrier was retained for standby. DF-1 cells in T75 flask were treated with trypsin-EDTA digestive solution, blown to dispersing, collected aseptically, and the cell concentration was measured by trypan blue staining method [15,16]. 3×10^6 cells per flask were inoculated, and

Table 1. The shaker control parameters at different culture stage

Culture Stage	Temperature (°)	CO_2 (%)	Shaker Speed (rpm)	Shaker On/Off (min)
Intermittent shaking	37	5	55	3x(1/30) 4x(1/60)
Continuous shaking	37	5	60	ON

then the volume of cell growth medium was fixed to 10ml. These cells in every flask were mixed with microcarrier and cultured in minitron culture shaker (CH-4103 BOTTMINGEN, Infors, Switzerland). 0~6 h after cell inoculation is intermittent shaking, the intermittent time is 30~60 min, and the shaking time is 1~2 min *(Table 1)*. Then, DF-1 cells were cultured by microcarrier suspension in the continuous shaking culture stage [17,18]. Compared with intermittent shaking culture, the shaker parameter was set with some change in continuous shaking culture stage.

CDV Proliferation and $TCID_{50}$ Determination

After suspension culture for 72 h, DF-1 grew into intact cell monolayer. Samples were taken to observe the morphology of cell microcarrier complex. The cell culture medium in three shake flasks of the exposed group were replaced with cell maintenance medium, and CDV (provided by the Institute of Special Animal and Plant Sciences of CAAS, China) was inoculated according to 2% (v/v) [19]. As healthy cell control, the other three shake flasks for cells count were also replaced with cell maintenance solution. After infected and healthy cells were grouped, they were cultured at 33°C, 35°C and 37°C. Compared with continuous shaking culture of DF-1 cells, other shaker parameters were unchanged except for the culture temperature at the CDV proliferation stage. Samples were taken every 24 h after virus inoculation, and $TCID_{50}$ of supernatants were measured every 24 h according to Reed-Muench method.

Cell Density and Glucose Consumption

In the suspension culture stage, three shake flask cells were taken for cell counting every 24 h, and the glucose (G8270, Sigma-Aldrich) content in the culture medium was measured by biosensor analyzer (SBA-40E, Shandong Academy of Sciences). The other three bottles of cells for CDV proliferation later were only measured the glucose content without cell counting. The initial concentration of glucose in the culture medium of 6 shake flasks was 4.0 g/L. When the glucose content in the culture medium was lower than 2.0 g/L, 100 μL solution contained 200 g/L glucose was added to the culture medium in time to meet the nutritional needs of high-density cell growth [20-22].

According to the initial concentration of glucose in the culture medium, the amount of glucose added to the culture medium and the glucose content in the culture medium measured every 24 h, the daily glucose consumption of cells was calculated, and the changes of daily glucose consumption of cells before and after exposure were observed [23].

Statistical Analysis

All statistical analyses were performed using statistical package SPSS (version 22.0 for Windows, IBM SPSS Statistics) and $P<0.05$ was considered statistically significant.

Results

Morphology of Cells on Cephodex in Growth Stage

DF-1 cell suspension prepared by trypsin digestion and dispersion was evenly mixed with Cephodex microcarrier. After 6 h intermittent shaking culture, the cells could adhere to the microcarriers. At the time, most of these cells on the surface of microcarriers not spread are round, and a few spread cells are spindle *(Fig. 1-A)*. The DF-1 cells growth was observed on the surface of each microsphere, and there were almost no microbeads without cells to culturing 24 h *(Fig. 1-B)*. After 48 h, the cells monolayer had formed on more than 80% the microspheres surface *(Fig. 1-C)*. At 37°C, due to the rapid growth of DF-1 cells, a complete cell monolayer was formed on the surface of microbeads at 72 h [24]. On the surface of some microspheres, The cells further grew into a dense multi-layer structure, and then formed an obvious protrusion *(Fig. 1-D)*.

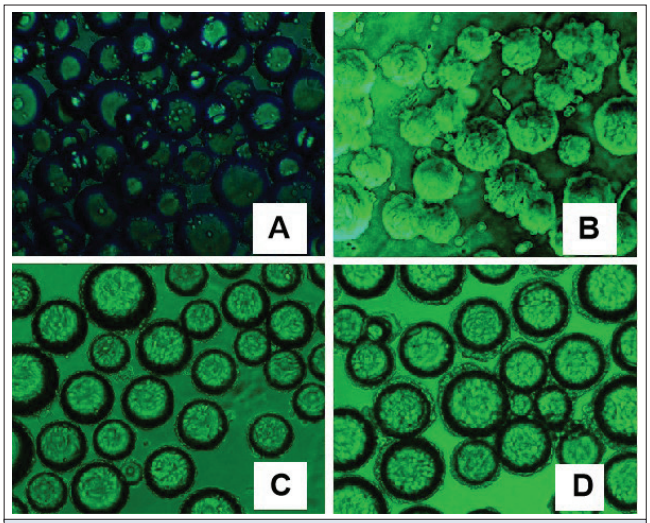

Fig 1. DF-1 cells on Cephodex in the different time at the growth culture stage. **A**- 6 h after cells inoculation; **B**- culture continuously for 24 h after cells inoculation; **C**- 48 h after cells grow; **D**- cells cultured for 72 h with Cephodex microcarrier

Growth Curve and Glucose Consumption in Growth Stage

For three flasks of DF-1 cells on microcarriers cultured at 37°C for 72 h, from the beginning of cell inoculation, samples was taken every 24 h for cell counting to determine cell density. At the same time, through the supernatant of all 6 flasks taken every 24 h, the glucose contents in the culture medium were measured to calculate the cell glucose consumption.

On the condition of 37°C, the cells in the three cells counted flasks grew rapidly, and the cell density increased rapidly too, reaching over 2.0×10^6 cells/mL at 48 h *(Fig. 2)*, and the cell glucose consumption also showed a rapid upward trend during this time *(Fig. 3)*. After 48 h, the cell density still increased, but the growth rate slowed down significantly. At 72 h, the cell density reached more than 3.0×10^6 cells/mL *(Fig. 2)*, and virus inoculation should be carried out at this time [25,26]. The cell growth curve and glucose consumption curve in this culture process showed that there was little difference in the cell growth curves among the three cells counted shake flasks ($P>0.05$), and their glucose consumption curves were also similar ($P>0.05$). Therefore, there were a high positive correlation between cell glucose consumption and cell density. In order to reduce the impact on the subsequent CDV proliferation experiment, the other three flasks of cells were not sampled for cell counting, but measured the glucose consumption. From the six flasks of cells glucose consumption curve, the cell density was very close to that of these cells counted flasks ($P>0.05$) *(Fig. 3)*.

The CPE of CDV Proliferation

Cytopathic effect (CPE) of DF-1 infected by CDV began to appear at 48 h post CDV inoculation, and a large number of cells shed from the surface of microcarriers. The cytopathy at different temperatures was observed. The cytopathy at 33°C was relatively light and the cell abscission was less *(Fig. 4-B)*. The lesion at 37°C was the most serious and the cell abscission was the most *(Fig. 4-D)*. The cytopathy and abscission at 35°C were between the two temperatures *(Fig. 4-C)*. At this time, there were still a large number of cells on the surface of the microcarrier, while DF-1 cells in the control group have no CPE or shedding *(Fig. 4-A)*.

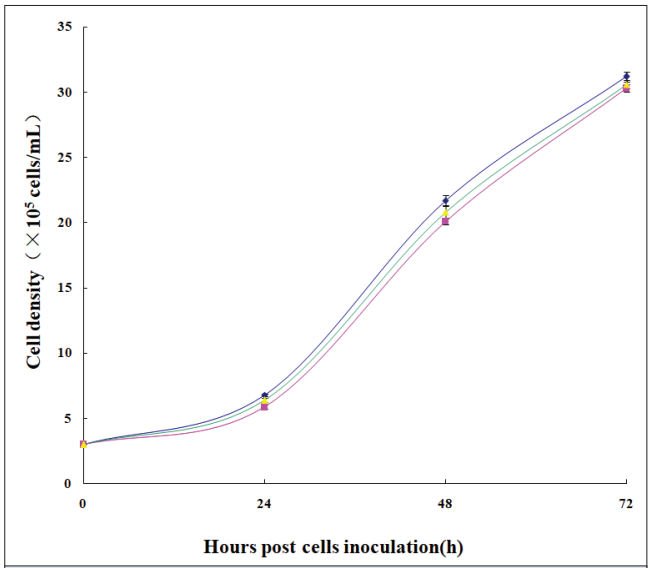

Fig 2. The DF-1 cells growth curve on Cephodex microcarrier at 37°C

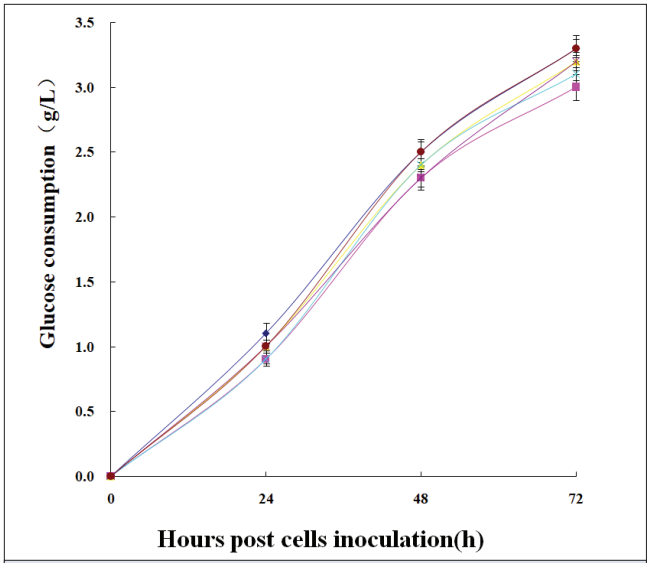

Fig 3. Glucose consumption curve of the DF-1 cells in the growth culture stage

To 72 h post CDV inoculation, the cytopathy and cell abscission further intensified, and some microspheres without cells began to appear [27]. Among the three culture temperatures, the rate of microspheres without cells at 37°C was the highest *(Fig. 4-H)*, more than 70%, followed by 35°C, nearly half of microcarriers without cells *(Fig. 4-G)*, while only a few microbeads were empty beads at 33°C *(Fig. 4-F)*. At the same time, DF-1 cells in the healthy control group were still in good condition, without CPE and obvious cell abscission, as shown *(Fig. 4-E)*. Therefore, after CDV infection, there are certain differences in the degree of cell lesions and cell abscission on the surface of microcarriers at different culture temperatures at the same time [28-30].

Cell Glucose Consumption and Virus Titer in CDV Proliferation

After the cells were infected by virus, samples were taken every 24 h to measure the viral titer. At the same time, the daily glucose consumption of cells was obtained according to measured the glucose content in the culture medium. From the results of $TCID_{50}$ determination, the viral titer showed an upward trend within 72 h at three culture temperatures, indicating that the virus maintained

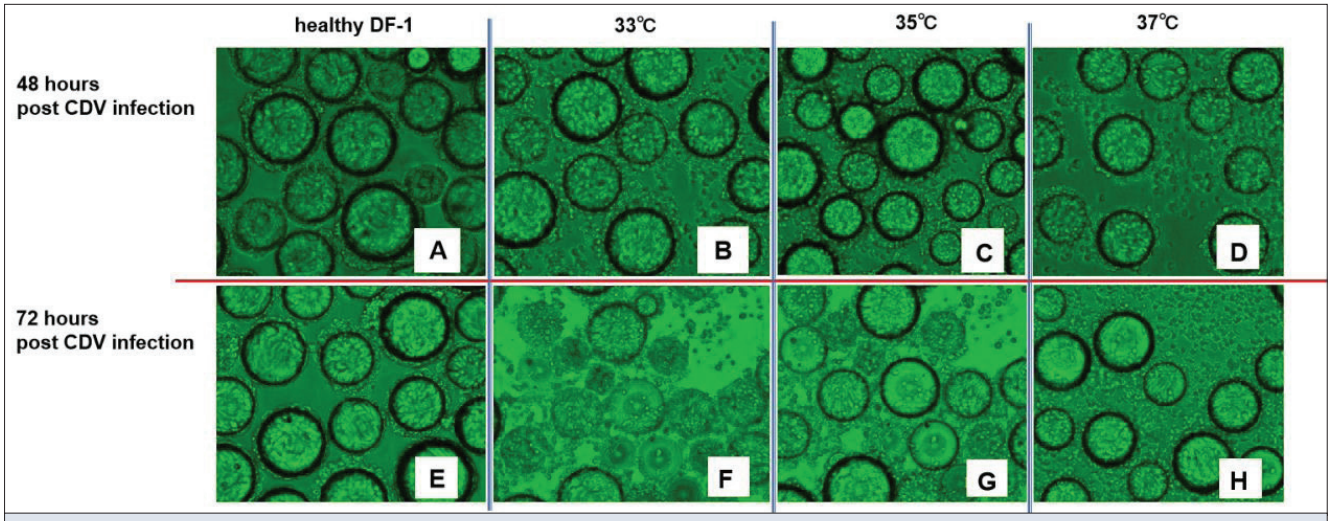

Fig 4. The CPE of DF-1 on the Cephodex after CDV infection in the different temperature. **A, B, C, D-** 48 h post CDV infection; **E, F, G, H-** 72 h post CDV infection; **A, E-** healthy DF-1 group; **B, F-** CDV infection group at 33°C; **C, G-** CDV infection group at 35°C; **D, H-** CDV infection group at 37°C

a high proliferation during this time *(Fig. 5)*. However, the virus titer at the same time was significantly different between different temperature conditions (P<0.05) *(Table 2)*. Among them, the titers of both 33°C and 37°C were lower than that of 35°C *(Fig. 6)*. Therefore, in the CDV proliferation stage, the 35°C was more conducive to CDV proliferation. The results of virus titer measurement showed that under the three temperature conditions, the viral titer increased rapidly within 48 h after virus exposure, indicating that the virus proliferated rapidly [31,32]. After 48 h, the virus titer still increased, but the rising rate slowed down slightly, indicating that the virus proliferation rate had begun to decline. From the point of view of cell glucose consumption at this stage, the rate of glucose consumption

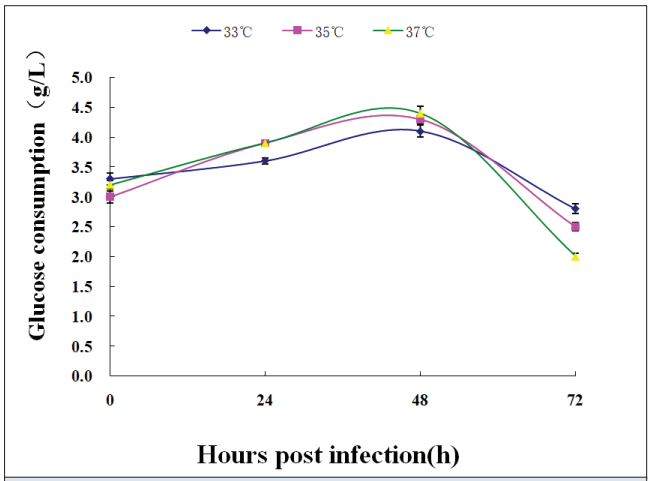

Fig 5. The glucose consumption curve of DF-1 cells infected by CDV at the different temperature

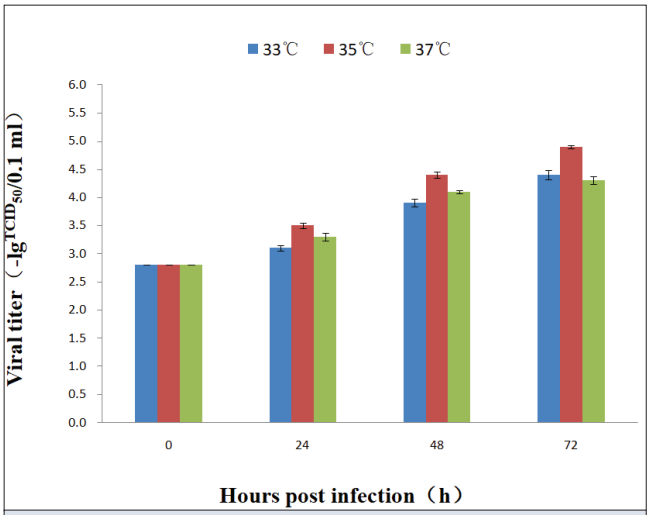

Fig 6. The viral titer in the culture at the different temperature

Table 2. *The CDV titer in different culture temperature*

Culture Temperature (°)	Culture Time				Viral Titer
	0 h	24 h	48 h	72 h	
33	2.8±0.03	3.1±0.05	3.9±0.07	4.4±0.08 ^{b}	lg^{TCID50}/0.1 mL
35	2.8±0.03	3.5±0.05	4.4±0.05	4.9±0.03 ^{a}	
37	2.8±0.03	3.3±0.07	4.1±0.03	4.3±0.07 ^{b}	

^{a,b} Values within a row with different superscripts differ significantly at P<0.05

increased continuously within 48 h after exposure, and the daily glucose consumption reached the highest level at 48 h. After 48 h, the glucose consumption of cells decreased significantly. After 72 h, the daily glucose consumption of cells in each group decreased to about half of the peak value, and the decrease was the fastest in the 37°C group *(Fig. 5)*. Therefore, the appropriate time to harvesting virus was 72 h post CDV inoculation.

Discussion

The cell compatibility of cell microcarriers is one of the key factors for the successful application of microcarrier suspension culture technology, and it is an important condition to determine whether cells can grow well in large scale and high density [33-35]. According to the results of this study, in the intermittent shaking stage, DF-1 cells adhered fast to Cephodex microcarriers, and had high adherent ratio and good morphology on Cephodex. In the continuous shaking stage, the cells had high vitality and vigorous growth on the surface of the microcarrier, forming a dense cell monolayer or multilayer structure. These data showed that Cephodex microcarrier had good compatibility with DF-1 cells and was very suitable to suspension culture DF-1 cells [36,37].

The quality and the number of cells have an important impact on the viral titer and the virus antigenicity [38,39]. For DF-1 cells growing rapidly and stably in a short time, 37°C was determined in the cell growth stage. The relationship between cell glucose consumption and cell density was investigated. The former had a significant positive correlation with the latter, and was verified in the cell counting results. After 48 h of cell culture, the growth rate of cell density and daily glucose consumption began to decline, indicating that the growth rate of cells began to slow down. The cell density reached the peak, and the daily glucose consumption almost did not increase at 72 h of cells culture which was the best time for virus inoculation. Of course, in order to determine a more accurate CDV inoculation time, we further investigated by subdividing the culture time [40]. As multiple sampling for cell counting could reduce the number of cells, it would inevitably affect the subsequent virus proliferation effect. It was little impact on virus proliferation that the supernatant was sampled to measure the glucose content because of generally no cell loss [41]. Therefore, only three shake flasks of cells were taken for cell counting and glucose consumption, which were used as healthy cell control. The other three flasks of cells for CDV inoculation were measured about the glucose consumption without cell counting. In this way, according to the daily glucose consumption of all 6 flasks of cells and the cell density in 3 flasks of control group, the cell density in 3 flasks of virus infection group could be predicted, which improved the accuracy and reliability of the experimental results. Of course, it might not be accurate to predict cell density only by cell glucose consumption which causing the experimental error, but this error was much smaller than the error led by cell loss by sampling for cell count. In order to accurately calculate the quantitative relationship between cell density and cell glucose consumption, the standard curves between them could be obtained by designed experiments in the future to reduce the error [42]. The virus titer increased rapidly within CDV inoculation 48 h, and the daily glucose consumption of cells also maintained a rapid increase. After 48 h, the glucose consumption decreased significantly, and the growth of viral titer slowed down. To 72 h the CDV proliferated, the sugar consumption decreased by nearly half, which in the 37°C group decreased especially more than 50%. Combined with microscopic observation of cytopathy, 72 h post virus inoculation could be determined as the best time for virus harvest. The virus titer at 35°C was about $10^{0.5}$ $TCID_{50}$/0.1 mL higher than that at 33°C and 37°C, which indicated 35°C was the best suitable temperature for CDV proliferation.

In conclusion, the new Cephodex microcarrier was applied to the high-density culture of DF-1 cells to CDV proliferate in this study. With a good cell compatibility, Cephodex microcarrier can be well used for large-scale culture of DF-1 cells and efficient proliferation of CDV. Moreover, CDV titer can be improved to a certain extent through the control of the key technical links, such as virus inoculation time, virus harvest time and culture temperature [43]. Since only several key technologies were discussed in the CDV suspension culture process with Cephodex microcarrier, there were more technical conditions to be optimized and improved for industrial vaccine production, which needed to be further studied in the future.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author (J. Zhuang) on reasonable request.

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Ethical Approval

The study was approved by the local Ethics Committee of Shandong Binzhou Animal Science and Veterinary Medicine Academy, Binzhou, China (Approval no: SDBZASVM-2020-002).

Competing Interests

The authors declared that there is no conflict of interest.

Author Contributions

Experimental design was conceived by Jianguo MEI, Yumao

WANG, Bing ZHANG, Shijun FU and Shijin GUO. Data were collected by Ling Mo and Lu Guo. Statistical analysis was conducted by Yan WANG and Jingjing SONG. Original draft was written by Jianguo MEI, Shijun FU and Jinqiu ZHUANG. All authors have contributed to the revision and final proof-reading of the manuscript.

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RESEARCH ARTICLE

The Status of Bovine Viral Diarrhea Virus (BVDV) in Western Türkiye: Detection of Three Subtypes

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Abstract: Bovine viral diarrhea virus (BVDV) is a viral pathogen that causes significant economic losses in cattle, especially by causing abortions. Globally, BVDVs are classified into three genetically distinct types: BVDV-1, BVDV-2 and BVDV-3. Despite the presence of all three groups in Türkiye, BVDV-1 is by far the most prevalent. The aim of the study was to determine the genetic diversity of BVDV detected in materials from aborted fetus between 2017 and 2020 in western Türkiye. Sequence and phylogenetic analyzes were performed based on the 5'-UTR and N^{pro} gene regions of BVDVs from samples, which tested positive using real time RT-PCR. According to pairwise similarity and cluster analysis the samples clustered into three different sub-types, with one dominant subtype 1d (n=4). The remaining samples clustered within subtype 1l (n=3) and 1f (n=2). In this study, different subtypes were found in abortion materials submitted from the same region. Since different subtypes of BVDV were identified even in a small geographical area of Türkiye, it is essential to prepare control and eradication programs through specific vaccination, diagnostic and mitigation programs coordinated by national government, to prevent the spread of these viruses.

Keywords: *Abortion, Bovine viral diarrhea virus, Cattle, Genetic variation, Türkiye*

Türkiye'nin Batısında Sığır Viral Diyare Virusunun (BVDV) Durumu: Üç Alt Grubun Tespiti

Öz: Bovine viral diyare virusu (BVDV), sığırlarda özellikle abortların görülmesiyle birlikte önemli ekonomik kayıplara neden olan viral bir patojendir. Küresel olarak, BVDV genetik olarak BVDV-1, BVDV-2 ve BVDV-3 olmak üzere farklı üç tipte sınıflandırılır. Türkiye'de her üç grubun varlığına rağmen, BVDV-1 açık ara en yaygın olanıdır. Bu çalışmanın amacı, Türkiye'nin batısında 2017-2020 yılları arasında sığırlardan elde edilen abort materyallerinde tespit edilen BVDV'nin genetik çeşitliliğini belirlemektir. Real Time RT-PCR kullanılarak pozitif test edilen abort örneklerinin 5'-UTR ve N^{pro} gen bölgelerine dayalı olarak dizi ve filogenetik analizleri yapıldı. İkili benzerlik ve küme analizine göre örnekler, bir baskın alt tip 1d (n=4) ile üç farklı alt tipte kümelendi. Kalan örnekler 1l (n=3) ve 1f (n=2) alt tipinde kümelenmiştir. Bu çalışmada aynı bölgeden gönderilen abort materyallerinde farklı alt tipler bulunmuştur. Türkiye'nin küçük bir coğrafi bölgesinde bile BVDV'nin farklı alt tipleri tespit edildiğinden, bu virusların yayılmasını önlemek için ulusal hükümet tarafından koordine edilen spesifik aşılama, teşhis ve hafifletme programları ile kontrol ve eradikasyon programlarının hazırlanması esas olmalıdır.

Anahtar sözcükler: *Abort, Bovine viral diyare virus, Sığır, Genetik varyasyon, Türkiye*

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Introduction

Bovine viral diarrhea virus (BVDV), which is in the *Pestivirus* genus, is of great importance due to the damage they cause to the world livestock economy. Until now, long-term programs for the control and eradication of pestivirus infections have not been developed and applied in many countries. Although BVDV frequently presents as subclinical cases, it causes respiratory, digestive and infertility disorders [1,2].

BVDV is transmitted horizontally by secretions and excreta (saliva and faces). In addition, the virus replicates in the placenta in susceptible pregnant cattle and vertically infects the fetus [3]. The consequences of intrauterine infection vary depending on the period of pregnancy, causing malformations, abortions, and immune-tolerant persistent infections. Chronically infected animals are either born weak or seemingly healthy but clinically undiagnosed, yet these animals are likely to develop Mucosal disease (MD) with anorexia, gastrointestinal erosion, and persistent diarrhea at some point in their lives [4,5].

This enveloped virus is 40-60 nm in diameter and contains a single-stranded RNA genome 12.3 kb in length. It belongs to the genus *Pestivirus* in the family *Flaviviridae* and has been divided in two biotypes: cytopathic (CP) and non-cytopathic (NCP) according to the morphological changes it produces in cell culture [6]. There is an untranslated region (UTR) at both the beginning (5') and end (3') of the genome [7]. The 5'-UTR is not capped and there is no poly(A)-tail at the 3' end of the viral RNA. The viral genome consists of four structural proteins (Core (C) and Envelope: Erns, E1 and E2) and seven non-structural proteins (N^{pro} NS2, NS3, NS4A, NS4B, NS5A, NS5B) [1]. Molecular characterization and phylogenetic studies of genetically diverse BVDVs are based on the 5'-UTR, N^{pro}, NS3 and E2 gene regions [8-12]. Recently, the International Committee on Taxonomy Viruses (ICTV) has renamed Pestiviruses and sub-divided them into 11 distinct species. These groups are called BVDV-1 (Pestivirus A), BVDV-2 (Pestivirus B), border disease (Pestivirus D), classical swine fever (Pestivirus C) and HoBi-like (Pestivirus H). In BVDV-1, 21 subtypes were identified and designated between 1a to 1u, whilst four subtypes of BVDV-2 were described and designated from 2a to 2d. In recent years BVDV-3, also called pestivirus H or HoBi-like pestivirus, has been identified as genetically and antigenically distinct disease in cattle and buffalo [13].

Previous studies identified BVDV-1 to be more prevalent than BVDV-2 in Türkiye [10-12]. In addition, it was recently reported that BVDV-3 was also present in Türkiye [12]. Whilst subtypes 1a, 1b, 1c, 1d, 1f, 1h have all been identified in Türkiye, it is reported that subtype 1l is the predominant subtype with the widest distribution [11,12].

Abortion cases due to BVDV infection are an important cause of economic loss to the livestock industry. No official vaccination, control and eradication program has been proposed or implemented for pestivirus cases in Türkiye, despite various studies indicating the presence and importance of BVDV in Türkiye. Therefore, early detection and subsequent elimination of diseases, such as BVDV, affecting herd health and well-being are critical in supporting a viable livestock industry. In order to achieve this, the fast and accurate identification of virus species, as well as the molecular characterization and phylogenetic classification of subtypes are necessary for BVDV in Türkiye.

In this study, the BVDV subtypes were determined based on sequence analysis using the 5'-UTR and N^{pro} gene regions of isolates obtained from abortion cases observed in western Türkiye.

Material and Methods

Samples and Preparation

In this study, nine pestivirus positive samples detected from 117 aborted fetus submitted to Izmir/Bornova Veterinary Control Institute for routine diagnosis between 2017-2020 were used. Internal organ samples of bovine abortions (spleen, liver, lung and lymph nodes) were transported and stored at 4°C. The samples were pooled and 1 g was crushed in a sterile porcelain mortar with sterile sand. It was homogenized by adding 5 mL of Eagle's Minimum Essential Medium (EMEM) and the homogenate was centrifuged at 3500 rpm at 4°C for 15 min. The supernatant was removed and used during subsequent RNA extraction. The provinces from where samples originated are indicated in *Fig. 1* and additional metadata of the samples are provided in *Table 1*.

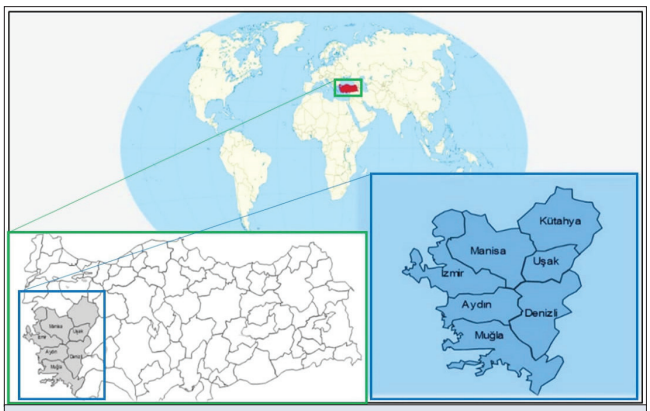

Fig 1. Geopolitical position of Türkiye and provinces where samples were submitted

Table 1. Information about BVDVs obtained from this study

Location	Sample Type	Year	Isolate Name
Uşak	Fetus	2017	BVDV-1_Bor1_Türkiye_2017
İzmir	Fetus	2019	BVDV-1_Bor2_Türkiye_2019
Aydın	Fetus	2018	BVDV-1_Bor3_Türkiye_2018
Muğla	Fetus	2019	BVDV-1_Bor4_Türkiye_2019
İzmir	Fetus	2018	BVDV-1_Bor5_Türkiye_2018
Denizli	Fetus	2020	BVDV-1_Bor6_Türkiye_2020
Kütahya	Fetus	2019	BVDV-1_Bor7_Türkiye_2019
Manisa	Fetus	2020	BVDV-1_Bor8_Türkiye_2020
Aydın	Fetus	2020	BVDV-1_Bor9_Türkiye_2020

RNA Extraction and Real Time PCR

Viral RNA extraction was performed using the Roche MagNA Pure LC 2.0 instrument and the MagNA Pure LC Total Nucleic Acid isolation kit according to the manufacturer's instructions (Roche, Basel, Switzerland). Real time RT-PCR were performed using primers and probe previously described by Hoffmann et al.[14] and the extracted RNA as template. For both the reverse transcription and DNA amplification reaction, the Real Time ready RNA Virus Master kit (Roche, Germany) was used. The RT-qPCR was performed in a 20 µL reaction volume containing 5 µL template RNA, 0.5 pmol of each primer and 0.25 pmol probe in a Roche LightCycler® 480. Following cDNA synthesis for 6 min at 50°C, the reaction progress for 40 cycles by 57°C annealing temperature.

Detection of Pestivirus Biotypes

The RT-PCR method described by Greiser-Wilke et al.[15] was used to determine biotypes (CP or NCP) of BVDV. For amplification, the Xpert One-Step RT-PCR Kit (Grisp Research Solutions, Porto, Portugal) was used. The reaction was performed in a reaction volume of 25 µL and primer final concentration as 0.4 mM.

Phylogenetic Tree and Molecular Characterization

Sequencing based on the 5'UTR were performed using the primers 324 and 326 as previously described by Vilcek et al.[8]. Additional analysis based on the N^{pro} gene region were performed using the primers BD1, BD2, and BD3 as described by Vilcek et al.[16]. All PCR products were visualized on an 1.5% agarose gel stained with ethidium bromide in TAE buffer and photographed using equipment from Vilber Lourmat (France). Amplified products were sequenced using both Forward and Reverse primers incorporated during the generation of the amplicons, at the commercial laboratory (Microsynth AG, Balgach, Switzerland). The nucleotide sequence results were evaluated and edited using DNADynamo Software. The consensus nucleotide sequences were verified using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI). Individual sequence alignments of the 5'-UTR (220 bp) and N^{pro} protein start (403bp) regions, containing the nine samples from Türkiye as well as sequences from GenBank (total n = 185 and n = 195), were generated using CLC Genomics Workbench v.9.5 (Qiagen, www.clcbio.com). Each of the alignments were used to determine the phylogenetic relatedness of the viruses, by generating individual maximum likelihood trees in Mega 6 [17]. Each individual phylogenetic tree was constructed under General Time Reversal (GTR) (G+I, G = 4) with 1000 bootstrap iterations.

Results

Nine pestivirus positive samples were identified as noncytopathic (NCP) biotype using RT-PCR. The nine pestivirus isolates originated from the aborted samples and the sequences corresponding to each of the samples were submitted to GenBank under the accession numbers provided in *Table 2*. These BVDV samples were submitted between 2017 and 2020 and their phylogenetic relatedness towards previously published BVDV sequences were analyzed by comparing the 220bp 5'UTR and 404bp N-terminal region of the polyprotein. The two maximum likelihood phylogenetic trees generated by utilizing each of the previously described data sets, displayed congruence concerning the clustering of the new BVDV samples *(Fig. 2, Fig. 3)*. While all nine samples were identified as BVDV-1, they were grouped to clusters BVDV-1d, BVDV-1f and BVDV-1l. Samples Bor6_Türkiye_2020, Bor7_Türkiye_2019, Bor8_Türkiye_2020 and Bor9_Türkiye_2020 grouped in cluster BVDV-1d, sharing a more recent common ancestor with BVDV samples from China in 2012 - 2013, than BVDV sample described in Türkiye in 2016 *(Fig. 1, Fig. 2)*. In contrast, samples Bor3_Türkiye_2018 and Bor4_Türkiye_2019 clustered phylogenetically closer to previously identified samples from Türkiye, than BVDV samples obtained in

Table 2. *Genetic classification and accession number of the field strains in the present study*

Samples	Subtype	Biotype	Gene Region	Accession no.
BVDV-1_Bor1_Türkiye_2017	1l	NCP	5'-UTR	OM223845
			N^{pro}	OM223836
BVDV-1_Bor2_Türkiye_2019	1l	NCP	5'-UTR	OM223846
			N^{pro}	OM223837
BVDV-1_Bor3_Türkiye_2018	1f	NCP	5'-UTR	OM223847
			N^{pro}	OM223838
BVDV-1_Bor4_Türkiye_2019	1f	NCP	5'-UTR	OM223848
			N^{pro}	OM223839
BVDV-1_Bor5_Türkiye_2018	1l	NCP	5'-UTR	OM223849
			N^{pro}	OM223840
BVDV-1_Bor6_Türkiye_2020	1d	NCP	5'-UTR	OM223850
			N^{pro}	OM223841
BVDV-1_Bor7_Türkiye_2019	1d	NCP	5'-UTR	OM223851
			N^{pro}	OM223842
BVDV-1_Bor8_Türkiye_2020	1d	NCP	5'-UTR	OM223852
			N^{pro}	OM223843
BVDV-1_Bor9_Türkiye_2020	1d	NCP	5'-UTR	OM223853
			N^{pro}	OM223844

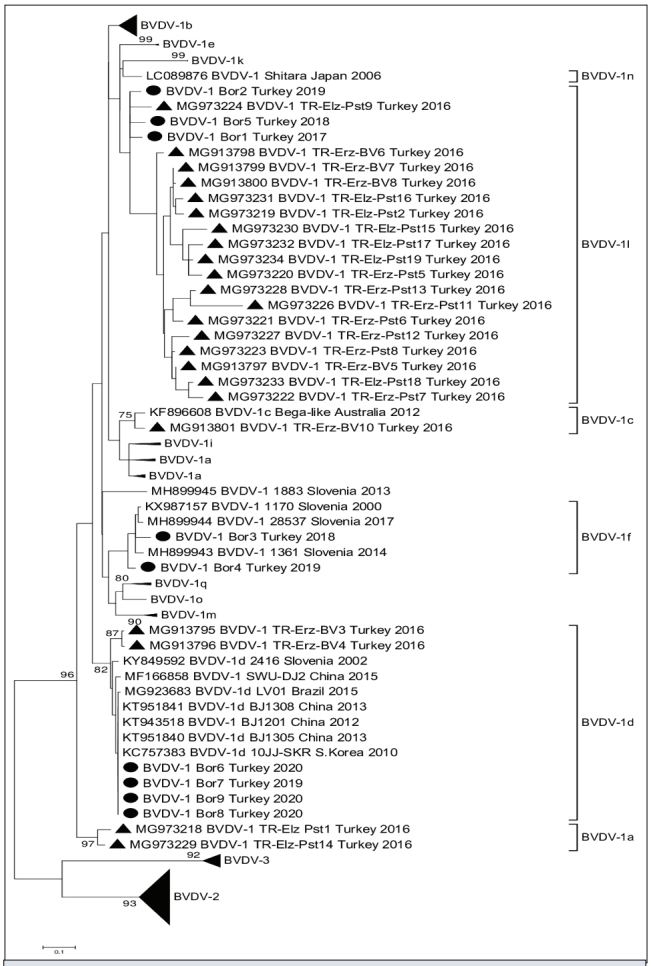

Fig 2. Maximum likelihood phylogenetic tree constructed with the 220bp 5'UTR region of BVDV-1, BDVD-2 and BVDV-3 sequences. The isolates were aligned by the ClustalW, and the phylogenetic tree was constructed using the MEGA6 package with a bootstrap value of 1.000 replicates. The sequences obtained in this study are marked with a round black spot (●), and the sequences obtained from different studies in Türkiye are marked with a black triangle (▲)

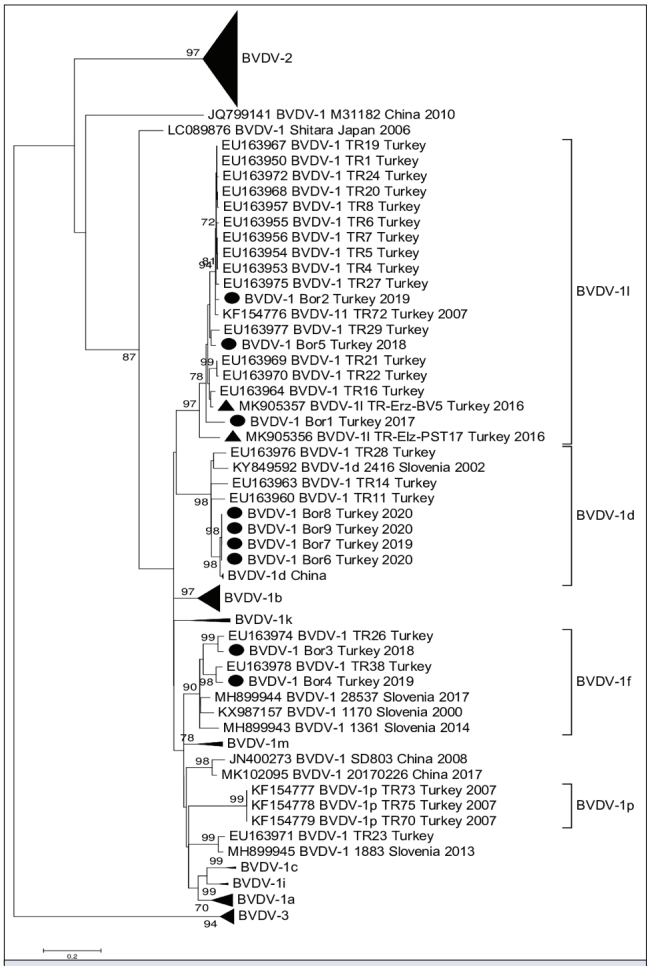

Fig 3. Maximum likelihood phylogenetic tree constructed with the 403bp N-terminal region of the polyprotein from BVDV-1, BDVD-2 and BVDV-3 sequences. The isolates were aligned by the ClustalW, and the phylogenetic tree was constructed using the MEGA6 package with a bootstrap value of 1.000 replicates. The sequences obtained in this study are marked with a round black spot (●), and the sequences obtained from different studies in Türkiye are marked with a black triangle (▲)

Slovenia belonging to cluster BVDV-1f *(Fig. 2, Fig. 3)*. The remaining three samples Bor1_Türkiye_2017, Bor2_Türkiye_2019 and Bor5_Türkiye_2018 grouped with the BVDV samples described in Türkiye between 2007 and 2017 in sub cluster BVDV-1l *(Fig. 2, Fig. 3)*. Subtyping based on phylogenetic study of the 5'-UTR and N-terminal region produced similar results.

Discussion

According to current statistical data, the cattle population in Türkiye is approximately 18 million [18]. Pestiviruses are endemic among livestock in Türkiye and causes significant economic losses by causing abortions in sheep, goats and cattle. In recent years, numerous studies on the typing of pestiviruses have been conducted in other parts of Türkiye, yet information concerning the subtyping of pestiviruses responsible for causing abortion in the west of the country is still deficient. This study was conducted to investigate the genetic diversity of pestiviruses circulating in western Türkiye, which cause abortions and subsequent economic losses in cattle. For this purpose, sequence analyzes were performed based on the 5'-UTR and N^{pro} gene regions of abortion samples, which were identified as BVDV positive using real time RT-PCR. Based on the resulting phylogenetic trees and pairwise percentage sequence identity, the nine samples were divided into three different subtypes. The most prevalent subtype identified from cattle abortion in the west of Türkiye was 1d (n=4), followed by 1l (n=3) and 1f (n=2).

Previous molecular characterization studies conducted in Türkiye, identified BVDV-1l as the dominant subtype amongst BVDV-1 types [19-22]. Yesilbag et al.[19] was the first to identify BVDV-1l as a unique subtype and that it is the most dominant subtype in Türkiye (in 5 out of 15 locations). This result was confirmed when 18 (45%) of the 40 positive samples, from 15 different farms representing 5 regions of Türkiye, identified BVDV-1l as the predominant genotype in Türkiye [22]. Similarly, 19 of the 28 samples (67.8%) described by Timurkan and Aydin [12], belonged to BVDV-1l subtype. Since all the previously mentioned studies were conducted in the Eastern Anatolia region of Türkiye, it was imperative to determine if the prevalence of BVDV-1l is restricted to the eastern Anatolia region or if it could be extrapolated to the whole of Türkiye.

In this study BVDV-1l (n=3), BVDV-1d (n=4) and 1f (n=2) subtypes were identified, originating from western Türkiye. Based on all the genetic and molecular characterization studies conducted in Türkiye, BVDV 1a, 1b, 1d, 1f, 1h, 1i, 1l as well as BVDV 2a, 2b and BVDV-3 have been identified [12,19-22]. Therefore, the 1d, 1l, 1f detected in this study correlates and clusters with sequences from previous identified samples in the country. It is also important to emphasize that the subtype, BVDV-1l, seems to be unique to Türkiye and circulates countrywide [10]. Considering the geographic position of Türkiye as a bridge between Europe and Asia, the importance of subtypes 1d and 1f detected in this study should be noted, since these two types were the dominant subtype identified in various Europe countries (Germany, Italy, Austria and Poland) [16,23-28]. Therefore, trade in animals and animal products between countries may increase the probability of observing different subtypes.

It has been reported that subtype BVDV-1b has a frequent and global distribution, yet upon considering the global spread of BVDV-1 the following observations are made. BVDV-1a and 1b occur in the Americas, 1c in Australia, 1a in Africa, whilst 1b, 1a, 1c, and 1m occur in Asia and 1a, 1b, 1e, 1f, 1h and 1d in Europe [11]. Although this explains the reason for 1a and 1b subtypes inclusion in the current vaccines, it is important to additionally evaluate the level of protection afforded by these vaccines, especially in the content of the isolates circulating in the western part of Türkiye, as described in this study.

BVDV-2 and BVDV-3 types were not detected in this study. Currently, BVDV-3 has only been described in a single study in Türkiye, which is not surprising considering the relatively recent global description of this virus as new species. In contrast, various studies have reported the identification of BVDV-2 in Türkiye [10,19,29]. These studies indicated that this type, which has a global distribution, not only occurs in Türkiye but has novel genetic differences based on spatial and temporal distributions.

Knowing the genetic heterogeneity and phenotypic differences of BVDV infection is important for a rapid diagnosis, control and eradication of the diseases caused by pestiviruses. In this study conducted in the west of Türkiye, 1d, 1l and 1f types were re-identified in cattle herds. The paucity of information on the true prevalence of this infection across Türkiye (cattle, sheep-goat and swine data) hinders efforts to estimate the true impact of pestiviruses on cattle populations. For this infection, systematic molecular epidemiological studies should be carried out throughout the country and prevention-control programs should be developed, including national trade regulations for live animals, animal products and biological products.

Both CP and NCP pestiviruses are involved in the pathogenesis of mucosal disease, a deadly disease induced by the development of CP virus in cattle that have been infected with NCP virus for a long time. Pestivirus biotypes are typically detected using cell cultures in which CP biotypes cause cytopathic effects. The creation of the non-structural protein NS3, which is antigenically linked to NS2-3 in cells infected with CP viruses alone, has been

found to constitute a major genetic difference between the CP and NCP biotypes [15,30]. Pestivirus cultivation in cell cultures is time-consuming and labor-intensive. As a result, the RT-PCR method described by Greiser-Wilke et al.[16] was used to differentiate biotypes in this study. Pestiviruses of the CP and NCP biotypes can cause abortion in cattle and small ruminants. However, based on the RT-PCR assay, all positive pestiviruses in this study were found to be the NCP biotype. This is consistent with previous research, which found that the majority of abortions in the field are caused by the NCP biotype of pestiviruses [31,32].

In conclusion, pestiviruses are an infection that causes serious economic losses (persistent infections) worldwide as well as in Türkiye. Since all pestivirus infections cause similar clinical symptoms, they will continue to cause significant economic losses as they enter new parts of the world. For this reason, it is necessary to prepare important control and eradication programs to prevent the spread of viruses by using specific diagnostic and control approaches that can be enforced by the state.

Availability of Data and Materials

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

Funding Support

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Ethical Approval

Ethics committee approval is not required as it is used with abortion material.

Competing Interests

There is no conflict of interest.

Author Contributions

AAÇ: design of the study; AAÇ, AS, MOT: drafting of the manuscript; AAÇ, MK, KP, FA: design of the study, performing the experiment, and drafting the manuscript; AAÇ, MK, KP, FA: sampling. All authors read and approved the final version of the manuscript.

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Research Article

Associations Between MSTN/HaeIII Polymorphism and Reproductive and Growth Characteristics in Morkaraman Sheep

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Abstract: The myostatin gene inhibits skeletal muscle growth in advanced animals, and a mutation in the gene coding region increases muscle formation. Hence, it is accepted to be a candidate gene for the selection of some production traits. The objective of the current research was to examine the genotypes of the myostatin gene and reveal the associations between the genotypes and a number of traits, including birth weight, weaning weight, weaning age, average daily weight gain, and productivity, in 110 Morkaraman sheep. Genotypes were determined by the PCR-RFLP method using the *Hae*III restriction enzyme, and the genotype frequencies were found to be 9%, 69%, and 22% for MM, Mm, and mm in the myostatin locus, respectively. The *M* allele frequency was 44%, whereas the *m* allele frequency was 56%. In the studied population, the myostatin locus was not in Hardy-Weinberg equilibrium. The association analysis revealed no statistically significant impact of the *MSTN* gene polymorphism in exon 3 on birth weight, weaning weight, and productivity (P>0.05) but found a significant effect on weaning age and average daily weight gain (P<0.05). As a result, the *MSTN* gene showed polymorphisms in Morkaraman sheep and can be regarded as a genetic marker for sheep selection according to the association analysis results.

Keywords: *MSTN gene, Polymorphism, Morkaraman, PCR-RFLP, Production traits*

Morkaraman Koyunlarında MSTN/HaeIII Polimorfizmi İle Dölverimi ve Büyüme Özellikleri Arasındaki İlişkiler

Öz: Miyostatin geni, gelişmiş hayvanlarda iskelet kası büyümesinin bir inhibitörüdür ve gen kodlama bölgesindeki bir mutasyon kas oluşumunu arttırır. Bu nedenle bazı verim özelliklerinin ıslahı için aday gen olarak kabul edilmektedir. Çalışmada, Morkaraman koyunlarından alınan genomik DNA örneklerinden MSTN/*Hae*III gen polimorfizmine ait genotiplerin araştırılması, genotip ve alel frekanslarının dağılımının belirlenmesi ve genotipler ile doğum ağırlığı, sütten kesim ağırlığı, sütten kesim yaşı, ortalama günlük canlı ağırlık artışı ve dölverimi gibi bazı özellikler arasındaki ilişkilerin belirlenmesi amaçlanmıştır. Kayıtlı 110 Morkaraman koyunundan kan örnekleri alındı ve her bir örnekten DNA ekstrakte edildi. Genotipler, PCR-RFLP yöntemi ile myostatin geninin polimorfik ekson 3 bölgesi için *Hae*III restriksiyon enzimi kullanılarak belirlendi. Sonuç olarak, miyostatin lokusunda MM, Mm ve mm için genotip frekansları sırasıyla %9, %69 ve %22 idi. M allel frekansı %44 ve m allel frekansı %56 idi. Popülasyonda miyostatin lokusu Hardy-Weinberg genetik dengesinde değildi. Sonuçlara göre *MSTN* geni ekson 3 polimorfizminin etkisi, doğum ağırlığı, sütten kesim ağırlığı ve dölverimi üzerine istatistiksel olarak önemli bulunamadı (P>0.05), ancak, sütten kesim yaşı ve ortalama günlük canlı ağırlık artışı üzerine etkisi önemliydi (P<0.05). Sonuç olarak, *MSTN* geni Morkaraman koyunlarında polimorfizm göstermiştir ve bu polimorfizm ıslah çalışmalarında genetik markör olarak kullanılabilir.

Anahtar sözcükler: *MSTN geni, Polimorfizm, Morkaraman, PCR-RFLP, Verim özellikleri*

Introduction

In recent years, many researchers have been trying to reveal the genetic structure of breeds by using new techniques at the molecular level and conducting studies at the DNA level to protect animal breeds as a genetic resource and help the breeding of indigenous breeds [1-6]. Sheep breeding is performed worldwide, and meat yield represents an important part of the income from sheep breeding. It is essential to increase productivity and quality

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per animal but not the number of animals in animal breeding. Production traits, such as growth, milk yield, multiple births, and meat yield, represent the most crucial economic features in sheep breeding. Since many genes affect the mentioned production traits, their improvement takes a long time [7-11].

The production of sheep meat can be increased even further by researching the impacts of the myostatin gene on sheep meat production and making the said gene available in sheep breeding.

MSTN is the growth differentiation factor 8 (GDF8) gene and regulates skeletal muscle growth negatively [12]. The polymorphisms of the said gene have been studied in various goat and sheep breeds [12,13]. The size of the sheep *MSTN* gene is 6756 bp, and it is located on chromosome 2 [14]. The myostatin protein belongs to the tumor growth factor (TGF-β) superfamily, and its synthesis is performed by a 376-amino acid precursor protein containing three domains, such as a C-terminal domain, N-terminal propeptide domain, and a signal sequence [15]. There is an association between single nucleotide polymorphisms (SNPs) within the coding region of the myostatin gene and double muscle [15,16]. Since it is nutritionally and economically important, extensive research has been done on various methods with the objective of determining the carcass and production traits of domestic animals [3,4,11].

Many studies have indicated significant associations between the myostatin gene and some yield traits in sheep, e.g., a study on muscle development in Belgian Texel sheep [17] and New Zealand Texel sheep [16,18], studies on muscle and fat thickness in English Texel sheep [19], Charollais sheep [20], and Ujumqin sheep [21] and on increased carcass amount in Norwegian White sheep [3], increased body weight and weaning weight in Baluchi sheep [22], on growth and carcass features in New Zealand Romney sheep [4,23], and on lamb birth weight and skeletal muscles in Dorper and Hu ewes [24]. Molecular methods, e.g., PCR-RFLP and PCR-SSCP, research the genetic variants in the myostatin *(MSTN)* gene associated with meat traits [3,11]. *MSTN* gene polymorphisms are associated with meat production and decreased total lean meat and an increased proportion of loin meat [4]. Marker-assisted selection (MAS) assists with the correct selection of DNA variations related to differences in growth and carcass traits and, thus, the selection of individuals with superior characteristics. Hence, sequencing of the livestock myostatin gene is essential to understand the structure, function, and evolution of the gene and generate genomic resources for the advancement of knockout technology.

The Morkaraman breed, the native breed dominant in the eastern and northeastern regions of Turkey, represents a fat-tailed breed, which has adapted to severe environmental conditions, including high altitude and harsh climatic conditions [25]. The Morkaraman breed takes an essential place as a domestic gene source due to its vicinity to the Fertile Crescent region, the first place of domestication with fewer selection studies. The work aimed to investigate the genotypic structures of the myostatin *(MSTN)* gene locus *Hae*III polymorphism and research the relationships between *MSTN* genotypes and a number of traits, e.g., birth weight, weaning weight, weaning age, average daily weight gain, and productivity, in Morkaraman sheep.

Material and Methods

Ethical Approval

The experimental protocol was approved by the Republic of Turkey Ministry of Agriculture Faculty Local Ethics Committee (AEC approval number: 3/2021).

Sampling and DNA Isolation

In the current research, blood samples were collected from unrelated 110 Morkaraman sheep raised as recorded in the pedigree and growth data in the Food and Livestock Application and Research Center (GHUAM), Sheep Breeding Branch at Ataturk University, Erzurum province. DNA was isolated from blood samples by utilizing the Qiagen genomic DNA purification kit.

Polymerase Chain Reaction (PCR)

The PCR reaction was carried out in a final reaction volume of 20 µL, including 4 µL of 10x buffer, 1 µL of every primer (10 pmol/µL, 1 µL$MgCI_2$, 0.5 µLDNTPs, 2.4 µL Taq DNA polymerase, 2 µL of total DNA (50-100 ng), and finally added ultrapure water until reaching a total volume of 20 µL. The amplification of a 337 bp fragment for exon 3 of the sheep *MSTN* locus was performed using the primer pairs reported by Smith et al.[26], forward primer 5'-CCG GAG AGA CTT TGG GCT TGA-3' and reverse primer 5'-TCA TGA GCA CCC ACA GCG GTC-3', with a thermal cycler. For polymerase activation, at the beginning of the PCR cycle, an incubation step was carried out at 95°C for 5 min, followed by 1 cycle of 95°C for 45 s, annealing at 59°C for 45 s, and an extraction step at 72°C for 45 s, followed by 35 cycles of 5 min at 72°C as a final extraction.

Genotyping of the *MSTN* Gene

The restriction endonuclease enzyme *Hae*III digested PCR products of the amplified *MSTN* gene. The incubation of the PCR products was carried out at a temperature of 37°C for a period of 10-12 h in a final volume of 20 µL, containing 8-10 µL of the PCR product, 5 µL of the buffer R, 2.5 µL of the buffer tango, and 6 U *Hae*III restriction enzyme. The digested products were run on 2% agarose gel stained with

EtBr (500 µL/mL in H_2O). Afterward, the digested PCR products were obviously envisioned under UV light.

Data Analysis

The allele gene and genotype frequencies and Hardy-Weinberg test for the examined population were computed in the GenAlEx 6.5 software [27].

One hundred ten pure Morkaraman sheep aged 2-4 years and raised in Atatürk University Food and Livestock Application and Research Center were utilized as the animal material of this study. The sheep were held under semi-extensive conditions. The associations between a number of yield traits and genotypic structures of Morkaraman sheep were studied. To this end, primarily their birth weight, weaning weights and weaning ages, and average daily weight gains were determined as the performance traits of sheep. As reproductive traits, annual lamb rates per sheep were determined, and the lambing rates (productivity) in birth for each ewe mated were calculated. Feeding and management practices were administered in an equal manner to all lambs. When analyzing the acquired data, SPSS statistical software (IBM SPSS 25.0 Corp. Inc.) was utilized based on the general linear model. The association analyses separately examined the impact of genotype on birth weight and productivity; the impacts of genotype and birth weight on weaning age, and average daily weight gain; the impacts of genotype, birth weight, and weaning age on weaning weight.

The statistical models below were employed in accordance with the yield traits in the study.

Yijk:µ+ai+(bj)+(ck)+eijkl

Yijk l: Value of any sheep for the considered performance (birth weight, weaning weight, productivity, weaning age and average daily weight gain) traits

µ: population mean

ai: effect of genotype i (i: 1-3; MM: 1, Mm: 2, mm: 3)

(bj): covariate effect of birth weight j (on weaning age and average daily weight gain)

(ck): covariate effect of weaning age k (on weaning weight)

eijkl: marginal error

In the model used, the genotype and the others' (covariate) effects were accepted as constant, while the error was accepted as random.

Results

Table 1 contains the observed and expected genotype results of the *MSTN* gene *Hae*III polymorphism and the Hardy-Weinberg genetic equilibrium test results in Morkaraman sheep.

The genotypes of the MSTN/*Hae*III polymorphism were found as MM, Mm, and mm, and the percentage frequencies were computed as 9%, 69%, and 22%, respectively *(Table 1)*. The m allele frequency was found as 56%, whereas the M allele frequency was found as 44% for the Morkaraman breed. The Hardy-Weinberg genetic equilibrium test demonstrated that the examined population was not in equilibrium with an X^2 test value of 17.37 and a probability of (P<0.001) *(Table 1)*.

Associations between MSTN/*Hae*III genotypes and a number of performance traits, including birth weight, weaning weight, weaning age, productivity, and weight gain, were researched. *Table 2* demonstrates the least squares mean and standard errors of the MSTN/*Hae*III genotypes concerning several yield traits.

Table 1. *Observed and expected genotypes of the MSTN gene and Hardy-Weinberg genetic equilibrium test results in Morkaraman sheep*

Genotype	(%)	Observed	Expected	X^2 Test	P
MM	(9)	10	20.7	17.37	**
Mm	(69)	75	53.6		
mm	(22)	24	34.7		
Gene frequencies: M: 44%, **m:** 56%					

*** P<0.01*

Table 2. *The least squares means and standard errors of MSTN/HaeIII genotypes in terms of some yield traits in Morkaraman sheep*

Genotype	N	Birth Weight (kg)		Weaning Weight (kg)		Weaning Age (Days)		Productivity		Average Daily Weight Gain (kg)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
MM	10	4.19	0.255	15.79	1.421	56.9^{a*}	4.20	0.849	0.045	0.202^{b*}	0.015
Mm	75	4.43	0.077	14.59	0.401	49.8^{ab}	1.34	0.871	0.030	0.204^{b}	0.006
mm	24	4.42	0.010	14.84	0.635	46.2^{b}	2.05	0.870	0.051	0.229^{a}	0.008
Total	109	4.41	0.062	14.76	0.333	49.7	1.12	0.867	0.024	0.209	0.005

*SE: Standard Error, * P<0.05*

Genotype did not have a significant impact on birth weight (P>0.05). The birth weight averages of the MM, Mm, and mm genotype groups were revealed to be 4.19 kg, 4.43 kg, and 4.42 kg, respectively, whereas the order was Mm>mm>MM. No significant difference was observed between the means.

Whereas the impact of genotype on weaning weight was insignificant (P>0.05), the impact of birth weight and weaning time was significant (P<0.01). Considering at what % the independent variable affected the dependent variable, the impact of genotype on weaning weight was 3%. The impact of weight was computed as 19%, while the impact of weaning time was computed as 50%. Whereas the mean weaning weights in the MM, Mm, and mm genotype groups were found to be 15.79 kg, 14.59 kg, and 14.84 kg, respectively, the order was inverse to birth weight as MM>mm>Mm. No significant difference was revealed between the means of the genotype groups *(Table 2)*.

While the impact of genotype on weaning age was found to be significant (P<0.05), the impact of birth weight was insignificant (P>0.05). The mean weaning age in the MM, Mm, and mm genotypes was determined to be 56.9 months, 49.8 months, and 46.2 months, respectively, and the order was MM>Mm>mm. The weaning age of the mm genotype was revealed to be shorter than that of the sheep with the MM genotype, and the said difference was statistically significant (P<0.05). However, no statistical difference was determined between the sheep with both genotypes and the heterozygous Mm genotype concerning the weaning age mean (*Table 2*).

The impact of genotype on productivity was determined to be insignificant (P>0.05). Whereas the mean productivity of MM, Mm, and mm genotype groups was acquired as 0.849, 0.871, and 0.870 kg, respectively, the order was Mm>mm>MM. The means of the genotype groups did not differ significantly *(Table 2)*.

The impact of genotype on the mean weight gain from birth to weaning was determined to be significant (P<0.05), while the influence of birth weight was insignificant (P>0.05). Whereas the mean weight gain in the MM, Mm, and mm genotype groups was acquired as 0.202 kg, 0.204 kg, and 0.229 kg, respectively, the order was mm>Mm>MM. The mean weight gain values of the mm genotype were revealed to be statistically significantly (P<0.05) higher compared to those of the sheep with the MM and Mm genotypes *(Table 2)*.

In this study, the polymorphic site of the *MSTN* gene exon 3 region was not associated with birth and weaning weight and productivity. However, there was an association with weaning age and mean daily weight gain, which may be caused by a breed-specific effect.

Discussion

The myostatin gene polymorphisms have been stated to differ in sheep. Soufy et al.[28] observed all of the three genotypes in Sanjabi sheep, while Bayraktar [29] indicated two genotypes, mm and MM, in Iraqi Avassi sheep, and others reported two genotypes, mm and Mm, in Kordi sheep [30], Kalehkoohi sheep [31], Farahani sheep [32], Mehraban sheep [33], and Teleorman Black Head sheep [34]. The studies on polymorphisms reported the higher frequency of the polymorphic *MSTN* gene m allele compared to the M allele gene frequency, and it was found to be consistent with the present research. However, a number of researchers indicated the absence of *MSTN* gene polymorphisms and reported that the mm genotype was monomorphic in Dalagh sheep [35], Zell sheep [36], and Bulgarian sheep [37].

Although some authors reported the Hardy-Weinberg genetic equilibrium in the populations of Mehraban sheep [33], Teleorman Black Head sheep [34], and Iraqi Avassi sheep [29], other authors reported the absence of the Hardy-Weinberg genetic equilibrium in Sanjabi sheep [28], Dalagh sheep [35], Zell sheep [36], and Mehraban sheep [33], which is consistent with the present study results. The above-mentioned discrepancy can be explained by environmental factors, breed differences, population and sampling size, mating strategies, geographical position effect, and genotypic distribution of genetic variants. This result can also be explained, especially by the imbalance caused by selection and migration. The examined population displayed a high degree of genotypic variability for the *MSTN* gene, which may be associated with the breeding plan applied.

Despite genotyping studies on MSTN/*Hae*III polymorphisms, a very low number of studies have been performed on the relationship between genotypes and yield traits. Nevertheless, several findings on the relationship between polymorphic exon 3 of the *MSTN* gene and growth traits have been indicated for weaning weight, 6-month weight, and some carcass traits in Batur sheep, and they have an insignificant effect [11]. Some research has been done on the other polymorphic regions of the *MSTN* gene. In case of the non-expression of myostatin, negative growth regulation fails, and an increase in the number of muscle fibers occurs, resulting in hyperplasia. Boman et al.[3] stated lower daily gain and weaning weight and higher carcass weight in homozygous c.960delG (AA) animals. Furthermore, the AG and GG genotypes led to significant (P<0.001) impacts as more meat and less fatty animals. Nevertheless, mutations in the *MSTN* gene exon 3 influence conformation and adiposity in NWS lambs, causing a carcass with less fat and more muscle mass. An insignificant effect of a mutation in *MSTN* exon 1 and intron 1 regions was revealed on birth, weaning

(3-month), and 6-month weight in Mecheri, Madras Red, and Nilagiri sheep in India [38]. In another PCR-SSCP study, genetic variants were found to be related to meat traits in the myostatin gene, whereas the *MSTN* gene was characterized by decreased total lean meat and increased waist meat ratio [4].

As a result, three genotypes, MM, *mm,* and *Mm*, were determined in the current research at a rate of 9%, 69%, and 22%, respectively. The *M* allele frequency was found to be 44%, whereas the *m* allele frequency was revealed to be 56%. There was a statistical association between the impact of the MSTN/*Hae*III polymorphism and weaning age and average daily weight gain. Nevertheless, no relationship was determined with birth weight, weaning weight, and productivity. The *MSTN* gene exhibits polymorphisms in Morkaraman sheep. However, for it to be regarded as an important genetic marker, further investigations on the *MSTN* gene polymorphisms are suggested in other sheep breeds all around the world to assess potential sheep breeds and use it as a genetic marker in improving growth traits.

Availability of Data and Materials

Data sets are not deposited in different repositories, and data from a third party were not used. The data are original, and users can get it from corresponding author (M. Özdemir).

Ethical Approval

The experimental protocol was approved by the Republic of Turkey Ministry of Agriculture Faculty Local Ethics Committee (AEC approval number: 3/2021).

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Competing Interest

All the authors declare that they have no conflict of interest.

Author's Contributions

Experimental design was conceived by MÖ and NE. ES and KE performed the laboratory experiments, and DT, UDT and SK collected the all data and arranged for the analysis. MÖ performed the statistical analysis and wrote the manuscript. All authors were ranked based on the contribution rates for performing lab studies, collecting the data and literature search and the corrections.

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RESEARCH ARTICLE

Feeding a Low-protein Maternal Diet Affects Qinghai Bamei Piglet Jejunal Structure and Microbial Function Response

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Abstract: This experiment investigated the impacts of feeding a maternal low-CP concentration diet having iso-essential amino acids on newborn suckling piglet's intestinal microbial composition and function. Forty randomly selected purebred Bamei sows were divided into two groups and fed a low dietary CP (12%, LP) or a normal CP (14%, CON) diet, respectively, but formulated to contain similar (iso-) essential amino acid concentrations per current recommendations. At 21 days, 12 piglets were randomly selected from each treatment and euthanized with jejunum content samples collected. The 16S rRNA gene sequencing was combined as an integrated approach for evaluating the functional impact of maternal CP concentrations on piglet intestinal microbiome. Even though piglets demonstrated similar 0 to 21 d ADG among treatments, the jejunum relative weight, villus width, crypt depth and muscular thickness were increased (P<0.05), while villus height, and villus height/crypt depth were reduced (P<0.05) for the material LP compared to the maternal fed CON diet. Maternal CP concentrations can modify the intestinal microbial composition of Bamei suckling piglets. The relative abundances of the bacterial species *Escherichia-Shigella*, *Actinobacillus*, *Clostridium*_sensu_stricto_1, *Veillonella*, and *Turicibacter* were increased (P<0.05) in the maternal LP fed diet compared with the maternal fed CON diet microbiota metabolites. Overall, LP diet contributed to improve piglet intestinal histomorphology, microbial composition and function.

Keywords: *Qinghai Bamei piglet, Low-protein maternal diet, Intestinal histomorphology, 16S rRNA, Bioinformatics*

Düşük Proteinli Maternal Diyet ile Besleme Qinghai Bamei Domuz Yavrularının Jejunal Yapısını ve Mikrobiyal Fonksiyon Yanıtını Etkiler

Öz: Bu çalışmada, izo-esansiyel amino asitlere sahip düşük CP konsantreli maternal bir diyetle beslenmenin, yeni doğmuş süt emen domuz yavrularının bağırsak mikrobiyal bileşimi ve işlevi üzerindeki etkileri araştırıldı. Rastgele seçilen kırk safkan Bamei domuzu iki gruba ayrıldı ve sırasıyla düşük CP (%12, LP) ve normal CP (%14, CON) diyetle beslendi. Ancak, her iki diyet de güncel tavsiyelere göre benzer (izo-) esansiyel amino asit konsantrasyonlarını içerecek şekilde formüle edildi. Her iki diyet grubundan 21. günde rastgele 12 domuz yavrusu seçildi, ötenazi yapıldı ve jejunum içerikleri toplandı. 16S rRNA gen sekans entegreli bir yaklaşım ile maternal CP konsantrasyonlarının domuz yavrularının bağırsak mikrobiyomu üzerindeki fonksiyonel etkisi değerlendirildi. Her iki diyet grubundaki domuz yavruları, 0 ile 21. günler arası benzer ADG göstermiş olsa da, CON diyetine kıyasla maternal LP diyeti ile beslenenlerde jejunum relatif ağırlığı, villus genişliği, kript derinliği ve kas kalınlığı artmış (P<0.05), villus yüksekliği ve villus yüksekliği/kript derinliği azalmıştı (P<0.05). Maternal CP konsantrasyonları, süt emen Bamei domuz yavrularının bağırsak mikrobiyal bileşimini değiştirebilir. Maternal CON diyetle beslenenlere kıyasla maternal LP ile beslenenlerde *Escherichia-Shigella*, *Actinobacillus*, *Clostridium*_sensu_stricto_1, *Veillonella* ve *Turicibacter* bakteri türlerinin relatif yoğunlukları artmıştı (P <0.05). Genel olarak, LP diyeti, domuz yavrularının bağırsak histomorfolojisinin, mikrobiyal bileşimin ve işlevinin iyileştirilmesine katkıda bulunmuştur.

Anahtar sözcükler: *Qinghai Bamei domuz yavrusu, Düşük proteinli maternal diyet, Bağırsak histomorfolojisi, 16S rRNA, Biyoinformatik*

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Introduction

The Bamei is a local swine breed in the Qinghai Province of the People's Republic of China. Even though Bamei is a slow growing breed, Bamei swines are known for their high meat quality and distinctive flavor [1,2]. The Qinghai plateau has used both natural and artificial selection practices for developing Bamei pigs that show a strong adaptability to the plateau, have high fat deposition, and good meat quality characteristics. However, Bamei's lower growth rate combined with the plateau's low feed quality/digestibility are important constraints limiting the Qinghai's growth potential of the Bamei swine industry [3].

The gastrointestinal tract's microbial ecosystem is dynamic and complex with the composition known to vary widely across healthy individuals [4]. In the human and animal gastrointestinal tract there is a large and diverse microbial community playing a vital role in host health [5], mucosal immunological environment maturation [6,7] and assisting with intestinal barrier integrity [8]. Over the last decade, numerous studies have reported that the intestinal microbiome composition plays an important role in regulating the metabolic health of both rodents and humans [9]. A recent study conducted on rodents suggests the major dietary factors regulating intestinal microbiome taxonomic composition are protein and carbohydrate intake [10].

The intestinal microbiome is in a continual state of flux and highly susceptible to numerous environmental factors, especially dietary nutrient supply. Reducing CP by 2 to 4 percentage units by adding crystalline amino acids (AA) to meet NRC (2012) nutrient recommendations has increased nitrogen utilization, reduced feed costs and nitrogen excretion, while promoting intestinal health and meat quality with similar growth performance [11,12]. Many studies demonstrate dietary CP concentrations versus CP source, have a greater impact on intestinal microbiota composition [13,14]. Previous studies have focused on changes in large intestinal microbiota, while ignoring the bacteria's role for the small intestine [15]. Moderate diet protein restriction may alter intestinal microbiota composition while improving adult pig ileal barrier function [16,17]. Chen reported that decreasing dietary CP concentration 3 % units reduced ileal *Streptococcus* spp., while increasing *Lactobacillus* spp. and *Bifidobacterium* spp. [18]. These ileal microbiota alterations improved intestinal stem cell proliferation and altered tight junction protein distribution resulting in similar intestinal barrier function. Therefore, feeding dietary LP concentrations has advanced while maintaining essential amino acid supply and has been applied to swine production. The purpose of this study was to explore the effects of low protein diet on the structure and function of intestinal microflora of Qinghai Bamei pigs, to lay a foundation for further exploration of the effects of maternal dietary intervention on jejunal microbiota composition and function to provide ideas for efficient breeding of Qinghai Bamei pigs.

Material and Methods

Ethical Approval

All procedures involving the use of animals were approved by the Animal Care Committee of Qinghai University, China (QHDX-17-02-12-06). Animal slaughtering was approved by the National Administration of Slaughtering and Quarantine regulations (Qinghai, China).

Animals and Diets

Forty (40) purebreds Huzhu Bamei well body condition (score 4) sows were sourced through the Qinghai Province Huzhu County Bamei Pig Seed Breeding Farm (Huzhu, China) having similar body weight (BW), health status, and 3 to 4 years of age being randomly assigned to one of two treatments (20/treatment). The LP treatment diet (12% CP) was balanced for the five EAA Lys, Met, Thr, Trp, and Val for their standardized ileal digestibility (SID) concentrations and then decreased CP by 2% compared to a control (CON; 14% CP) diet balanced for the same SID EAA according to Chinese feeding standards for a 90 kg heavy body conditioned sow. The complete diet composition is given in *Table 1*. After 5 d of facility and diet

Table 1. *Ingredient and nutrient composition of maternal diets (DM basis) containing 12% (LP) or 14% crude protein (CON). DM basis) %*

Items	Groups	
	LP	CON
Ingredient composition		
Corn	50.60	44.90
Soybean meal	4.50	9.80
Rapeseed meal	2.50	2.70
Wheat bran	37.78	38.14
Lys	0.34	0.20
Met	0.07	0.05
Thr	0.15	0.10
Trp	0.02	0.01
Val	0.04	0.10
4% premixb	4.00	4.00
Nutrient concentrations, calculated via formulation		
DE (MJ/kg) a	11.72	11.72
CPb	12.04	12.04
Lys	0.81	0.81
Met+Cys	0.33	0.33
Thr	0.35	0.35
Trp	0.08	0.08
Val	0.26	0.26
Total Ca	0.62	0.62
Total P	0.51	0.51
Solt	3.20	3.20

[a] DE=digestible energy; [b] CP=crude protein; [b] The premix during pregnancy provided the following per kilogram of diets: Vit. A: 3.52 kIU; Vit. E: 20 kIU; Vit. D_3: 0.76 kIU; Vit. K_3: 2.6 mg; Vit. B_2: 9.52 mg; Vit. B_3: 24 mg; Vit. B_5: 45 mg; Cu: 4 mg; Fe: 10 mg; Zn: 40 mg; Mn: 16 mg; Ca: 15 %; Total P: 1.8%; NaCl: 8%; Water: 10 %

acclimation, the sows were fed the assigned treatment diet while skipping one estrous cycle (21 days) during natural estrus and then mated. The newborn piglets were housed with their mothers prior to weaning with litter size, live birth %, birth weights, and diarrhea rates being published previously [19]. Throughout the study all the sows had ad libitum access to feed and fresh water.

Sample Collection

Randomly, 12 piglets were selected from each treatment group, fasted for 12-h, weighed, and euthanized with 50 mg/kg sodium pentobarbital on day 21 of age. The small intestine was ligated at the pylorus, duodenum, jejunum, and ileum and dissected. The ligated jejunum was weighed. The jejunal contents were sampled at approximately the half-way point of the jejunal length, placed into 1.5 mL sterile polypropylene tubes, and stored in liquid nitrogen until analyses were conducted for intestinal microbiome. An approximate 1.5 cm jejunal tissue sample was collected, washed, and placed in 4% paraformaldehyde for histomorphometric analysis at the same time.

Histomorphometric Analysis

Jejunal tissue samples fixed in 4% paraformaldehyde were embedded in paraffin (5 μm) and stained with HE (hematoxylin-eosin). In each jejunal section, 12 intact villi were randomly selected from each piglet. The jejunum villus height, villus width, crypt depth, and muscular layer thickness were measured using an image analysis system (Caseviewer 2.0 software, 3DHISTECH, Hungary).

gDNA Extraction, 16S rRNA Gene Sequencing and Microbial Function Prediction

The jejunal content samples were extracted to harvest total bacterial DNA using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA samples were stored at -80°C until outsourced for analyzing the 16S rRNA gene sequencing by BIOMARKER (Beijing, China). The 16S rRNA gene sequence (Illumina HiSeq 2500) was used to measure microbial diversity and bacterial community composition. The extracted DNA was used as a template and PCR was performed using barcode primers located on both sides of the V3-V4 hypervariable region of the bacterial 16S rRNA gene. The primer sequences used were: 338F: 5'-ACTCCTACGGGAGGCAGCA-3' and 806R: 5'-GGACTACHVGGGTWTCTAAT-3'. Amplification was performed for 30 cycles using a DNA thermal Cycler (Bio-Rad, Hercules, CA, USA). The first cycle was at 98°C for 2 min followed by 30 subsequent cycles of 98°C x 30 s, 50°C x 30 s, then 72°C x 1 min, and the last cycle at 72°C for 7 min.

Statistical Analyses

All data were checked for outliers before any statistical analyses were conducted. Data were either plotted or the box and whisker plots and the Shapiro Wilk Test were used to verify that the data were normally distributed ($P>0.15$). All data were subjected to least squares analysis of variance (ANOVA) for a completely random design (CRD; Steel and Torrie, 1980) having 2 treatments using SPSS 21 software (SPSS Inc., Chicago, IL, USA). Least squares means were separated using the Least Significant Difference (LSD) and significant was declared at $P<0.05$.

The OTU were rarified based on several metrics for alpha diversity analysis including OTU rank curves, rarefaction, and Shannon, along with Shannon, Chao1, Simpson, and ACE calculated indices. Principal Coordinates Analysis (PCoA) and unweighted pair group method with arithmetic mean (UPGMA) were performed using QIIME based weighted UniFrac distance for beta diversity analysis [20]. Finally, PICRUSt [21] was used to predict microbial function. Bacterial domains, phyla, and genera were compared using Wilcoxon rank-sum test, with the FDR adjusted P value <0.05 being considered as significantly different. Finally, Spearman's rank correlations among jejunal microbiome changes, histomorphometric, and shifted metabolome were calculated to examine functional impacts of material LP diet concentrations on the small intestinal microbiome.

Results

Piglet Performance

Piglet birth BW (day 0) was greater for sows fed LP compared with piglet birth BW for sows fed CON ($P>0.05$), while 21 d piglet BW tended ($P<0.05$) to be greater for piglets from sows fed LP compared with sows fed CON *(Table 2)*. However, these initial and final piglet BW differences did not affect piglet ADG, which was similar among both treatments ($P>0.05$).

Table 2. *Piglet body weight (BW) and average daily gain (ADG) when feeding maternal diets containing 12% (LP) or 14% crude protein (CON)*

Items		LP	CON	SDM	P-value
Piglet BW, kg	Day 0	0.90	0.88	0.02	0.020
	Day 21	3.85	3.78	0.09	0.067
	ADG, 0 - 21, g/d	135.8	134.0	1.38	<0.37

Jejunal Morphology

Intestinal HE staining demonstrated that piglets nursing sows fed a maternal LP diet demonstrated reduced (P<0.05) villus height and ratio of villus height to crypt depth, while jejunum relative weight, villus width, crypt depth, and muscle thickness were increased (P<0.05) compared with piglets from sows fed the maternal CON diet *(Table 3)*.

The Diversity and Composition of Jejunal Microbiota

The 16S RNA jejunal microbiota samples after data filtering, quality control, and low-confidence singletons removal resulted in an average of 42.718 reads being obtained for the 21 d samples. The Good's coverages exceeded 99% demonstrating excellent sequence accuracy and reproducibility *(Table 4)*. Of the 482 total OTU numbers, 452 OTU were detected in both groups. Based on the Shannon (P<0.001), and Simpson (P=0.001) indices piglets from the maternal fed LP diet demonstrated more diversity and greater evenness compared with piglets from the material fed CON diet. The Chaol (P=0.519) and Ace (P=0.435) indices were similar for piglets from the maternal fed LP compared with the maternal fed CON. Taxonomic analysis revealed the predominant phyla *Firmicutes* and *Proteobacteria* being 67.21% and 24.97%, respectively of total reads identifying 16 bacterial phyla *(Fig. 1-A)*. At the genus level, 232 genera were identified in the jejunal samples. The predominant genera were *Lactobacillus* (51.11%), *Escherichia*-Shigella (9.00%), *Actinobacillus* (7.41%), *Clostridium*_sensu_stricto_1 (5.60%), *Romboutsia* (4.35%), and *Buchnera* (3.54%), respectively *(Fig. 1-B)*. Furthermore, using a PCoA plot illustrated microbial community dissimilarity and revealed distinct structures between piglets from the maternal fed LP compared with maternal fed CON *(Fig. 1-C)*. The PCoA plot uses a weighted method for UniFrac similarity, which revealed PC1 and PC2 explained 55.61% and 13.98% of sample variation, respectively. Similarly, the jackknifed beta diversity and hierarchical clustering analysis via the Unweighted Pair-group Method with Arithmetic Mean (UPGMA) demonstrated that different piglets fed different maternal CP diets were clustered in their individual groups *(Fig. 1-D)*. In addition, piglets from maternal fed CON diets in the PCoA plot were clustered into two subgroups and UPGMA hierarchical clustering analysis, which was attributed to individual variations of jejunum microbiome profiles.

Differences in Jejunal Bacterial Community Composition

Relative phylum abundances of *Firmicutes, Proteobacteria, Bacteroidetes,* and unknown were > 1% for both treatments *(Table 5)*. Firmicutes relative abundance was decreased (P=0.002) and Proteobacteria (P=0.001) was increased for piglets from the maternal LP treatment compared with piglets from the sows fed maternal CON. Thirty-two (32) specific genera demonstrated relative abundances >0.1%. The relative bacterial community abundances of *Escherichia*-Shigella (P=0.050), *Actinobacillus* (P=0.050), *Clostridium*_sensu_stricto_1 (P=0.003), *Veillonella* (P=0.015), and *Turicibacter* (P=0.011) were higher, and *Lactobacillus* was lower (P<0.001) for piglets from the

Table 3. *Jejunum weight and tissue morphology by 21-day old suckling piglets when feeding maternal diets containing 12% (LP) or 14% crude protein (CON)*

Items	LP	CON	SDM	P-value
Jejunum weight, g	123.22	109.95	17.12	0.074
Jejunum relative weight, %	3.42	3.17	0.30	0.048
Villus height, μm	318.58	385.44	17.99	<0.001
Villus width, μm	96.44	83.43	3.62	<0.001
Crypt depth, μm	150.15	99.01	6.58	<0.001
Villus height: Cryptdepth	2.13	4.62	0.19	<0.001
Muscular thickness, μm	65.17	60.75	2.24	<0.001

Table 4. *Alpha diversity measures of bacterial communities by 21-day old suckling piglets when feeding maternal diets containing 12% (LP) or 14% crude protein (CON)*

Items	LP	CON	SDM	P-Value
Chao1	218.08	208.89	33.48	0.519
Ace	216.58	205.47	33.66	0.435
Shannon	2.72	1.67	0.68	<0.001
Simpson	0.16	0.45	0.13	0.001
Coverage	0.9996	0.9996	<0.001	0.898

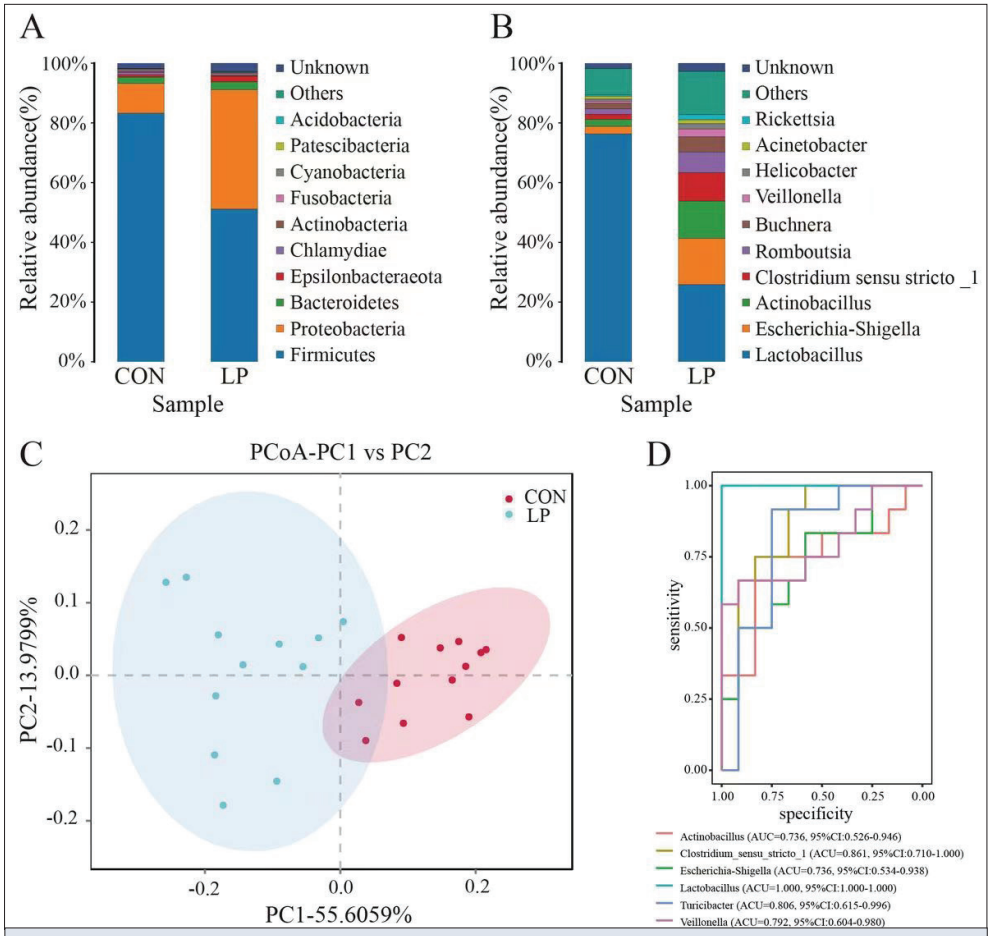

Fig 1. Classification of the bacterial community composition) by 21-day old suckling Bamei piglets when feeding maternal diets containing 12% (LP) or 14% crude protein (CON). **A**- Phylum level, **B**- genus level, **C**- PCoA plot, **D**- UPGMA tree. The relative abundance of the top 10 phylum and genus of jejunal microbiome composition profiles revealed by 16S rRNA sequencing (each color represents one bacterial). PCoA plot and UPGMA tree using the weighted unifrac similarity method

Table 5. *Phylum-level taxonomic composition of the jejunal bacterial communities by 21-day old suckling piglets when feeding maternal diets containing 12% (LP) or 14% crude protein (CON)*

Phylum	LP	CON	SDM	P-value
Firmicutes	0.51169	0.83253	0.17449	0.002
Proteobacteria	0.39987	0.09948	0.15060	0.001
Bacteroidetes	0.02626	0.02173	0.03188	0.299
Chlamydiae	0.00004	0.00804	0.01304	0.686
Epsilonbacteraeota	0.01906	0.00739	0.02340	0.166
Cyanobacteria	0.00210	0.00414	0.00565	0.773
Fusobacteria	0.00397	0.00372	0.00485	0.525
Actinobacteria	0.00452	0.00332	0.00593	0.356
Patescibacteria	0.00176	0.00111	0.00204	0.817
Acidobacteria	0.00110	0.00032	0.00140	0.840
Tenericutes	0.00070	0.00014	0.00112	0.544
Cloacimonetes	0.00009	0.00010	0.00035	0.544
Chloroflexi	0.00048	0.00007	0.00072	0.312
Verrucomicrobia	0.00008	0.00005	0.00020	0.356
Planctomycetes	0.00024	0.00002	0.00037	0.908
Gemmatimonadetes	0.00022	0.00002	0.00056	0.470
Unknown	0.02785	0.01781	0.02738	0.156

maternal fed LP treatment compared with piglets from the maternal fed CON treatment (genus level; *Table 6)*. The receiver operating characteristic curve (ROC) predicted different microorganisms for piglets from maternal fed LP compared to maternal fed CON piglets for inducing jejunal development. The area under the curve (AUC) judged via diagnosis test [22] that *Lactobacillus* is the most likely biomarker (0.9< AUC <1.0) for piglets from both treatments, while *Clostridium*_sensu_stricto_1 and *Turicibacter* are more likely biomarkers (0.8< AUC <0.9) for piglets from maternal fed LP sows.

Predicted Function of Jejunal Microbiota

The PICRUSt analyzed pathway compositions for evaluating jejunal bacterial community functional capacity is a functional-gene-count matrix. Second level KEGG (levels) metabolism pathway analysis via global and overview maps demonstrated that biosynthesis of other secondary metabolites was enriching amino acid, cofactors, and vitamins metabolism (P<0.05), while lipid and nucleotide metabolism were decreased (P<0.05) for piglets when maternal sows were fed LP diet compared with piglets from the maternal fed CON *(Fig. 2)*.

Table 6. *Genus-level taxonomic composition of the jejunal bacterial communities by 21-day old suckling piglets when feeding maternal diets containing 12% (LP) or 14% crude protein (CON)*

Genus	LP	CON	SDM	P-value
Lactobacillus	0.25881	0.76331	0.13670	<0.001
Escherichia-Shigella	0.15483	0.02514	0.12003	0.050
Actinobacillus	0.12509	0.02318	0.07921	0.050
Buchnera	0.05169	0.01920	0.05861	0.488
Romboutsia	0.06841	0.01856	0.06543	0.166
*Clostridium*_sensu_stricto_1	0.09503	0.01698	0.07304	0.003
Acinetobacter	0.01295	0.00957	0.01571	0.248
Prevotella_7	0.00384	0.01020	0.02064	0.436
Chlamydia	0.00004	0.00804	0.01298	0.686
Helicobacter	0.01813	0.00691	0.02292	0.094
Veillonella	0.02581	0.00659	0.01388	0.015
Turicibacter	0.00703	0.00440	0.01058	0.011
Rickettsia	0.01763	0.00407	0.01963	0.686
Uncultured_bacterium_f_Muribaculaceae	0.00853	0.00352	0.00993	0.326
Fusobacterium	0.00326	0.00329	0.00419	0.644
Pseudomonas	0.00922	0.00300	0.01422	0.106
Terrisporobacter	0.01388	0.00331	0.01267	0.299
Bacteroides	0.00514	0.00264	0.00618	0.184
Enterobacter	0.00117	0.00237	0.00358	0.603
Megasphaera	0.01073	0.00276	0.01537	0.386
Streptococcus	0.00261	0.00183	0.00164	0.149
Pasteurella	0.00642	0.00150	0.00635	0.194
Uncultured_bacterium_f_Lachnospiraceae	0.00161	0.00105	0.00270	0.795
Epulopiscium	0.00100	0.00116	0.00153	0.225
Citrobacter	0.00164	0.00093	0.00206	0.453
*Prevotellaceae*_UCG-001	0.00160	0.00064	0.00226	0.149
Lachnoclostridium	0.00174	0.00070	0.00185	0.100
Uncultured_bacterium_f_Clostridiales_vadinBB60_group	0.00295	0.00067	0.00352	0.260
Wolbachia	0.00205	0.00058	0.00233	0.624
Acidaminococcus	0.00419	0.00065	0.00800	0.386
Sutterella	0.00240	0.00023	0.00299	0.356
Others	0.05272	0.03520	0.01200	0.150
Unknown	0.02785	0.01781	0.02738	0.156

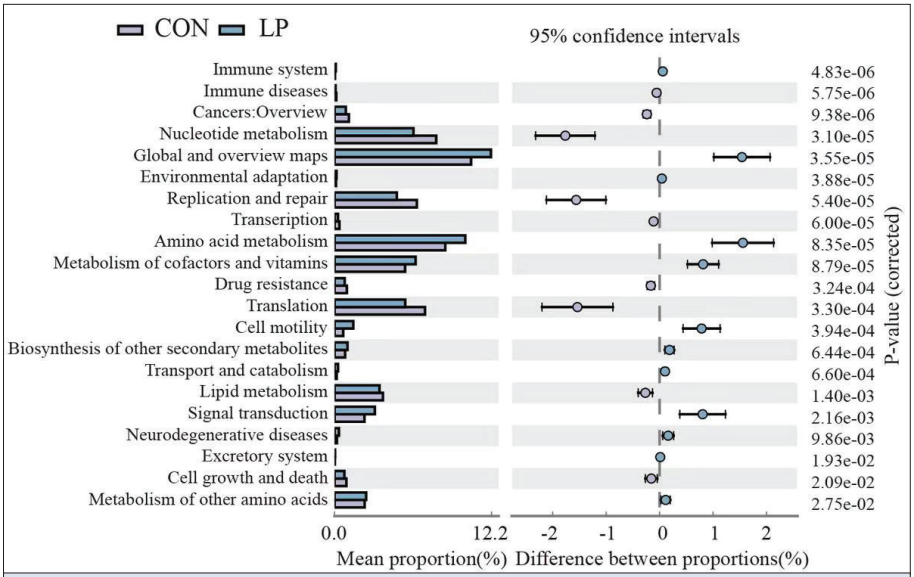

Fig 2. Predicted microbial functions using PICRUSt by 21-day old suckling piglets when feeding maternal diets containing 12% (LP) or 14% crude protein (CON) when bacteria differed

Correlations Between Intestinal Microbial Species and Jejunum Morphological Traits

Numerous correlations via Spearman's correlation analyses (P<0.05, *Fig. 3*) were investigated between the different genera (n=6) relative abundances and morphological parameters (n=7). *Clostridium*_sensu_stricto_1 was positively correlated with villus width, crypt depth, and muscular thickness, while being negatively correlated with villus height, and ratio of villus height: crypt depth. *Escherichia-Shigella* was positively correlated with muscular thickness and negatively correlated with villus height. *Turicibacter* was positively correlated with crypt depth and muscular thickness, while *Veillonella* was positively correlated with villus width. *Lactobacillus* was positively correlated with villus height, and villus height: crypt depth, and negatively correlated with jejunum weight, villus width, crypt depth, and muscular thickness.

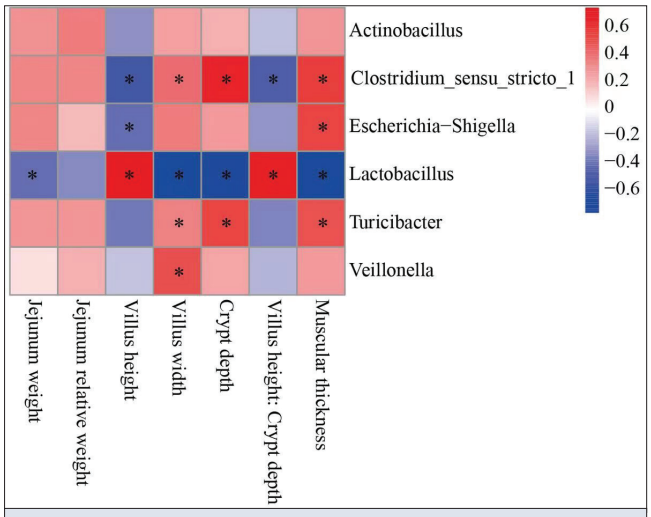

Fig 3. Correlations between differential genera and morphological traits at the jejunum by 21-day old suckling Bamei piglets when feeding maternal diets containing 12% (LP; N=12) or 14% crude protein (CON; N=12). Each row in the graph represents a genus, each column represents a morphological trait, and each lattice represents a Spearman correlation coefficient between a genus and a morphological trait. Red represents a positive correlation, while blue represents a negative correlation. *Significant correlation between the LP and CON groups (P<0.05)

Discussion

The small intestine has an important role in defense against health challenges in addition to nutrient digestion and absorption. The main nutrient digestion and absorption site is the jejunum [23]. Maternal suckled milk enters the piglet's gastrointestinal tract, thereby promoting crypt cell proliferation and proliferation. Suckling piglet jejunal development directly affects post-weaning growth performance [24]. In this study, reducing maternal dietary protein concentrations by 2% units resulted in similar 21 d ADG. The small intestinal growth rate before and after birth of the piglet is greater than the whole body [25]. The small intestine relative weight 24 h after birth is 50% greater than at birth [26]. Intestinal crypt depth increases 40% and villus height increases 35% within 3d [27]. These crypt stem cells divide and differentiate to form intestinal epithelial cells that gradually migrate to the villi tip for nutrient absorption [28]. Through this process, the digestive and absorption functions of intestinal epithelial cells are gradually improved [29].

After the piglet's birth, there are 2 sources of gut microbes with one being the maternal microbes, which are vertically passed, while the 2nd source is environmental, which are horizontally passed. The combined data using Bamei piglets demonstrated that maternal dietary LP concentrations

resulted in significant changes in intestinal microbiome composition compared with CON piglets. Alpha diversity metrics (Shannon and Simpson index) demonstrated a higher piglet bacterial diversity from sows fed lower maternal dietary CP concentrations compared with piglets from sows fed the CON CP concentrations, suggesting that altering CP concentration has a direct impact on jejunal microbial composition of Bamei suckling piglets. In agreement with previous pig studies [30,31], the Bamei piglet's dominant jejunum core microbiome was the phyla Firmicutes, *Proteobacteria*, and *Bacteroidetes*. The dominant genus level Bamei suckling piglet jejunum bacteria were: *Lactobacillus, Escherichia-Shigella*, *Actinobacillus, Buchnera, Romboutsia,* and *Clostridium*_sensu_stricto_1. The bacterial community diversity and richness are known to be influenced by dietary intervention [32].

The correlation analysis between intestinal bacteria *(Clostridium*_sensu_stricto_1, *Lactobacillus,* and *Turicibacter)* and intestinal histomorphology demonstrated that feeding a maternal LP diet can induce shifting abundance changes in the piglet's intestinal microbiome. Equally important, dietary interventions may not always alter the piglet's bacterial species and abundance but may alter the intestinal histomorphology produced by these bacterial species thru influencing their metabolism and physiology. *Lactobacilli* are beneficial bacterial members of the small intestinal microbiota that were reduced for piglets from sows fed the LP diet. The intestinal bacterial environment can protect the intestine from toxic dietary ingredients [33]. The reduction of Lactobacillus spp. abundance may result from decreased oligosaccharide ingestion (less soybean meal inclusion), which reduces nutrient availability, which relates to reduced piglet weight [34]. These results indicate that maternal dietary LP concentration alters Bamei piglets' intestinal microbiota through altering the beneficial bacterial colony structure [35]. Therefore, it is reasonable to hypothesize that intestinal microbiota differences are the result of early dietary intervention, host-microbe interactions, and/or host physiological state. The most important host-microbe interaction may occur on or at the intestinal barrier. These data demonstrated that dietary CP concentrations altered the intestinal microbiome composition and associated function in Bamei piglets. This could be an exciting research field with the potential to solve many important problems.

Availability of Data and Materials

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

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Ethical Approval

All procedures involving the use of animals were approved by the Animal Care Committee of Qinghai University, China (QHDX-17-02-12-06). Animal slaughtering was approved by the National Administration of Slaughtering and Quarantine regulations (Qinghai, China).

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Cui YF and Zhang HX: the hypothesis of this study; Cui YF and Zhang LP: work management, article writing; Cui YF, Chen Q and Ren L: experimental procedure follow-up, statistical analysis; Cui YF, Chen Q and Ren L: literature review, review of results; Jia JL: final decision, experimental design.

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RESEARCH ARTICLE

Hypothetical study of Small Hive Beetle *Aethina tumida* Infestation in Honeybees, Risk Commodities and Probabilities for Its Introduction in Türkiye

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Abstract: Türkiye is the second-largest honey producer globally; however, the export of honey and bee products does not adequately support the beekeeping industry. Pests account for the largest share of expenditure for agents found in honeybees in the country. Although the Small Hive Beetle (SHB) has not been detected in Türkiye, a risk assessment was performed to determine what happens if it enters the country. The risk assessment included: a) hazard identification; b) risk pathway determination; c) risk assessment for entry via the identified pathways; and d) outcome assessment for becoming endemic in Türkiye. The Risk AMP add-in program was used to assess the probability of distribution for each method of entry, pathway, and simulation. According to the simulations, the probability of SHB introduction in Türkiye varies from 0.17 per 1000 events/days (1.7 per 10000 days or 27 years) to 0.6 per 1000 events/imports (6 per 10000 days or 27 years). The highest likelihood of introduction comes from fruit import (11/15) and soil/compost import (4/15). The mean probability of introducing SHB infestation after 1000 iterations of the constructed model is 0.37 per 1000 events/days (3.7 within 10000 days or 27 years). Finally, the simulated average cost of SHB after the possible introduction is 523 million US $ for Türkiye. With these simulated data, risk assessment of a non-detected pest, SHB, was determined for Türkiye.

Keywords: *Aethina tumida, Risk, Small hive beetle, Türkiye*

Bal Arısında Küçük Kovan Kurdu *Aethina tumida*'nın Hipotetik Çalışması: Türkiye'ye Girişi İçin Riskler ve Olasılıklar

Öz: Türkiye, dünyanın en büyük ikinci bal üreticisidir. Fakat bal ve arı ürünleri ihracatı, arıcılık endüstrisini tam olarak destekleyememektedir. Ülkede bal arılarında bulunan etkenler için yapılan harcamalarda en büyük payı zararlılar oluşturmaktadır. Küçük Kovan Kurdu *(Aethina tumida)* (KKK) Türkiye'de bulunmamasına rağmen, bu çalışmada risk değerlendirmesi ve ülkeye girişi durumunda verebileceği ekonomik zarar değerlendirmesi yapılmıştır. Risk değerlendirme süreci birkaç aşamada gerçekleştirilmiştir: a) tehlikenin tanımlanması; b) risk yollarının belirlenmesi; c) tanımlanmış yollara giriş için riskin değerlendirilmesi, d) ekonomik zararın değerlendirilmesi ve e) hastalıkların Türkiye'ye girişinden sonra endemik hale gelme riskinin değerlendirilmesi. Bu amaçla, Excel için Risk AMP eklentisini kullanan bir elektronik tablo, benimsenen yollara göre her bir giriş yolu için olasılık dağılımı ile oluşturulmuş ve simülasyonlar Monte Carlo yöntemi kullanılarak yapılmıştır. Simülasyonlar için verilen varsayımlarla, Küçük Kovan Kurdunun Türkiye'ye giriş olasılık değeri, 1000 olay/gün için en düşük 0.17 (10.000 gün veya 27 yıl içinde 1.7) ile 1.000 olay/ithalat için en yüksek 0.6 (10.000 gün veya 27 yıl içinde 6) arasında değişmektedir. Giriş yolu olarak en yüksek olasılık meyve ithalatından (11/15), ikinci olarak toprak/kompost ithalatından (4/15) olabilecektir. Oluşturulan modelin 1000 yinelemesinden sonra KKK istilasının ortaya çıkma olasılığı ortalama 1.000 olay/gün başına 0.37'dir (10.000 gün veya 27 yılda 3.7). Son olarak, KKK'nun ülkeye girişinden sonra simüle edilmiş ortalama ekonomik zararı 523 milyon $ olarak bulunmuştur. Bu çalışmayla, Türkiye'de henüz görülmeyen KKK zararlısının, bulaşma risk olasılıkları ve ülkeye girişi halinde verebileceği ekonomik zararlar ortaya konmuştur.

Anahtar sözcükler: *Aethina tumida, Küçük kovan kurdu, Risk, Türkiye*

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Introduction

The insect *Aethina tumida* Murray (Coleoptera, Nitidulidae), also called the small hive beetle (SHB), is native to Africa and causes relatively little harm as a minor scavenger pest of honey bee colonies. It is a fruit pest that feeds on fruit remains and was first identified in 1940 in South African beehives [1]. This pest spread to many regions of the world by the end of the 1990s, particularly on the American continent and Australia, by importing fruit and beehives. When the imported beetle was discovered, it was so well established that there was little or no chance of eradication. The colony is damaged by the larvae burrowing through the brood combs and consuming the brood and honey. The amount of harm depends on the infestation level. If SHB infests an apiary and is not treated, up to 100% hive mortality is expected [2,3].

The adult *A. tumida* is 5-7 mm long, has three pairs of legs, two pairs of strong wings, and is a dark brown, almost black colour. The strong chitin layer on the beetle's body prevents the bees from stinging them. They can fly up to 24 km, rapidly spreading throughout the environment. The eggs are fusiform and laid irregularly into the deep, hard-to-reach corners of the hives and cells. Larvae are long, oval, approximately 11 mm long, and have a whitish-light brown colour [4]. Although the SHB larvae have a similar appearance to the wax moth larvae, they have three underdeveloped legs and well-developed dorsal spikes, distinguishing them from the wax moth. The larvae feed on pollen and honey and defecate in the cells. As the larvae feed, they leave a sticky repellent substance on the combs, which may cause the bees to abandon the hive. So, they damage the honey in combs and cause the fermentation of nectar/honey. Adult beetles prefer feeding on a brood instead of honey and pollen. Weakened and stressed colonies collapse within two weeks [2,5].

The World Organization for Animal Health (OIE) [1] states that currently, *A. tumida* is present on the Northern/Central/Southern American continent, in Cuba, Jamaica, Hawaii, South Korea, Australia, Portugal, and Italy. There is no verifiable information about its localisation in Africa. However, the pest is endemic in that region [1,6]. Although the pest is not officially reported to the OIE, the Terrestrial Animal Health Code from the OIE states the pest is present in Egypt (2000), and Portugal (2004) [1]. It has also been recorded in the Calabria region of Italy without wide establishments [2,7].

African bee species are considered very aggressive and have strong self-cleaning and defensive traits, which prevent beetle access to the colony by aggressively harassing them. They remove beetle larvae from the hive and confine beetle to a 'propolis prison'. This behaviour of African bees limits SHB reproduction and keeps its population below the damaging threshold [5]. Therefore, SHB is considered a minor economic pest of weak honey bee colonies in Africa [1].

Unlike African honeybees, Western honeybee races have less aggressivity behaviour against SHB. Consequently, SHB could reproduce much more effectively [8], and their population would overgrow if introduced in Türkiye.

The economic consequences of the SHB infestation are significant and multiple. In 11 states of the USA between 1998 and 2004, the disease killed almost one in three colonies [5]. From those days to now, SHB is spread across 48 states in the continental United States and continues to suffer economically [9]. In addition, there were substantial and valuable losses in plant production because these bees were used in the pollination process. The USA has to import bee colonies due to the contamination of honey, fermentation and pesticides used to control the disease. State support per hive was 35 $ which increased 2-3 fold due to colony losses [4,5,10].

A spreadsheet with the Risk AMP add-in (Structured Data, LLC), a Monte Carlo simulation engine for the Microsoft Excel® programme, was created with a probability distribution for each entry method per the adopted pathways - imported commodities. The simulation uses a computational algorithm with repeated random sampling to generate numerical results within a user-specified range and distribution [11,12]. The technique accounts for biological variability and diversity in health events. Such methods are able to see future results under current risks and can be used in similar studies [13,14].

This infestation is not present in Türkiye, where migratory beekeeping is performed [15]. Also, the disease is not present in the country's immediate neighbourhood. There is no particular geographical area at the current time that is considered at greater risk than others. Therefore, this study aimed to evaluate whether SHB infestation is introduced in Türkiye by economic modelling.

Material and Methods

To determine the risk commodities and probabilities, gained parameters and their values were entered into the simulation engine programme. All the parameters and their values are obtained from the Statistical Institute of Türkiye, the Central Beekeepers Association of Türkiye and the American Ministry of Agriculture *(Table 1)*.

Risk is significantly dependent on the imported quantities, especially their origin, i.e., only from an infested country. However, the lack of official information on these quantities and sources prevents such delineation of the calculated risk. Therefore, the possibility of disease

Table 1. Türkiye's beekeeping parameters and their values	
Parameter	**Value (2021)**
Unit price of a colony	62.5 $ [a]
Average honey production per hive	14.62 kg [b]
Minimum honey production per hive	6 kg [b]
Maximum honey production per hive	55 kg [b]
The average unit price of 1 kg honey	3 $ [b]
The average unit price of 1 kg wax	5 $ [b]
Min number of infested apiaries	30% [c]
The average number of infested apiaries	35% [c]
Maximum number of infested apiaries	50% [c]
No. Professional beekeepers	81 000 [b]
Months/year utilized for professional beekeeping	9 [b]
Average monthly salary	355 $ [b]
Average Total colonies in Türkiye	8.4 million [b]

[a] *According to the Central Beekeepers Association of Turkey (2021)*
[b] *According to the Turkish Institute of Statistics (2020)*
[c] *According to the American Ministry of Agriculture (2020)*

introduction via imports from infested countries has been evaluated and included as an additional risk.

The considered criteria and direct and indirect losses due to the infestation are; i) Loss of colonies, honey, and frames within the infested colonies, and ii) damaged equipment. Costs for disease control are; i) Time and labour to detect and treat the disease, ii) Treatment materials and consumables, and iii) Additional costs for control of the disease. Possible invasion routes of this pest in Türkiye are migratory beekeeping, queen importations, and fruit imports from infested continentals.

Results

The risk of introducing SHB through the legal import of bees and apiculture products was considered negligible. The Turkish national legislation on prohibiting imported bees and honey products from countries with reported SHB and prescriptions indicated with the International veterinary health certificate was considered. However, the disease cannot be detected in exporting countries until it is well spread; a very low probability was allocated. In addition, the possible illegal import of small quantities was considered in the risk assessment *(Fig.1)*.

According to the data in *Table 2,* assumptions and line with the results of the risk assessment process, if the infestation is introduced in Türkiye, it could happen in any region of the country since importing those commodities is not regionalised. Further and detailed investigation describing the likelihood of occurring above assumptions and measures for prevention is required.

This simulation reported the probability of SHB introduction in Türkiye varied from 0.17 per 1000

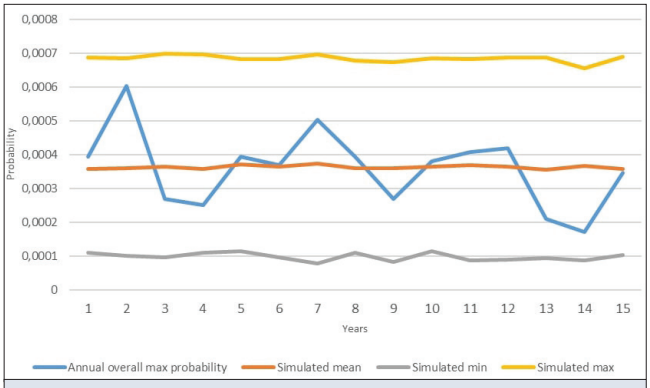

Fig 1. Introduction probability of SHB infestation in Türkiye using Monte Carlo procedure with 1000 iterations

events/days (or 1.7 per 10000 days or 27 years) to 0.6 per 1000 events/imports (or 6 per 10000 days or 27 years). The simulation results suggested that importing fruit (11/15) and importing soil/compost (4/15) had the highest likelihood of causing the infestation. The mean probability of SHB introduction after 1000 iterations of the constructed model was 0.37 per 1000 events/days (or 3.7 in 10000 events/days or 27 years) *(Table 3)*.

According to the Monte Carlo procedure after 1000 simulations, the consequence assessment of direct losses after introducing SHB in Türkiye is 523 million US $ on average, with a minimum of 482 million US $ and a maximum of 565 million US $ range. Labour loss was calculated only for the professional beekeepers since the hobby beekeepers are mainly professionally involved with other jobs, and they spare minimal time (on average, 20 days/year).

Discussion

Climate change is a global environmental problem that can interact by changing the impact and distribution of invasive species. Although invasive species play a role in endangering the health of honey bees, the effects of climate change on the severity are unknown [16]. In June 1998, *A. tumida* was first reported in the Northern Hemisphere in honey bee colonies *(Apis mellifera)* in Florida [17]. It was previously known only in sub-Saharan Africa. By October 2001, the SHB had been found in 30 states, most of which were east of the Mississippi River. Migratory beekeepers carry bee colonies from SHB-infested areas, contributing to their spread. This pervasiveness is expected due to the immigrant pollination demands in the USA. States with SHB report occasional problems with insects invading and destroying beehives. However, more issues occur with SHB damaging stored honey [4,5,18]. International trade also facilitates biological invasions, but the role of the wax trade-in SHBs is not fully understood. Adequate mitigation measures are needed to address the critical role

Table 2. *Risk assessment of SHB in Türkiye*

Entry Way	Biologic Stages	Risk	Current Practices in Türkiye	Probability of Entry (%)
Wax	Adult	The wax represents an attractive medium for the beetle and therefore, its storage after heat treatment poses a risk of additional infestation. Raw wax presents a great risk for the introduction of adult beetle	Minimal quantities are imported	Very low to negligible Min: 0.00001 Mean: 0.0005 Max: 0.0001
Queen bees or package bees (workers)	Adult	Packing queen bees is associated with the risk of packing an adult insect. This has proven to be a model for the spread of the disease in the United States	Minimal quantities are imported	Very low to negligible Min: 0.00001 Mean: 0.0005 Max: 0.0001
Used hives and apiculture equipment	All biological stages	If they are previously infested or infested during storing, they present a risk of introducing all beetle stages	Very limited or no import	Very low to negligible Min: 0.00001 Mean: 0.0005 Max: 0.0001
Comb honey or honey in drums	All biological stages	If imported generally illegally, honey is given as feed for bees	Very limited or no import	Very low to negligible Min: 0.00001 Mean: 0.0005 Max: 0.0001
Colonies	All biological stages	No colony imports but can be brought in illegally	Limited or no import	Very low to negligible Min: 0.00001 Mean: 0.0005 Max: 0.0001
Bumble bees for pollination purposes	Adult	Packaging of has a risk of unintentional packaging of an adult beetle	No. They are produced in Turkey for a lower price	Negligible to low Min: 0.000001 Mean: 0.00005 Max: 0.00001
Soil or compost associated with the plant trade	All biological stages	In the insect diapause stage, it poses a great risk for the initiation of all stages	Yes, insufficient information on quantities and origin	Low Min: 0.000005 Mean: 0.0001 Max: 0.0005
Fruit imports – especially tropical fruits	All biological stages	Ripe fruit will be considered a risk when imported or stored from infested countries. Low sensitivity of visual inspection of such commodities increases the probability of introduction. Turkish legislation on the import of fruits does not cover the risk of the entry of SHB	Yes, but no information on the quantities and origin of ripe fruit if imported	Low due to the assumption of ripe fruit is not imported Min: 0.00007 Mean: 0.00025 Max: 0.0007

Table 3. *Risky products and probabilities of SHB entry into Türkiye*

Year	Route of Introduction	Probability of Introduction (per 1000)
1	Fruit	0.393
2	Fruit	0.603
3	Fruit	0.267
4	Soil or compost	0.249
5	Fruit	0.394
6	Soil or compost	0.368
7	Fruit	0.502
8	Fruit	0.394
9	Soil or compost	0.268
10	Fruit	0.380
11	Fruit	0.408
12	Fruit	0.418
13	Soil or compost	0.210
14	Fruit	0.171
15	Fruit	0.346

of wax imports in the further spread of SHBs. Combining genetics with trade data may be an effective tool to better track and reduce biological invasions [10,18,19].

Currently, SHB infestation is a notifiable disease of honey bees in the European Union and an OIE-listed pest [7]. The best defence of a beekeeper against SHB is to protect strong colonies, keep apiaries free of abandoned hive material (especially wax combs), and implement preventive measures when insects are detected in the colonies. Unfortunately, no economic threshold (number of insects to act in a hive) has been established for SHB. Their reproduction and damage to the colonies depend on factors such as colony strength and the prevalence of other pest pathogens.

Migratory beekeeping practices in the United States of America and Türkiye are very similar in many ways. Turkish professional beekeepers move an average of 2000 km annually in the country. Therefore, introducing the SHB factor into the country is a severe problem. To date,

the eradication of *A. tumida* in the USA and Italy does not show an apparent success as in the other ten infested countries [6,20]. The existence of 8.4 million colonies and 80 thousand professional beekeepers (have 50 hives and more) in Türkiye poses a severe threat of possible infestation for sustainable beekeeping [2,21].

Different risk assessment studies are based on meteorological variables [22] or their biology and migratory beekeeping [14,23]. However, there is no study about the simulated economic risk assessment of SHB, even in infested or uninfested countries.

In countries without SHB infestations, strict import regulations and an early warning system are needed to prevent the pest's introduction and to detect it as soon as possible if it is introduced. Once introduced and well established, SHB cannot be eradicated. The international experience from the USA and Australia suggests good pest control management is the best defence. This management begins with maintaining strong colonies that can control the beetle [24].

As a result, the cost of SHB introduction and contamination risk probabilities was documented for Türkiye with statistical models. Our study is the first documentation of an economic price for an undetected parasite of honeybees in Türkiye. These data can lead to having an economic cost model for other uninfested countries.

Availability of Data and Materials

All the data and materials are kept in the laboratory of the Department of Parasitology, Veterinary Faculty, Bursa Uludağ University.

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Competing Interests

The authors declared that there is no competing interest.

Author Contributions

LA conceived and designed the analysis, and interpreted the outputs; AOG and YEY contributed the data and analysis tools; AOG finalized the presentation of the work.

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RESEARCH ARTICLE

Prediction of Marketing Live Weights in Hair Goat Kids Using Artificial Neural Network

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Abstract: In this study, marketing live weights (120th day) were predicted using artificial neural network model according to the herd, gender, birth type, maternal age, birth weight, body weight at 60th day and weaning weight (90th day) measurements of 12983 hair goat kids born between 2018-2021 years. Artificial neural networks (ANN) have been frequently used as an alternative to classical regression analysis in recent years, especially in future estimation studies in the field of livestock, and also in many different fields. In this study, it was aimed to predict the marketing weights of hair goats according to the holding, gender, birth type, maternal age, birth, 60th day and weaning weights with the ANN model. For this purpose, the multi-layer feed-forward backpropagation algorithm the ANN model, in which the number of hidden layers is one and the numbers of hidden neurons are three, was used. This model performance metrics were obtained for training set as 0.98, 0.62 and 0.55; for validation set as 0.97, 0.62 and 0.55, respectively. According to these results, it was determined that ANN can be used successfully in terms of estimation of marketing live weight in Hair goat kids. Estimating the marketing weight will enable the economic cost calculations to be obtained from kids to be evaluated both based on Turkey and on the farm basis, and to reveal future projections.

Keywords: *Artificial neural network, Marketing live weight, Weight prediction, Hair goat kid*

Kıl Keçisi Oğlaklarında Pazarlama Canlı Ağırlığının Yapay Sinir Ağları Kullanılarak Tahminlenmesi

Öz: Bu çalışmada 2018-2021 yılları arasında doğan 12983 baş Kıl keçisi oğlaklarının sürü, cinsiyet, doğum tipi, ana yaşı, doğum ağırlığı, 60. gün canlı ağırlık ve sütten kesim canlı ağırlık (90. gün ağırlığı) ölçümlerinin dikkate alındığı yapay sinir ağları modelinde pazarlama canlı ağırlıkları (120. gün ağırlığı) tahminlenmiştir. Yapay sinir ağları (YSA) pek çok farklı alanda olduğu gibi son yıllarda hayvancılık alanında özellikle de geleceğe yönelik tahminleme çalışmalarında klasik regresyon analizine alternatif olarak sıklıkla kullanılmaya başlanan bir veri madenciliği yöntemidir. Çalışmada işletme, cinsiyet, doğum tipi, anne yaşı, doğum, 60. gün ve sütten kesim ağırlıklarına göre kıl keçisi pazarlama ağırlıklarının YSA modeli ile tahmin edilmesi amaçlanmıştır. Bu amaçla, gizli katman sayısının bir ve gizli nöron sayısının üç olduğu çok katmanlı ileri beslemeli geri yayılım algoritması YSA modeli kullanılmıştır. Bu model performans kriter değerleri eğitim seti için sırasıyla 0.98, 0.62 ve 0.55 ve doğrulama seti için 0.97, 0.62 ve 0.55 olarak elde edilmiştir. Bu sonuçlara göre, Kıl keçisi oğlaklarında pazarlama canlı ağırlığının tahmini bakımından YSA yönteminin başarıyla kullanılabileceği belirlenmiştir. Pazarlama ağırlığının tahmin edilebilmesi, oğlaklardan elde edilecek ekonomik maliyet hesaplarının hem Türkiye hem de çiftlik bazında önceden değerlendirilmesine ve geleceğe yönelik projeksiyonların ortaya çıkarılmasına olanak sağlayacaktır.

Anahtar sözcükler: *Yapay sinir ağları, Pazarlama ağırlığı, Ağırlık tahmini, Kıl Keçisi oğlağı*

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Introduction

Hair goat is the dominant goat breed, which has adapted to almost all regions in Turkey and is resistant to adverse environmental conditions [1-3]. According to TUIK data, in 2021, the number of Hair goats was 12.051.957 which establish 99% of total goat population. Hair goats, which are the socio-economic and cultural richness of Turkey, are mostly raised in forests and villages, in village herds, on highland or nomads [4]. It constitutes an important source of food and livelihood for low-income families in rural and forested areas [5]. In the Aegean region, it is primarily consumed as kid meat. Hair goats, 40-45 kg live weight of mature, have low fertility and relatively low production of milk [1]. There are 1.248.977 head goats, of which approximately 11% and approximately 18% of the Aegean Region is in İzmir [4]. Especially in the Aegean Region, in recent years, it is seen that there is a tendency towards intensive or semi-intensive breeding in order to increase the production of kid meat or to make more income for the breeders. These transitions affect the physiology of the animal according to the breeding system of the goats and may have some consequences in human nutrition and health as a result of their consumption as a final product. This distinction may have a genetic aspect as well as a relationship with the feeding regimen. On the other hand, with the right relationship between increasing population and nutritional needs, domestic production remains insufficient, especially for red meat. Habits in animal feeding are changing and as a result, differences in yield and quality of red meat emerge among animal products obtained according to production systems. Therefore, hair goats are important for Turkey in terms of closing the red meat deficit and offering natural and healthy animal products as their nutrition is based on pasture. As a matter of fact, the average meat production per goat is 19.26 kg, and the milk yield is around 105 kg [6]. Since free breeding is generally applied in the herds held by the public and the oestrus is not synchronized, pregnant, giving birth, pregnant for the first and large and small kids can be found in the herd in the same period. This situation also creates problems for the breeders who make their living by putting their kids on the market. When the breeders market their kids without knowing the slaughter weight of their kids, they make a loss before they reach the slaughter age. However, the covid epidemic in the world and in Turkey and the accompanying economic problems have put goat breeding in a bottleneck. In this context, estimating the marketing weight of kids in the early period is important for breeding. Whereas the heritability of meat yield is low-medium. However, in estimating the adult live weight, the 120-day-old body weight has a higher heritability than the birth weight and weaning age weight [2]. Since the marketing weight can be determined in the early period with artificial neural networks, it will be possible to select the breeder for animal breeding in the earlier period. Important economic values that can be measured for the development of small ruminant breeding and increasing meat production are growth characteristics such as birth weight, live weight at 3 months, live weight at 6 months, live weight at 9 months and live weight at 12 months. The live weight at the age of 3 months, where weaning is frequently performed, is considered as one of the most important production characteristics in lambs. The decrease in maternal effects on lamb live weight at the age of 3 months has a high share in this. For this reason, 3-month age is accepted as one of the important selection criteria for lambs [7]. It is possible to determine the marketing weight in the early period with the measurements taken before 120 days and with various mathematical approaches including the relevant environmental factors, and thus the breeder selection can be made in the earlier period. However, knowing the body weight of the sheep in various periods is very important for determining the amount of feed to be given to the animal, the drug dose to be applied and the marketing weight [8]. Moreover, body weight (BW) values, which is one of the most important measurements used to describe the variation among animals and to reveal their growth characteristics, are frequently used criteria for both scientific research and selection applications. BW estimation, which is an important measure of animal performance, not only provides informative measures for animal nutrition, health care, selection, but also provides important information for research on economic growth, reproductive efficiency and meat production per animal [9,10]. In other words, the estimation of BW for successful breeding is indispensable for both increasing the reproductive performance of animals and thus both meat production per animal and obtaining superior offspring [11,12].

However, classical methods such as simple linear regression or multiple linear regression analysis were used in these estimation studies. In these studies, it was emphasized that when the effects of many factors on body weight should be examined simultaneously, multicollinearity problems may arise between the factors and the effect of the features affecting the estimated parameter with the body weight estimates could not be interpreted correctly [10,13-15]. On the other hand, it is reported that data mining algorithms are not affected by the current multi-collinearity problem [10,16,17]. In addition, data mining techniques can process fast, accurate, low-cost and also non-linear complex data, which is not possible using traditional techniques [18]. Many researchers [15,19-24] used the artificial neural network method in sheep, goat and cattle breeding, and they reported that artificial neural

networks had a lower error rate than classical statistical methods.

In this study, the estimation efficiency of Hair goat marketing weights was investigated with the artificial neural network model, which takes into account the farm, sex, birth type, maternal age, birth, 60th day and weaning weights, which are known to be effective on live weight.

Material and Methods

Material

The total of 12983 kids born in 28 goat farms from 2018 to 2021 years in İzmir province were used as material. In general, semi-intensive breeding system is applied in an extensive part of the herds in the farm. The herd capacities of the farms vary between about 150-1000 heads. İzmir provience is under the influence of Mediterranean climate, population density is 369/ km^2. In this study, herd, gender (male or female), birth type (single or twin), age of dam (1-10 year) factors, and birth, 60th day, and weaning weights were considered for predicting marketing live weights of kids.

The mean and standard deviation values of the 60th day, weaning and marketing body weights and frequencies of the herd, gender, birth type, age of dam effects together with the percentages were given in *Table 1*.

Method

ANN of many neurons in its structure work similarly to biological neurons. The main components of an artificial neuron are inputs, weights, transfer function, activation function and output [25]. Artificial neural networks consist of artificial neurons that systematically join in each of the input layer, hidden layer, and output layer [25-27].

The task of ANN is to produce an output as given in *Fig. 1* in response to the information given to it as an input set. In order to do this, input information and output

Table 1. *The description of the data set*

Herd	Frequency	Percent	Gender	Frequency	Percent
1	481	3.7	Male	6455	49.7
2	274	2.1	Female	6528	50.3
3	516	4.0			
4	274	2.1			
5	608	4.7	**Birth Type**	**Frequency**	**Percent**
6	256	2.0	Single	11446	88.2
7	288	2.2	Twin	1537	11.8
8	646	5.0			
9	780	6.0			
10	604	4.7			
11	308	2.4	**Age of Dam (Year)**	**Frequency**	**Percent**
12	662	5.1	1	181	1.4
13	178	1.4	2	746	5.7
14	234	1.8	3	1450	11.2
15	1059	8.2	4	1606	12.4
16	149	1.1	5	2167	16.7
17	652	5.0	6	1831	14.1
18	535	4.1	7	2405	18.5
19	266	2.0	8	1465	11.3
20	273	2.1	9	811	6.2
21	391	3.0	10	321	2.5
22	494	3.8			
23	649	5.0			
24	829	6.4			
25	656	5.1	**Weights**	**Mean**	**Std. Deviation**
26	240	1.8	Weight at 60th Day	11.34	2.32
27	323	2.5	Weaning Weight	15.00	2.85
28	358	2.8	Marketing Weight	18.74	3.84

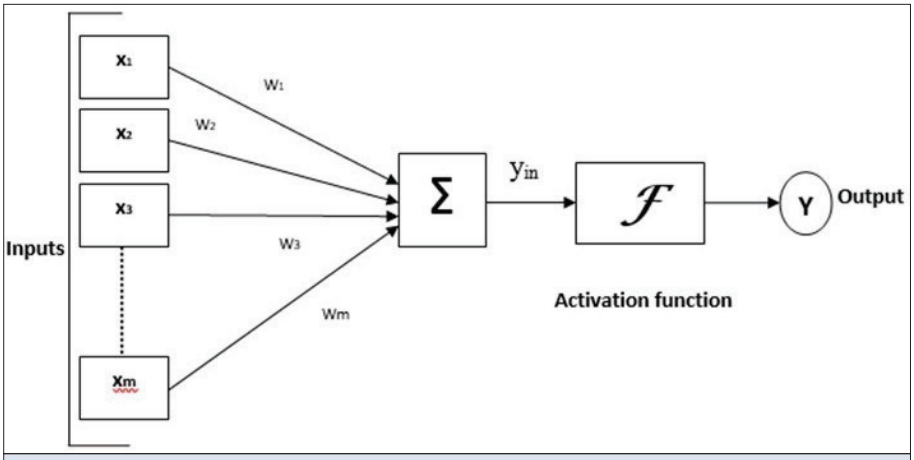

Fig 1. The architecture of artificial neural network

information corresponding to the inputs are given to the ANN, and the network is provided to learn the relationship between input-output, thus training the network. Then, the network reaches the level where it can generalize and decide, and determine the outputs with this acquired ability [27-30].

Artificial neural networks operate differently from the calculations of traditional processors. While a computer's processor (CPU) does the task assigned to it within the framework of a certain algorithm, each artificial neural network processes only a small part of a major problem nonlinearly and achieves a result [25,31].

Some goodess of fit criteria such as The Adjusted coefficient of determination (R^2_{adi}), Root Mean Square Error (RMSE), and Mean Absolute Error (MAE) are used to determine performance of ANN models and even compare with different model predictions [29,32,33]. According to these criteria, the model that provides the highest R^2_{adj} and the lowest RMSE, and MAE values is accepted as the most suitable. The mainly used equations and their definitions were given in the *Table 2*. In equations y_i denotes observed data, $\hat{y}_i$ is predicted data, n is the number of observations and p is the numbers of independent variables.

In this study, preliminary analysis with the general linear model was carried out for the effects of herd, gender, birth type and age of dam, which are thought to be effective in estimating the marketing age. Finally, these effects were found to be statistically significant (P<0.05). Therefore, these effects were used in the ANN model.

Moreover, marketing live weigths were predicted using a multi-layer feed-forward backpropagation algorithm ANN architecture. In this architecture, 7 variables as farm, gender, birth type, maternal age, birth weight, 60th day body weight and weaning weights were used as input data, and marketing weight was used as output data set. The data set was randomly divided into two groups by 70% training and 30% as the validating set. Therefore, artificial neural networks were modeled with different number of hidden layers and neurons training algorithms on (n=9088) training and (n=3894) validation sets. Hyperbolic Tangent activation function was used between layers. The convergence criterion was 1.10-6, the maximum number of iterations was 50, and 20 epochs were taken at the termination of the algorithm in each run. JMP Pro 16.0.0 program was used in all analyzes.

Table 2. *Definition and equation of criteria for model evaluation*

Definition	Equation
Adjusted coefficient of determination The coefficient of how well the values fit compared to the original values	$R^2_{adj} = 1 - \left[(1 - R^2).\left(\frac{n-1}{n-p-1}\right)\right]$
Root Mean Square Error The difference between the original and predicted values extracted by squared the average difference over the data set	$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{n}}$
Mean Absolute Error The difference between the original and predicted values extracted by averaged the absolute difference over the data set	$MAE = \frac{1}{n}\sum_{i=1}^{n}\lvert y_i - \hat{y}_i\rvert$

Results

In this research, the aim was to predict marketing live weight of Hair goat kids using an artificial neural network. Based on this aim the most suitable hidden layer number in the designed artificial neural network was found to be "1" and the neuron number in this layer was found as '3' *(Fig. 2)*.

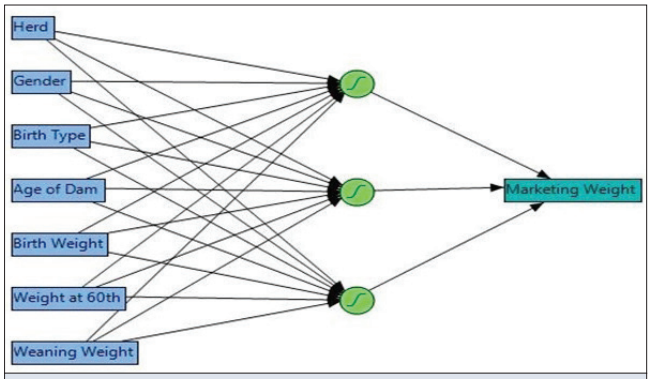

Fig 2. The diagram of the ANN for prediction of marketing weigh

In this study, R^2_{adj} and the RMSE, and MAE were adopted to evaluate the quality of fit for ANN in terms of the distance of the predictors from the actual training points and the results of model performance metrics were shown in *Table 3*. This model performance metrics were obtained for training set as 0.98, 0.62 and 0.55; for validation set as 0.97, 0.62 and 0.55, respectively. As can be seen, the validation set gave the same results as the training set.

Table 3. *The results of model performance metrics*

Metrics	Training Set	Validation Set
Adj. R^2	0.975	0.973
RMSE	0.623	0.618
MAE	0.546	0.553

The actual and residual by predicted marketing live weight plots of the best ANN model for training and validation sets were visualized in *Fig. 3* and *Fig. 4*, respectively.

As can be seen from *Fig. 3* and *Fig. 4*, the preditcs were very well adapted to the observations and the error levels were found to be quite low.

According to these results, it was determined that ANN has a good fit and can be used successfully. It has been revealed also, the 7-input, single-output and single-layer 3-hidden node network structure can be used safely in estimating the marketing live weight for Hair goats.

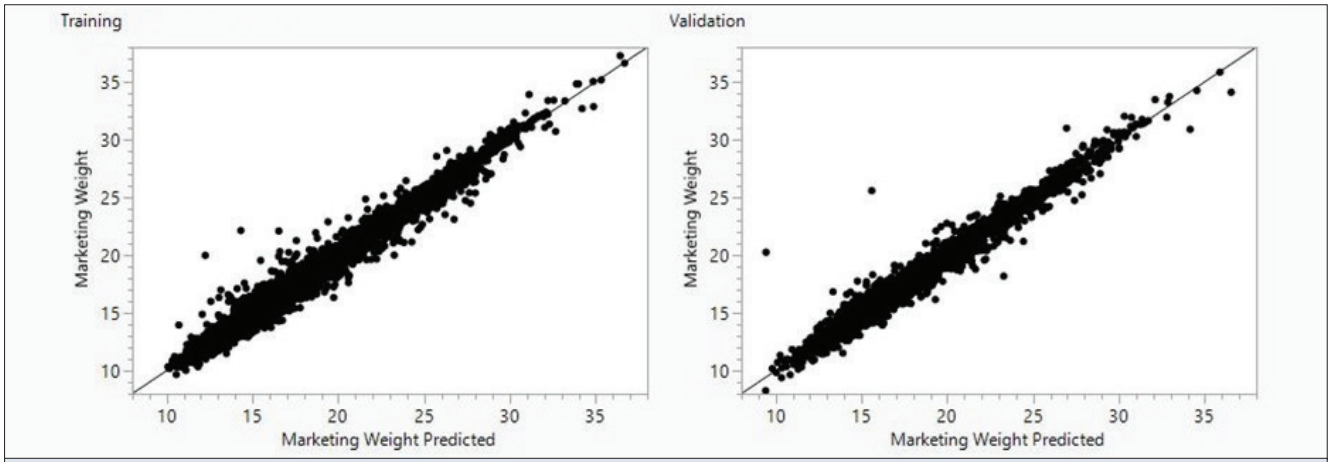

Fig 3. Actual by predicted marketing live weight of the best ANN model

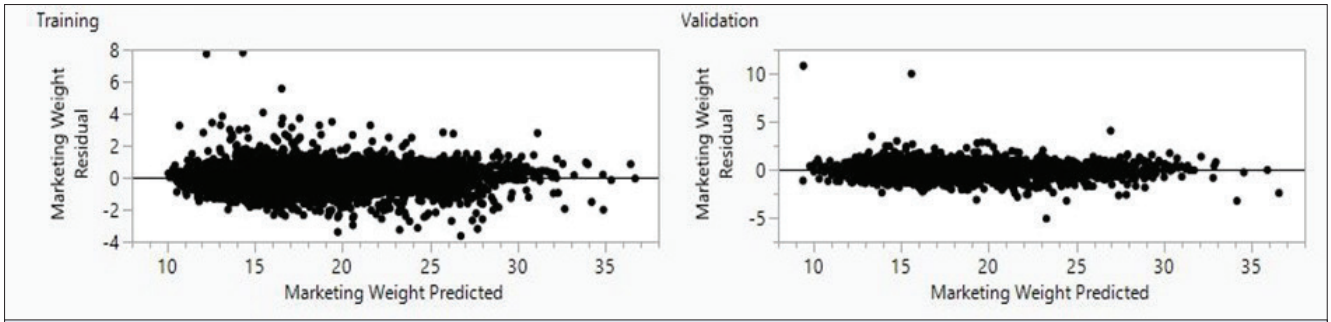

Fig 4. Residual by predicted marketing live weight of the best ANN model

Discussion

Taşdemir and Özkan [34] used ANN modeling, which includes 4 inputs and 1 output, to estimate body weight in cattle using body measurements, and they found the correlation coefficient between the estimated body weight values and the body weight values determined from the scale as 0.995. As a result of their studies, researchers reported that ANN can be used successfully in body weight estimation. Tırınk [35] showed that artificial neural network algorithms can be successfully applied in estimating BW based on measured values in estimating body weight in Thalli sheep breed. However, the R^2 value obtained from the ANN algorithm was found to be lower than the values obtained in this study.

Race and assessed traits may account for these differences. Behzadi and Aslaminejad [36] reported that in estimating the growth characteristics of Baluchi sheep, ANN provides the most accurate estimation by creating a better descriptive sheep growth curve compared to nonlinear models. They reported an MSE of 0.06 for the artificial neural network, which is similar to our research results. They also stated that ANN is a valuable tool in estimating body weight in lambs. Ali et al.[37] compared artificial neural networks and other methods in estimating weaning body weight of lambs with the help of some morphological features in Harnai sheep. The correlation coefficient for actual and estimated body weight with ANN was determined as 0.91, MAE value as 0.0594, and RMSE value as 1.589. However, in their study of data mining algorithms, they suggested that the SD ratio of the algorithm applied should be lower than 0.40 for a good prediction performance. Norouzian and Vakili Alavijeh [38] compared ANN and multiple regression models for tail fat estimation by using birth weight and weaning weights in Balouchi sheep and found that ANN had higher R^2 value (0.93) and lower MSE (0.51) value compared to multiple regression model. The same values were reported as 0.81 and 1.24 in the multiple regression model, respectively. As a result of the study, the researchers revealed that the ANN model gave more accurate predictions than the multiple regression model in estimating the tail fat weight in sheep. Lactation milk yield was estimated by ANN model in terms of province, number of sheep, total amount of feed, veterinary costs, total labor costs, amount of salt, grazing period, lambing month, age of ewe, lactation length and number of milking factors in Akkaraman sheep [39]. As a result of the study, they determined R^2, MAE and RMSE in the ANN model as 0.791, 14.360, and 18.78, respectively. Ghotbaldini et al.[21] estimated the body weight of Kermani sheep at 6 months of age with ANN. For this purpose, they used the records of 867 lambs including lamb gender, maternal age, birth weight, weaning weight and 3-month live weight. They found the correlation coefficient as 0.864 and MSE=0.015 in the multilayer ANN model with nine input variables and seven neurons. As a result of the study, they reported that ANN has a good ability to predict the growth characteristics of sheep with an acceptable speed and accuracy. It has been reported that ANN can be used successfully as an alternative method to linear regression in estimating the effect of herd, lambing month and lactation period on lactation milk yields [40]. In addition, it has been stated that ANN provides valuable contributions to animal husbandry studies in terms of estimating body weight from testicular measurements and defining body weight in sheep [41].

It has been reported that the determination coefficients between the estimated and actual body weights in goats were positive and highly significant and ranged from 90.27% to 93.69%. The highest R^2 value in estimating body weight in goats using body measurements was obtained in 0-3 month-old kids [42]. However, they reported that ANN gave more successful results than multiple regression analysis in body weight estimation in all age groups. According to Eyduran et al.[33] stated that artificial neural networks and other algorithms can be successfully applied in the estimation of live weight according to various body measurements in Beetal goats and they obtained estimates that are very close to the truth. In addition, they found that the gender factor was the most determinant body weight predictor in all algorithms. It has been reported by Kaygısız and Sezgin [43] that ANN can be used safely in terms of prospective estimates of goat milk production data and their compatibility. According to Kannan et al.[44] reported that ANN models predicted responses more accurately than regression models in predicting physiological stress responses in post-transplant goats. Khorshidi-Jalali et al.[45] stated that ANN (R^2=0.86, RMSE=19.86) provides realistic data in body weight estimation using various morphological measurements in Raini goats and that it can be successfully used instead of traditional methods for this purpose.

On the other hand, Cihan et al.[46] used and compared Artificial Intelligence Methods and Immunoglobulin G prediction in lambs, ANN, multivariate adaptive regression curves (MARS), support vector regression (SVR) and fuzzy neural network (FNN) models. FNN was found to be the most successful method for estimating the IgG value. According to Ekiz et al.[47] compared chi-squared automatic interaction detection (CHAID) and ANN methods in estimating carcass tissue composition in Gokceada kids and found that the results were close to each other in estimating bone ratio, and the CHAID model gave better results in estimating subcutaneous fat and intermuscular fat ratios. They explained the reason for this as the small size of the data set in the study is a limiting factor for ANN estimation. When these studies are evaluated, it is seen that

the ANN model can be used instead of multiple regression models in estimating live weight by using many different measurements in livestock, because it has a higher R^2 and correlation coefficient, a lower standard deviation, and thus performs more effectively.

In this study, the obtained results proved that the ANN can be used successfully for the prediction of marketing live weight. ANNs also have an important role because it does not need large data sets to design a quite reliable neural network. The usage of ANN modeling may be highly recommended to predict marketing live weight. With the estimation of the marketing weight, it will be possible to pre-evaluate the economic cost calculations to be obtained from the kids based on both the country and the farm, and to reveal the future projections, especially for supporting red meat production in Turkey or using it as an alternative. The importance of keeping records is seen again in this study, that proper and continuous records will increase the degree of accuracy in breeder selection and breeding for meat purposes. It is essential for the future of small cattle breeding and its contribution to the country's economy to put into operation systems that will record long-term data and monitor herd management programs. ANN will enable early selection of animals in terms of meat yield with low heritability as well as other yield characteristics.

Due to the developing technology and livestock herd management systems and parallel to the increase in the number of records, it has become necessary to evaluate large data sets with data mining methods. As long-term and large-scale data are obtained in the hands of the public with this study, it has been revealed that it is necessary to evaluate the environmental factors important in terms of agriculture at the same time and to prefer the ANN, which is an unbiased and reliable estimation method. It is necessary to continue to work on estimating marketing weights with different ANN models in larger data sets.

Availability of Data and Materıals

The authors declare that the data supporting the study findings were obtained from the corresponding author (F. Erdoğan Ataç).

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No financial support was received for conducting this study.

Competing Interests

The author declared that there is no conflict of interest.

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.

Authors' Contributions

F.E.A and Ç.T. planned the study; F.E.A. collected datas; F.E.A., Ç.T., Ş.Ö.A., Y.G., designed the experiments and drafted manuscript; Ç.T., Y.G., analyzed all data; F.E.A., Ç.T., Ş.Ö.A. reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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RESEARCH ARTICLE

Molecular Characterization of *Pseudomonas aeruginosa* Isolated From Clinical Bovine Mastitis Cases

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Abstract: This study aimed to investigate the presence and distribution of virulence determinants and their antimicrobial susceptibilities of 44 *Pseudomonas aeruginosa* isolates obtained from clinical bovine mastitis cases. In addition, selected 6 *P. aeruginosa* isolates were further characterized using whole-genome sequencing (WGS). Based on the presence of T3SS-related genes, 25% of the isolates were found to carry *exoU* and/or *exoS* genes belonging to invasive *(exoU*-/exoS+, 18.2%*)* and cytotoxic *(exoU*+/*exoS*-, 6.8%*)* strains. But, none of the isolates carried both *exoU* and *exoS* genes. In terms of other virulence genes examined, various virulence gene profiles were observed among the isolates. The majority of the isolates (72.7%) were susceptible to all tested antimicrobials. Resistance rates to ciprofloxacin and carbapenems (imipenem and meropenem) were determined as 25% and 4.5%, respectively. WGS analysis indicated the presence of different resistome, but *fos*A genes in all isolates, and different combinations of mutations in *gyr*A, *par*C, *opr*D, efflux pump, and genes playing a role in the regulation of *ampC* gene expression. Different sequence types (STs) and serotypes were found in representative isolates with the occurrence of the O11-ST235 clone, which is a worldwide multidrug-resistant high-risk clone representing a serious public health threat. The findings of this study provide valuable information on *P. aeruginosa* isolated from clinical bovine mastitis cases and current antimicrobial resistance levels and virulence determinants.

Keywords: *Antimicrobial resistance, Clinical bovine mastitis, Pseudomonas aeruginosa, Virulence, Whole-genome sequencing*

Klinik İnek Mastitis Vakalarından İzole Edilen *Pseudomonas aeruginosa* Suşlarının Moleküler Karakterizasyonu

Öz: Bu çalışmada klinik sığır mastitis vakalarından izole edilen 44 *Pseudomonas aeruginosa* izolatının virülans genlerinin varlığı ve dağılımı ile antimikrobiyal duyarlılıklarının araştırılması amaçlandı. Ayrıca, seçilen 6 *P. aeruginosa* izolatının tüm genom dizileme (WGS) ile taslak genomları elde edilerek daha detaylı karakterizasyonları yapıldı. T3SS ilişkili genlerin varlığına dayalı olarak, izolatların %25'inin invaziv *(exoU*-/*exoS*+, %18.2*)* ve sitotoksik *(exoU*+/*exoS*-, %6.8*)* suşlara ait *exoU* ve/veya *exoS* genleri taşıdığı belirlendi. Ancak izolatların hiçbirinde *exoU* ve *exoS* genleri birlikte tespit edilmedi. İncelenen diğer virülans genleri açısından ise izolatlar arasında çeşitli virülans gen profilleri gözlendi. İzolatların çoğu (%72.7) incelenen tüm antimikrobiyallere duyarlı bulundu. Siprofloksasin ve karbapenemlere (imipenem ve meropenem) direnç oranları sırasıyla %25 ve %4.5 olarak belirlendi. WGS analizi tüm izolatlarda farklı rezistomun, *gyrA*, *parC*, *oprD*, efflux pump ve *ampC* gen ekspresyonunun regulasyonunda rol oynayan genlerde farklı mutasyon kombinasyonlarının varlığını gösterdi. Seçilen izolatlarda ciddi bir halk sağlığı tehdidi oluşturan ve dünya çapında çoklu ilaç dirençli yüksek riskli O11-ST235 klonu dahil farklı sekans tipleri (ST) ve serotipleri bulundu. Bu çalışmanın bulguları, klinik sığır mastitis vakalarından izole edilen *P. aeruginosa* suşlarının mevcut antimikrobiyal direnç düzeyleri ve virülans determinantları hakkında değerli bilgiler sunmaktadır.

Anahtar sözcükler: *Antimikrobiyal direnç, Klinik inek mastitis, Pseudomonas aeruginosa, Virulens, Tüm genom dizileme*

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Introduction

As a versatile and adaptable opportunistic pathogen, *Pseudomonas aeruginosa* inhabits diverse ecological niches including environments, plants, and mammals, due to having a relatively large genome (5-7 Mb) encoding diverse metabolic pathways and defense mechanisms that enable the bacteria to cope with these hostile conditions [1]. *P. aeruginosa* is one of the most common nosocomial pathogens worldwide, causing life-threatening nosocomial infections especially among immunocompromised and critically ill patients [1,2]. Therefore, this pathogen was included in the ESKAPE pathogens group *(Staphylococcus aureus*, *Acinetobacter baumannii*, *Enterococcus faecium*, *Pseudomonas aeruginosa, Klebsiella pneumoniae*, and *Enterobacter* species*)* by World Health Organization (WHO) [3]. *P. aeruginosa* has also veterinary importance, and causes different clinical manifestations in livestock and companion animals, and clinical mastitis is one of the most encountered infections in dairy cows [2].

The frequency of other mastitis pathogens differs between herds, geographic locations, and whether or not mastitis control programs are implemented [4]. The overuse and misuse of antimicrobials for the prevention and treatment of mastitis increased antimicrobial resistance (AMR) in bacteria implicated, and increasingly undermined the sustainable use of antimicrobials [5,6]. The presence of such strains in dairy animals also implies a potential risk for their transfer to humans through the food supply chain, direct animal contact, or through environmental routes [7]. Currently, β-lactams combined with beta-lactamase inhibitors (amoxicillin-clavulanic acid) and fluoroquinolones are the most frequently used antimicrobial classes used for intramammary infusion in Türkiye. In addition, in recent years, last generation cephalosporins have been used in the treatment of dry mastitis in cows. The studies conducted in Türkiye are based on specifically selected animal populations and represent estimates of AMR in specific geographic regions at a given time, but they do not provide an overall picture of the situation. In addition, the proportion of AMR in bovine mastitis pathogens indicated differences in AMR proportions according to mastitis pathogens, regions and populations. The highest proportions of resistance were observed for penicillins and tetracyclines due to their long-term use in Türkiye [8-11]. Although resistance rates to quinolones (enrofloxacin and danofloxacin) and third-generation cephalosporins classified as critically important antimicrobials by the World Health Organization (WHO) [12] are not very high, in a recent study, higher rates of resistance against enrofloxacin and ceftiofur were observed in various bacteria species isolated from clinical bovine mastitis cases [13].

P. aeruginosa-related mastitis cases were mostly related to outbreaks [14-16]. Ohnishi et al.[17] isolated 116 *P. aeruginosa* strains from milk samples obtained from 115 cows affected by clinical mastitis in 89 dairy herds in Japan. Park et al.[18] isolated 116 *P. aeruginosa* without any bacteria from 35 625 raw milk samples collected from nine provinces in Korea. Ibrahim et al.[19] isolated 34 *P. aeruginosa* from 100 milk samples collected from cows with clinical mastitis in Egypt. In addition to being intrinsically resistant to many antimicrobials, *P. aeruginosa* can acquire new resistance mechanisms, resulting in the emergence of multidrug-resistant (MDR) *P. aeruginosa* strains, which are resistant to fluoroquinolones, carbapenems, and aminoglycosides. Acquired resistance occurs either by chromosomal mutations on target sites or by the acquisition of resistance genes via horizontal transfer [20]. Another mobile genetic element (MGE) that plays an important role in the acquisition and transfer of antimicrobial resistance is integrons. These elements can capture, integrate, and express gene cassettes conferring resistance to different classes of antimicrobials [21]. The integrons were transferred between species by hitchhiking with other MGEs [22].

The pathogenesis of *P. aeruginosa* infections is mainly associated with a large arsenal of both cell-associated (lectins, flagella, pili, lipopolysaccharide, alginate/biofilm) and extracellular (hemolysins, proteases, cytotoxin, pyocyanin, siderophores, exotoxin A, exoenzyme S, exoenzyme U, etc.) virulence factors [23]. Some of the virulence factors are coordinated by a cell density recognition mechanism called Quorum Sensing (QS) [24]. The type III secretion system (T3SS) is among the major virulence factors in *P. aeruginosa*. The T3SS is an injectisome that delivers its four exotoxins (ExoS, ExoT, ExoU, and ExoY), which have different functions and action mechanisms, into the cytoplasm of target eukaryotes [25,26].

This study aimed to investigate the current status of antimicrobial resistance among *P. aeruginosa* isolates from clinical bovine mastitis cases and their virulence characteristics, to further characterize the representative isolates by whole genome sequencing (WGS).

Material and Methods

Bacterial Strains

The study was conducted on 226 dairy farms located in two provinces (Aydın and Şanlıurfa) between 2017-2021. Throughout the study period, 12964 lactating cows were examined and 822 cows were found to have clinical mastitis manifestations such as swollen udder quarters, entirely swollen udders, abnormal milk secretion, and loss of appetite. A total of 1546 milk samples were collected aseptically from the infected quarters and inoculated onto Blood Agar (Merck, Germany) supplemented with 5% defibrinated sheep blood, and incubated at 35°C for 24-48 h. The isolates were identified by classical biochemical

methods (Triple Sugar Iron and oxidase reactions), and species identification was carried out using MALDI-TOF MS (Bruker Daltonics, Billerica, MA, United States) and 16S rRNA based PCR amplification of *Pseudomonas* spp. and *P. aeruginosa* [27].

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by the disc diffusion method following Clinical and Laboratory Standards Institute (CLSI) criteria (CLSI, 2021) [28]. Eleven anti-pseudomonal agents were used: gentamicin (CN, 10 µg), tobramycin (TOB, 10 µg), amikacin (AK, 30 µg), piperacillin/tazobactam (TZP, 100/10 µg), aztreonam (ATM, 30 µg), meropenem (MEM, 10 µg), imipenem (IPM, 10 µg), ciprofloxacin (CIP, 5 µg), cefepime (FEB, 30 µg), piperacillin (PIP, 100 µg), and ceftazidime (CAZ, 30 µg). The *P. aeruginosa* ATCC 27853 was used as a quality control strain. Following the screening of carbapenem resistance using disc diffusion method, MICs values of carbapenem resistant isolates were determined using E-test strips (Bionalyse, Ankara, Türkiye). The isolates that have acquired resistance to at least one antimicrobial in three or more antimicrobial classes were defined as multi-drug resistant (MDR) [29].

DNA Extraction

Genomic DNA was extracted from overnight culture using Qiagen DNeasy Kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. The isolated DNA was stored at -20°C until use.

Detection of Virulence and Integron Genes

ß-hemolytic, greenish on blood agar, lactose negative mucoid, and grape-like fruity odor on MacConkey agar were detected. A total of 44 isolates determined to be Gram negative, motile, oxidase +, catalase +, citrate +, urease +, VP - and MR - were evaluated as *Pseudomonas* spp. The presence of the following virulence and integron genes was investigated by polymerase chain reaction (PCR) among *P. aeruginosa* isolates: adhesion *(alg*U, *alg*D, and *alg*L*)*, oxidative stress *(phz*I, *phz*II, *phz*M, and *phz*S*)*, quorum sensing (QS)/regulation *(las*A, *las*B, *las*I, *las*R, *rhl*AB, *rhl*I, and *rhl*R*)*, T3SS *(exo*S, *exo*U, *exo*T, and *exo*Y*)*, biofilm *(psl*A, *pel*A, and *ppy)*, alkaline protease *(aprA)* and flagellin *(fli*C*)*, exotoxin A *(exoA),* pilin *(pil*A*)* and type IV pili *(pil*B*)*, phospholipases C *(plc*H, *plc*N*)*, L-ornithine N5-oxygenase, and class I, II, and III integrons [30-35]. Primers used for the detection of virulence and integron genes genus and species determination are compiled in *Table 1*.

Table 1. *Primers used for the detection of virulence genes, integron types, genus and species determination*

Target	Primer	Sequence (5'→3')	PCR Conditions	Product Size (bp)	Reference
16S rRNA *(Pseudomonas* spp.)	PA-GS-F	GACGGGTGAGTAATGCCTA	95°C 2 min; 25 cycles x [94°C 20 sec, 54°C 20 sec, 72°C 40 sec]; 72°C 1 min	618	[25]
	PA-GS-R	CACTGGTGTTCCTTCCTATA			
16S rRNA *(P. aeruginosa)*	PA-SS-F	GGGGGATCTTCGGACCTCA	95°C 2 min; 25 cycles x [94°C 20 sec, 58°C 20 sec, 72°C 40 sec]; 72°C 1 min	956	
	PA-SS-R	TCCTTAGAGTGCCCACCCG			
Biofilm	*psl*AF	TCCCTACCTCAGCAGCAAGC	95°C 5 min; 35 cycles x [94°C 1 min, 60°C 1 min, 72°C 1 min]; 72°C 5 min	656	[28]
	*psl*AR	TGTTGTAGCCGTAGCGTTTCTG			
	*pel*AF	CATACCTTCAGCCATCCGTTCTTC		786	
	*pel*AR	CGCATTCGCCGCACTCAG			
	*ppy*RF	CGTGATCG CCGCCTATTTCC		160	
	*ppy*RR	ACAGCAGACCTCCCAACCG			
Alginate	*alg*UF	CGATGTGACCGCAGAGGATG		292	
	*alg*UR	TCAGGCTTCTCGCAACAAAGG			
	*alg*LF	CCGCTCGCAGATCAAGGACATC		432	
	*alg*LR	TCGCTCACCGCCCAGTCG			
	*alg*DF	AGAAGTCCGAACGCCACACC		550	
	*alg*DR	CGCATCAACGAACCGAGCATC			
Type III Secretion System	*exo*SF	GCGAGGTCAGCAGAGTATCG	94°C 2 min; 36 cycles x [94°C 30 sec, 58°C 30 sec, 68°C 1 min]; 68°C 7 min	118	[29]
	*exo*SR	TTCGGCGTCACTGTGGATGC			
	*exo*UF	CCGTTGTGGTGCCGTTGAAG		134	
	*exo*UR	CCAGATGTTCACCGACTCGC			
	*exo*YF	CGGATT CTATGGCAGGGAGG		289	
	*exo*YR	GCCCTTGATGCACTCGACCA			
	*exo*TF	AATCGCCGTCCAACTGCATGCG		152	
	*exo*TR	TGTTCGCCGAGGTACTGCTC			

Table 1. *Primers used for the detection of virulence genes, integron types, genus and species determination (continued)*

Target	Primer	Sequence (5'→3')	PCR Conditions	Product Size (bp)	Reference
Quorum sensing genes	*las*A-F	GCAGCACAAAAGATCCC	94°C 3 min; 30 cycles x [94°C 30 sec, 55°C 30 sec, 72°C 1.5 min]; 72°C 5 min	1075	[30]
	*las*A-R	GAAATGCAGGTGCGGTC			
	*las*B-F	GGAATGAACGAAGCGTTCTCCGAC		284	
	*las*B-R	TGGCGTCGACGAACACCTCG			
	*las*I-F	CGTGCTCAAGTGTTCAAGG	95°C 2 min; 30 cycles x [95°C 40 sec, 60°C 1 min, 72°C 2 min]; 72°C 10 min	295	[31]
	*las*I-R	TACAGTCGGAAAAGCCCAG			
	*las*R-F	AAGTGGAAAATTGGAGTGGAG		130	
	*las*R-R	GTAGTTGCCGACGACGATGAAG			
	*rhl*I-F	TTCATCCTCCTTTAGTCTTCCC		155	
	*rhl*I-R	TTCCAGCGATTCAGAGAGC			
	*rhl*R-F	TGCATTTTATCGATCAGGGC		133	
	*rhl*R-R	CACTTCCTTTTCCAGGACG			
	*rhl*AB-F	TCATGGAATTGTCACAACCGC		151	
	*rhl*AB-R	ATACGGCAAAATCATGGCAAC			
Alkaline metalloproteinase	*aprAF*	GTCGACCAGGCGGCGGAGCAGATA	95°C 2 min; 30 cycles x [95°C 40 sec, 65°C 1 min, 72°C 2 min]; 72°C 10 min	993	
	aprAR	GCCGAGGCCGCCGTAGAGGATGTC			
Initial colonization factor flagellin	*fli*CF	GGCAGCTGGTTNGCCTG	95°C 2 min; 30 cycles x [95°C 40 sec, 55°C 1 min, 72°C 2 min]; 72°C 10 min	1.02 kb (Type A) 1.25 kb (Type B)	
	*fli*CR	GGCCTGCAGATCNCCAA			
Major pilin	*pil*AF	ACAGCATCCAACTGAGCG	94°C 3 min; 30 cycles x [94°C 30 sec, 55°C 1 min, 72°C 1.5 min]; 72°C 5 min	1675	[30]
	*pil*AR	TTGACTTCCTCCAGGCTG			
Type IV fimbrial biogenesis protein	*pil*BF	TCGAACTGATGATCGTGG		408	
	*pil*BR	CTTTCGGAGTGAACATCG			
Phenazine operon I	*phz*IF	CATCAGCTTAGCAATCCC		392	
	*phz*IR	CGGAGAAACTTTTCCCTC			
Phenazine operon II	*phz*IIF	GCCAAGGTTTGTTGTCGG		1036	
	*phz*IIR	CGCATTGACGATATGGAAC			
Phenazines	*phz*MF	ATGGAGAGCGGGATCGACAG		875	
	*phz*MR	ATGCGGGTTTCCATCGGCAG			
	*phz*SF	TCGCCATGACCGATACGCTC		1752	
	*phz*SR	ACAACCTGAGCCAGCCTTCC			
Hemolytic phospholipase C	*plc*HF	GCACGTGGTCATCCTGATGC	94°C 3 min; 30 cycles x [94°C 30 sec, 58°C 30 sec, 72°C 1 min]; 72°C 7 min	608	
	*plc*HR	TCCGTAGGCGTCGACGTAC			
Non-hemolytic phospholipase C	*plc*NF	TCCGTTATCGCAACCAGCCCTACG		481	
	*plc*NR	TCGCTGTCGAGCAGGTCGAAC			
Exotoxin A	*tox*AF	CTGCGCGGGTCTATGTGCC		270	
	*tox*AR	GATGCTGGACGGGTCGAG			
L-ornithine N5-oxygenase	*pvd*AF	GACTCAGGCAACTGCAAC	94°C 3 min; 30 cycles x [94°C 30 sec, 55°C 1 min, 72°C 1.5 min]; 72°C 5 min	1281	
	*pvd*AR	TTCAGGTGCTGGTACAGG			
Integron	*int*1F	CCTCCCGCACGATGATC	95°C 5 min; 30 cycles × [95°C 15 sec, 56°C 15 sec, 72°C 1 min]; 72°C 7 min	280	[33]
	*int*1R	TCCACGCATCGTCAGGC			
	*int*2F	TTATTGCTGGGATTAGGC	95°C 5 min; 30 cycles x [95°C 15 sec, 50°C 15 sec, 72°C 1 min]; 72°C 7 min	233	[32]
	*int*2R	ACGGCTACCCTCTGTTATC			
	*int*3F	AGTGGGTGGCGAATGAGTG		600	
	*int*3R	TGTTCTTGTATCGGCAGGTG			

Whole-Genome Sequencing

For whole-genome sequencing, 6 isolates were selected based on antimicrobial resistance phenotype and isolation site. The genomic DNA of selected 6 *P. aeruginosa* strains was extracted using a MagAttract HMW DNA extraction kit (Qiagen, Hilden, Germany). Genomic DNA was quantified on a Qubit 2.0 fluorometer using the dsDNA BR assay kit (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA). The sequencing libraries of the bacterial genomes were prepared using The Illumina Nextera XT DNA library preparation kit (Illumina Inc, San Diego, CA, USA). The paired-end (2x150 bp) sequencing run was performed on the NovaSeq platform (Illumina Inc., San Diego, CA).

Quality Control, Trimming, Assembling and Annotation

The raw sequenced reads were checked for quality using FastQC v.0.11.5 [36]. After trimming for low-quality reads and adapter regions using Trimmomatic v.0.36 [37], *de novo* genome assembly was performed using the SPAdes algorithm (version 3.1.14) [38], and contigs less than 200 bp were filtered out using BBmap 38.06 *(https://github.com/BioInfoTools/BBMap)*. Assembly metrics were calculated using QUAST v.5.0.0 [39]. Gene predictions and annotations were performed using the National Center for Biotechnology Information's (NCBI) Prokaryotic Genome Automatic Annotation Pipeline (PGAAP) [40].

Identification of The Resistome

The detection of the acquired antimicrobial resistance genes (ARGs) was conducted using the Center for Genomic Epidemiology (CGE) *(http://www.genomicepidemiology.org/)* tools and ABRicate v.0.8.13 *(https://github.com/tseemann/abricate)* with the default settings. In addition, mutations involved in fluoroquinolone and carbapenem resistance were also analyzed using SNIPPY software (v4.4.3) *(https://github.com/tsemann/snippy)*, mapping all assemblies against the *P. aeruginosa* PAO1 reference genome (GenBank Accession No. NC_002516.2).

Multi-Locus Sequence Typing and Serotyping

Serotypes of the isolates were determined using the *P. aeruginosa* serotypes (PAst) script available on CGE [41]. *In slico,* multi-locus sequence typing (MLSTs) was performed using the MLST 2.0 server *(http://www.cge.cbs.dtu.dk/services/MLST/)*.

Results

Isolation and Identification

Fourty four (2.84%) *P. aeruginosa* were isolated and identified from 1546 milk samples examined *(Fig. 1)*.

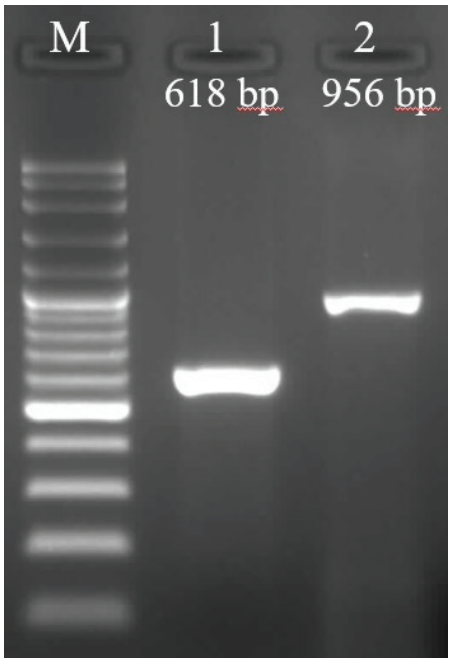

Fig 1. Agarose gel electrophoresis image of amplified products of the PCRs. Lane M: 100 bp plus molecular marker, Lane 1: 618 bp size amplified product of 16S rRNA *Pseudomonas* spp., Lane 2: 956 bp size amplified product of 16S rRNA *Pseudomonas aeruginosa*

Antimicrobial Susceptibility

Antimicrobial resistance phenotypes of *P. aeruginosa* are given in *Table 2*. The test results revealed that most of the isolates (n=32) were susceptible to all tested antimicrobials. Only 12 (27.3%) isolates were resistant to at least one of the antimicrobials tested. One isolate displayed the MDR phenotype. Of the meropenem and imipenem resistant four *P. aeruginosa* isolates by disc diffusion method, two isolates (ADU_VET_ST3 and

Table 2. *Antimicrobial resistance phenotypes of Pseudomonas aeruginosa isolated from bovine clinical mastitis cases*

Isolate ID	Resistance Phenotype[a]
ADU_VET_ST3[b]	CIP
ADU_VET_ST5[b]	CIP
ADU_VET_ST6	CIP
ADU_VET_ST11	CIP
ADU_VET_ST22[c]	MER, IPM, CIP
ADU_VET_ST24	CN, TOB, AK, CIP, CAZ
ADU_VET_ST30[c]	MER, IPM
ADU_VET_ST32	CN, CIP
HARRAN_VET_OK7	CIP
HARRAN_VET_OK11	CIP
HARRAN_VET_OK12	CIP
HARRAN_VET_OK28	CIP

[a] ***MER:*** *meropenem,* ***IMP:*** *imipenem,* ***CIP:*** *ciprofloxacin,* ***CN:*** *gentamicin,* ***TOB:*** *tobramycin,* ***AK:*** *amikacin,* ***CAZ:*** *ceftazidime;* [b] *These two isolates had meropenem and imipenem MIC of ≤2 µg/mL,* [c] *These two isolates had meropenem and imipenem MIC of ≥16 µg/mL*

ADU_VET_ST5) were found to be susceptible to both meropenem and imipenem by E-test (MIC values of ≤2 µg/mL), other two isolates (ADU_VET_ST22 and ADU_VET_ST30) were also found to be resistant to both meropenem and imipenem by E-test (MIC values of ≥16 µg/mL). Antimicrobial resistance rates for ciprofloxacin, gentamicin, tobramycin, amikacin and ceftazidime were recorded as 22.7% (10), 4.5% (2), 2.3% (1), 2.3% (1) and 2.3% (1), respectively.

Detection of Virulence Genes

Several virulence-associated genes with various combinations were detected in all isolates *(Table 3, Fig. 2, Fig. 3)*. Of the examined 44 *P. aeruginosa* isolates, all carried at least one of the four T3SS-related genes. The eleven isolates (25%) harboured *exo*U and/or *exo*S genes, including non-cytotoxic/invasive strain *(exo*U-/*exo*S+, 18.2%*)* and cytotoxic/non-invasive strain *(exo*U+/*exo*S-, 6.8%*)*. The non-cytotoxic/non-invasive strain genotype *(exo*U-/*exo*S-*)* was detected in 33 (75%) isolates, of which 30 (68.2%) isolates carried both *exo*Y and *exo*T, and 3 (6.8%) carried only *exo*T gene.

The distribution of QS genes revealed the presence of five different gene combinations among the isolates. The thirty (68.2%) isolates were positive for all investigated QS genes.

Oxidative stress-related phenazine genes were observed among isolates being *phz*I$^+$/*phz*II$^+$/*phz*M$^+$/*phz*S$^+$ (16/44, 36.4%) and *phz*I$^+$/*phz*II$^+$/*phz*M$^+$ (15/44, 34.1%) as most common gene combination. While all isolates were positive for alginate genes, half of the isolates were positive for all examined biofilm-related genes. Of biofilm-related genes, *pel*A and *ppy*R genes were detected in 16 isolates, *psl*A and *pel*A in three isolates, and *pel*A in three isolates. Fimbria genes were only presented in 34.1% (15) of the isolates, of which 12 isolates carried *pil*A and 3 had both *pil*A and *pil*B. Of the investigated toxin gene, the *exo*A and *pvd*A genes were detected in 17 and 13 isolates, respectively. The investigated phospholipase genes, *plc*H, and *plc*N were simultaneously detected in 34 isolates, and *plc*N was detected alone in 10 isolates.

Detection of Integrons

Eighteen (40.9%) isolates were found to be integron positive. Class II integron was detected in eleven (25%)

Table 3. *Virulence genes detected in Pseudomonas aeruginosa isolated from clinical bovine mastitis cases*

Related Function	Gene or Gene Combination	Number of the Isolates (%)
T3SS	*exoS, exoY, exoT*	8 (18.2)
	exoU, exoY, exoT	3 (6.8)
	exoY, exoT	30 (68.2)
	exoT	3 (6.8)
QS/regulation	*lasA, lasB, lasI, lasR, rhlI, rhlR, rhlAB*	30 (68.2)
	lasB, lasI, lasR, rhlI, rhlR, rhlAB	8 (18.2)
	lasA, lasB, lasI, lasR, rhlI, rhlAB	3 (6.8)
	lasA, lasB, lasI, rhlI, rhlR, rhlAB	1 (2.3)
	lasB, lasI, rhlI, rhlR, rhlAB	2 /4.5)
Adhesin	*algU, algL, algD*	44 (100)
Oxidative stres	*phzI, phzII, phzM, phzS*	16 (36.4)
	phzI, phzM, phzS	15 (34.1)
	phzI, phzM	11 (25)
	phzI, phzII, phzM	1 (2.3)
	phzM, phzS	1 (2.3)
Biofilm related genes	*pslA, pelA, ppyR*	22 (50)
	pelA, ppyR	16 (36.4)
	pslA, pelA	3 (6.8)
	pelA	3 (6.8)
Exotoxin A	*exoA*	17 (38.6)
L-ornithine N5-oxygenase	*pvdA*	13 (29.5)
Pilus	*pilA, pilB*	3 (6.8)
	pilA	12 (27.3)
Phospholipase	*plcH, plcN*	34 (77.3)
	plcN	10 (22.7)
Initial colonization factor flagellin	*fliC type A*	24 (54.5)
	fliC type B	18 (40.9)

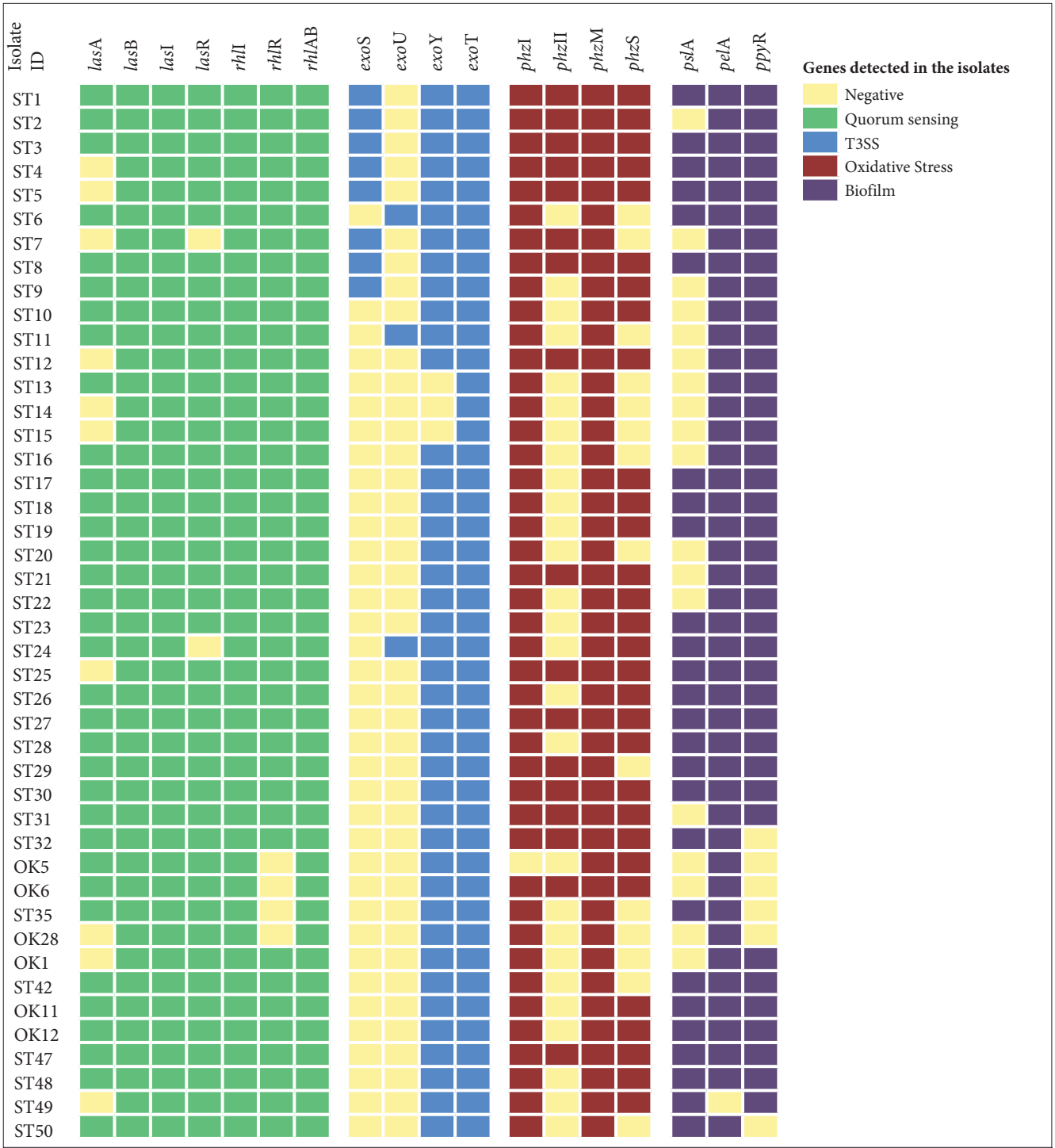

Fig 2. Heatmap shows presence and absence of quorum sensing, T3SS, oxidative stress and biofilm genes of *Pseudomonas aeruginosa* isolates

isolates, class I integron in one (2.3%) isolate, and both class I and II integron in 6 (13.6%) isolates. Whereas no isolate carried class III integron.

Sequencing Statistics

The median length of genome assembly of the isolates was 6.78 Mbp with an average GC% of 66.07±0.176. The average N_{50} of the assembled contigs was 3.93 Mbp. Detailed sequence statistics and genome features are summarized in *Table 4*.

Serotypes and Multi-Locus Sequence Types

While five *P. aeruginosa* isolates were serotyped, one isolate (ADU_VET_ST32) was found to be non-typeable by this approach due to the absence of a single definitive hit to the gene encoding the entire O-specific antigen (OSA). Four

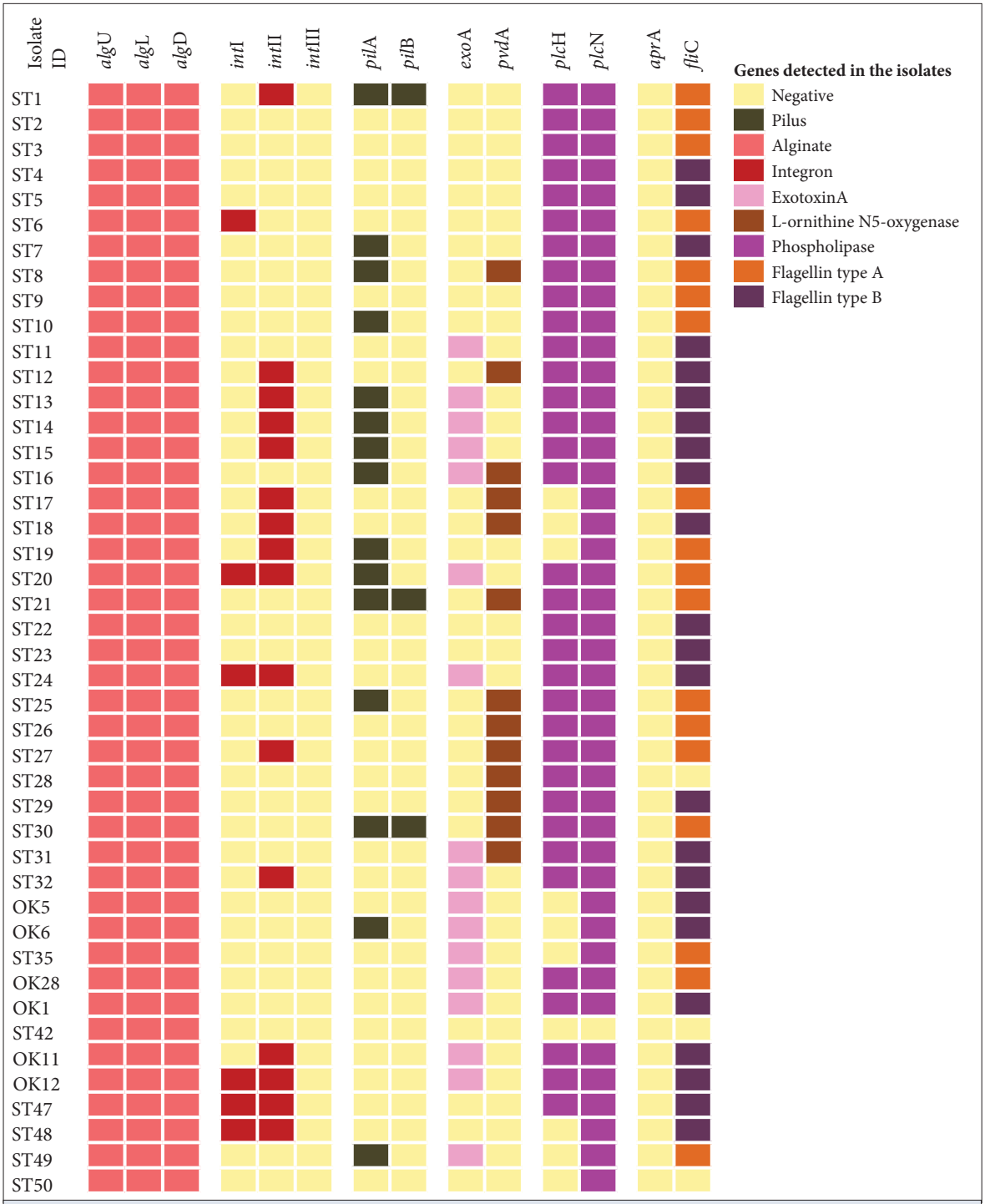

Fig 3. Heatmap shows presence and absence of pilus, alginate, virulence, integron, exotoxinA, L-ornithine N5-oxygenase, phospholipase, flagellin type A and B genes of *Pseudomonas aeruginosa* isolates

different serotypes were detected among the five isolates, i.e., O5 (HARRAN_VET_OK28), O6 (ADU_VET_ST3), O7 (ADU_VET_ST30), and O11 (ADU_VET_ST11, ADU_VET_ST24). The isolates were assigned to the respective sequence types (STs) with ST3246 (ADU_VET_ST3), ST235 (ADU_VET_ST11), ST235 (ADU_VET_ST24), ST1247 (ADU_VET_ST30), ST865 (ADU_VET_ST32), and ST591 (HARRAN_VET_OK28).

In Slico Detection of Acquired and Mutation Mediated Resistance

Based on the WGS analyses, 20 different types of acquired resistance genes found against various classes of antimicrobials including beta-lactams (*bla*$_{PAO}$, *bla*$_{PDC-374}$, *bla*$_{OXA-488}$, *bla*$_{OXA-396}$, *bla*$_{OXA-494}$, *bla*$_{GES-1}$, *bla*$_{OXA-904}$, *bla*$_{PDC-55}$, and *bla*$_{OXA-50}$), aminoglycosides (*aph(3')-IIb*, *aad*A6, *aac(6')-Ib4*, and *aph(3')-XV*), phenicols (*cat*B7 and *flo*R2),

Table 4. *The sequence statistics and genome features of whole-genome sequencing analysis*

Characteristic	Data for Isolate					
	ADU_VET_ST3	ADU_VET_ST11	ADU_VET_ST24	ADU_VET_ST30	ADU_VET_ST32	HARRAN_VET_OK28
Genome coverage (×)	83	83	83	83	83	83
No. of the contigs	89	310	362	168	4973	121
Total length (bp)	6 776 179	6 866 621	6 797 270	6 368 118	6 790 887	6 749 145
Largest contig	1 344 433	907 132	700 981	1 225 473	94 170	1 340 126
GC content (%)	65.9	65.99	66.04	66.41	66.02	66.04
N_{50} (bp)	781 852	272 482	355 003	431 554	29 306	469 602
N_{75} (bp)	362 714	185 975	224 305	239 081	13 490	306 699
L_{50} (bp)	4	7	7	4	71	5
L_{75} (bp)	7	15	13	9	158	9
GenBank accession no	JAKGDU000000000	JAKGDT000000000	JAKGDS000000000	JAKGDR000000000	JAKGDQ000000000	JAKGDP000000000
BioSample no.	SAMN25050669	SAMN25050670	SAMN25050671	SAMN25050672	SAMN25050673	SAMN25050674

Table 5. *Genetic characteristics of Pseudomonas isolates*

Characteristics	ADU_VET_ST3	ADU_VET_ST11	ADU_VET_ST24	ADU_VET_ST30	ADU_VET_ST32	HARRAN_VET_OK28
ST Serotype/Type	O6/ST3246	O11/ST235	O11/ST235	O7/ST1247	ND[a]/ST865	O5/ST591
Resistance phenotype	CIP	CIP	CIP, CN, TOB, AK, CAZ	MER, IPM	CIP, CN	CIP
Antimicrobial resistance genes	$bla_{PDC-374}$, bla_{OXA-50}, bla_{PAO}, *aph(3')-IIb*, *crp*P, *cat*B7, *fos*A	$bla_{PDC-374}$, $bla_{OXA-488}$, bla_{PAO}, *aph(3')-IIb*, *aad*A6, *crp*P, *cat*B7, *sul*1, *fos*A, *qac*E	$bla_{PDC-374}$, $bla_{OXA-488}$, bla_{GES-1}, bla_{PAO}, *aph(3')-IIb*, *aad*A6, *aac(6')-Ib4*, *aph(3')-XV*, *aac(6')-Ib-cr*, *cat*B7, *flo*R2, *tet*G, *fos*A, *qac*E	$bla_{PDC-374}$, $bla_{OXA-494}$, $bla_{OXA-396}$, bla_{PAO}, *aph(3')-IIb*, *cat*B7, *fos*A	$bla_{OXA-904}$, $bla_{OXA-396}$, *aph(3')-IIb*, *cat*B7, *fos*A	$bla_{OXA-494}$, bla_{PDC-55}, bla_{PAO}, *aph(3')-IIb*, *cat*B7, *fos*A
Amino acid substitutions[b]						
AmpC regulators						
*amp*R	M288R, G283E	M288R, G283E	M288R, G283E			M288R
*amp*D	D183T, P162L, G148A	G148A	G148A		D183T	G148A
*amp*Dh2		A239V, A196T				
*amp*Dh3		A208V	A208V	A219T		A219T
QRDR						
*gyr*A	T83I	T83I	T83I		N652T, N87T	T83I
gyrB				S466F		
*par*C			S87L		P595L	S87L
*par*E	E459V	D533E, S457R	D533E			
Efflux pumps regulatory genes						
MexAB-OprM regulators						
*mex*R						V126E
*nal*C	G71E	G71E, E153Q, S209R	G71E, E153Q, S209R	G71E	G71E	G71E, S209R
*nal*D		A162E				
MexCD-OprJ regulator						
*nfx*B				R21H, D56G, V134L		
MexEF-OprN regulators						
MexS						
MexT	P60S, Q80fs, F172I	P60S, Q80fs, F172I	P60S, Q80fs, F172I	P60S, Q80fs, R164H, F172I	F172Ile	P60S, Q80fs, F172I
MexXY-OprA regulator						
MexZ						
OprD mutations	D43N, S57Glu, E202Q, I210A, E230L, S240T, N262, A267S, A281G, L296Q, Q301E, R310G	T103S, L115T, P185Q, F170L, V189T, R310E, A315G, G425A	T103S, L115T, Q185G, F170L, V189T, R310E, A315G, G425A	Frameshift (Δnt 402/fs)		D43N, S57E, E202Q, I210A, E230L, S240T, N262T, A267S, A281G, K296Q, Q301E, R310G

[b] ***ND:*** *not detected;* [a] *Amino acid substitution compared to the sequences of* Pseudomonas aeruginosa *strain PAO1 (GenBank accession number NC_002516.29)*

sulphonamides *(sul*1*)*, tetracycline *(tet*G*)*, fosfomycin *(fos*A*)*, and quinolones *(crp*C and *aac(6')-Ib-cr)* in the study strains. Carbapenem-resistant isolates did not harbor metallo-β-lactamase genes *(bla_{IMP}*, *bla_{NDM}*, *bla_{VIM}*, and *bla_{GES})*. Additionally, the *qac*E gene conferring resistance to quaternary ammonium compounds (QACs) was also detected. Different amino acid substitutions in *gyr*A, *par*C, *opr*D, efflux pump, and AmpC β-lactamase regulatory genes were identified among representative isolates *(Table 5)*.

Data Availability

The genomic sequences for these isolates are available at NCBI under the BioProject number PRJNA798228.

Discussion

In this study, 72.7% of the isolates were susceptible to all tested antimicrobials, and resistance rates for aminoglycosides and cephalosporins were less than 4.5%, and for carbapenems and fluoroquinolones were 9.1% and 25%, respectively. In a previous study conducted in Japan [17], no resistance for this class of antimicrobials was reported, another study carried out in South Korea [18], resistance rates for meropenem and ciprofloxacin were reported as 4.9% and 0.8%, respectively. In Egypt, Ibrahim et al.[19] found that 11.76% of 34 *P. aeruginosa* from clinical mastitis were resistant to meropenem, and 3 of these isolates were positive for the *bla_{VIM}* gene.

Carbapenems are a class of ß-lactam antibiotic with broad-spectrum activity used as a last resort for treating infections caused by multidrug-resistant bacteria. Carbapenems are not used in veterinary medicine in Türkiye. The presence of carbapenem-resistant isolates among the isolates might be attributed to environmental contamination rather than the selective pressure exerted by these antimicrobials. Resistance to carbapenems in *P. aeruginosa* is mainly due to a contribution of several genetic mechanisms including horizontally acquired carbapenemases, low permeability or loss of oprD, overexpression of efflux pumps (mainly MexAB-OprM and MexXY-OprM), and overexpression of intrinsic AmpC beta-lactamases [42]. Previous studies demonstrated that *oprD* mutation together with other intrinsic mechanisms (overexpression of *mex*AB and *amp*C) might play an important role in carbapenem resistance [43,44]. Although amino acid substitutions in OprD have not been well elucidated in carbapenem resistance development, *oprD*-mediated carbapenem resistance can be achieved by the downregulation of *opr*D expression or the inactivation of this porin through a frameshift by the insertion/deletion mutation and/or a premature stop codon [44]. In this study, carbapenem-resistant isolate (ADU_VET_ST30) had frameshift mutation together with *nal*C and *nfx*B (involved in the regulation of efflux pumps) and *amp*C regulator genes *(Table 5)*. Similar observations were also reported by Hayashi et al.[45]. However, further molecular studies are needed to elucidate evolutionary mechanisms for carbapenem resistance in *P. aeruginosa* isolates.

Fluoroquinolones are one of the most prescribed antimicrobials to treat various bacterial infections in both human and veterinary medicine in Türkiye. Misuse and overuse of this class of antimicrobials have contributed to the selection and spread of resistant bacteria species isolated from animals and humans. Fluoroquinolone resistance rates against some bovine mastitis pathogens were reported as 23.17% in *E. coli*, 41.67% in *P. aeruginosa*, and 29.63% in *K. pneumonia* in Burdur province [46]; 25% in *S. aureus*, 26.32% in *E. coli*, 25% in *P. aeruginosa* and 100% *K. pneumonia* in Bursa province [13]; 3.7% in *S. aureus*, 4.5% in CoNS, and 36.5% in *E. coli* in Kars province [8]. The differences in fluoroquinolone resistance rates could be explained by the usage rates of this group of antimicrobials.

Major resistance mechanisms of fluoroquinolones in *P. aeruginosa* isolates are associated with mutations in the quinolone resistance-determining regions (QRDRs) of *gyr*A, *gyr*B, *par*C, and *par*E [47]. Consistent with previous studies, analysis of WGS data revealed that the main mechanism of resistance in ciprofloxacin-resistant isolates was related to mutations in gyrase and topoisomerase encoding genes, especially DNA gyrase II *(gyrA)* and topoisomerase IV *(parC)* [48-50]. Other resistance mechanisms, such as decreased intracellular accumulation regulated by the *nal*C and *nfx*B genes, are also important in clinical resistance to fluoroquinolones in *P. aeruginosa* but were mostly found in strains with high-level resistance together with mutations in DNA replicating enzymes [47]. In addition, horizontally transferred *aac(6')-Ib-cr* and *crp*P genes responsible for decreased susceptibility to ciprofloxacin were detected in two isolates. *crp*P (plasmid-encoded ciprofloxacin resistance protein) is the novel resistance determinant, which was recently described in Mexico within the plasmid named pUM505 in *P. aeruginosa*, conferring resistance to decreased susceptibility to ciprofloxacin through the enzymatic phosphorylation of the antibiotic [51]. Recently, the presence of *crp*P (variant crpP-2) was reported in two carbapenemase-producing *P. aeruginosa* isolates in a tertiary hospital in Türkiye [52].

Integrons are genetic elements that have the ability to capture, exchange, and express resistance genes called gene cassettes, and hence, they play a key role in the horizontal transmission of resistance genes [21]. Integrons have been reported to be closely related to MDR, especially class I integrons, and are widely distributed in clinical *P. aeruginosa* isolates [53]. The class I and II integrons were only observed in 5 of twelve resistant isolates. However,

it is not possible to ascertain an association between integron carriage and resistance phenotype due to the not known genetic context of class I and II integrons. Further studies are therefore needed to elucidate the genetic context of integrons.

P. aeruginosa possesses a large and variable arsenal of virulence factors that contribute to its pathogenesis. Most of *P. aeruginosa* virulence factors are regulated by a cell density-dependent mechanism called Quorum Sensing (QS) [23]. In this study, all isolates harbored various combinations of QS system genes, including *las*I and *rhl*I genes, which are responsible for the autoinducers (PAI-1 and PAI-2) biosynthesis and upregulation of virulence genes.

Based on T3SS related genes, 25% of the isolates were found to harbour *exo*U and/or *exo*S genes, including the invasive *(exo*U-/*exo*S+, 18.2%*)* and cytotoxic *(exo*U+/*exo*S-, 6.8%*)* strains. In contrast, higher rates of *exo*S or *exo*U were reported by Park et al.[18], who found that 82.7% of the isolates had *exo*U and/or *exo*S genes. The researchers also suggested that *P. aeruginosa* isolates having either *exo*S or *exo*U genes should be considered as pathogenic for dairy cattle. Similarly, Tartor et al.[54] examined 8 *P. aeruginosa* isolates from clinical and subclinical milk samples and found that 5 isolates carried both *exo*S and *exo*U and 2 isolates had only *exo*S and one isolate *exo*U. Horna et al.[55] investigated the presence of the *exo*U+/*exo*S+ genotype in 189 *P. aeruginosa* clinical isolates and found that all isolates had *exo*S, *exoT* and *exo*Y genes, but 22.8% (43/189) of the isolates carried *exo*U. The authors stated that *exo*U was significantly associated MDR as well as with higher level quinolone resistance. Indeed, *exo*U carrying 3 isolates had ciprofloxacin-resistant phenotype; and one of these isolates had also MDR phenotype.

Epidemiological studies have revealed that some sequence types (ST111, ST175, ST235, ST244, and ST395) of *P. aeruginosa* are distributed worldwide and are frequently associated with outbreaks [56]. Of these clones, ST235 (serotype O11) has been reported to be the predominant globally high-risk clone with MDR profiles [57]. It has been also reported that ST235 is highly associated with *exo*U and infections caused by this clone have a highly unfavorable prognosis than infections with other strains [58]. Detection of the ST235/O11 serotype associated with *exoU* in two *P. aeruginosa* isolates from clinical bovine mastitis suggests that mastitis pathogens may have adverse public health implications.

In conclusion, this is the first comprehensive study to date to determine the presence and distribution of virulence traits in *P. aeruginosa* isolated from clinical bovine mastitis. The findings of the study also indicate a low rate of resistance to tested antimicrobials and the presence of high-risk clone ST235 among the isolates. Comprehensive studies are needed to better understand the molecular epidemiology of *P. aeruginosa* isolates at the human-animal-environment interface and to assess their clinical implications on humans and animals. In addition, considering the importance of virulence determinants, anti-virulence strategies should be developed to combat infections caused by *P. aeruginosa*.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available from the corresponding author (Ö. Aslantaş) on reasonable request.

Ethical Statement

The study does not require ethical approval from Animal Experiments Local Ethics Committee

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Conflict of Interest

The authors declared that there is no conflict of interest related to this study.

Author Contributions

ÖA, ST, OK and KB planned, designed, and supervised the research procedure, ÖA, ST and AGY performed all microbiological and molecular experiments, ÖA and KB performed bioinformatic analyses, and ÖA wrote the manuscript. All authors have read and approved the manuscript.

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RESEARCH ARTICLE

Effects of Crude Fiber Level on Growth Performance, Serum Biochemical Indicators, and Digestibility in Zhedong White Geese

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Abstract: The purpose of this study was to investigate the feasibility and supplementation level of rice hull as a dietary fiber source for geese. The effect of rice hull addition level on growth performance, serum biochemical indices, and digestive performance of geese was explored. Three hundred 28-day-old Zhedong white geese (half male and half female) with similar body weights were selected and divided into three groups. The crude fiber (CF) level of the groups was 4.8%, 6.1% and 6.9%, respectively. The pre-feeding period was 7 days, and the formal test period was 21 days. The growth performance and serum biochemical indexes, amylase, lipase, and protease activities, and apparent digestibility were determined. CF level of 6.1% and 6.9% were higher than 4.8%, but the feed/gain at 6.9% CF level was higher than that at 4.8% ($P<0.05$). Serum total cholesterol and high-density lipoprotein cholesterol concentrations decreased at CF levels of 6.1% and 6.9%, and insulin or insulin-like growth factor-1 levels were increased. The intestinal amylase activity at 6.9% CF level was lower than that at 6.1%. In contrast, pancreatic amylase activity at 6.9% CF level was higher than that at 4.8%. The digestibility of crude ash and crude fat in CF level of 6.9% and 6.1% were lower than that at 4.8%, whereas the digestibility of crude protein increased. Rice hull as the main fiber source, with fiber level between 6.1% and 6.9%, maintains growth performance and improves some beneficial serum biochemical indicator levels and crude protein digestibility.

Keywords: *Crude fiber, Digestive enzymes, Goose, Growth performance, Rice hull, Serum*

Zhedong Beyaz Kazlarında Ham Lif Seviyesinin Büyüme Performansı, Serum Biyokimyasal İndikatörleri ve Sindirilebilirlik Üzerine Etkileri

Öz: Bu çalışmada, kazlarda pirinç kabuğunun bir diyet lifi kaynağı olarak uygulanabilirliği ve katkı düzeyinin araştırılması amaçlandı. Pirinç kabuğu ilavesinin kazların büyüme performansı, serum biyokimyasal indeksleri ve sindirim performansı üzerine etkileri araştırıldı. Benzer vücut ağırlıklarına sahip 300 adet 28 günlük Zhedong beyaz kaz seçildi (yarısı erkek ve yarısı dişi) ve üç gruba ayrıldı. Grupların ham selüloz (CF) düzeyleri sırasıyla %4.8, %6.1 ve %6.9 olarak belirlendi. Hayvanlar uygulama öncesi 7 gün ve uygulama süresince 21 gün süreyle beslendi. Büyüme performansı, serum biyokimyasal indeksleri, amilaz, lipaz ve proteaz aktiviteleri ve saptanabilir sindirilebilirlik test edildi. Ham selüloz oranı, %6.1 ve %6.9'luk gruplarda yüksekti, ancak %6.9'luk gruptaki yem kazanç oranı %4.8'lik gruba göre daha yüksekti ($P<0.05$). Serum total kolesterol ve yüksek yoğunluklu lipoprotein kolesterol konsantrasyonları, %6.1 ve %6.9'luk gruplarda azaldı ve insülin ve insülin benzeri büyüme faktörü-1 seviyeleri yüksekti. %6.9'luk gruptaki bağırsak amilaz aktivitesi, %6.1'lik gruptan daha düşüktü. Buna karşılık, %6.9'luk gruptaki pankreas amilaz aktivitesi, %4.8'lik gruptan daha yüksekti. Ham kül ve ham yağın sindirilebilirliği %6.9 ve %6.1'lik grupta %4.8'lik gruba göre daha düşük iken, ham proteinin sindirilebilirliği artmıştı. Temel lif kaynağı olarak pirinç kabuğunun %6.1 ile %6.9 arasındaki lif konsantrasyonları, büyüme performansını sürdürülebilir kılmakta ve bazı faydalı serum biyokimyasal indikatörlerinin düzeylerini ve ham proteinlerin sindirilebilirliğini artırmaktadır.

Anahtar sözcükler: *Ham selüloz, Sindirim enzimleri, Kaz, Büyüme performansı, Pirinç kabuğu, Serum*

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Introduction

In recent years, due to the impact of war and COVID-19, the prices of raw materials such as corn and soybean meal have risen [1,2]. Many countries are facing food challenges [3,4]. Therefore, the use of corn-soybean meal-based feed in animal husbandry should be reduced. It is important to reduce food competition between humans and animals [5,6]. Rice is a staple food in many countries. Rice hull is the main byproduct of rice processing. It is rich in cellulose and lignin, but the content of fat and protein is low, and its nutritional value is not as high as that of rice bran [7]. Rice hulls are commonly used as fermentation beds [8] and fertilizers [9]. Zhedong White Goose is a native species of China [10] being widely bred in the southern provinces due to its good flavor. Geese are herbivorous poultry and have a great advantage over other poultry in digesting crude fiber (CF). Under natural conditions, green grasses are the preferred feed of geese under natural conditions [11]. The upper and lower beaks of the goose have serrations, the tongue has barbs, and the stomach and cecum contain cellulolytic bacteria [12,13]. Currently, there are different reports for geese nutritional requirements. The American NRC (1994) poultry feed nutrition standard recommends a CF level of 4% to 10% for geese over 4 weeks of age [14]. If the CF level is too low, the goose intestinal microbial flora will be disordered, which will affect the absorption and utilization of nutrients, thereby affecting its growth rate and increasing mortality. A high CF level will affect the digestion and absorption of other nutrients, such as proteins [5]. The source and level of fiber in the diet will affect the intestinal digestive enzyme activity, along with glucose and lipid absorption. Thatcher et al.[15] pointed out that different fiber sources can affect the digestive enzyme activity of livestock and poultry intestinal chyme. Zhan et al.[16] used ryegrass as the dietary fiber source of Yangzhou geese and found that ryegrass can reduce the content of serum triglyceride and total cholesterol in Yangzhou geese. Although the use of forages with different fiber sources in geese has received much attention [17,18], there are few reports on rice hulls as a fiber source. We explored the possibility and level of rice hull as a dietary fiber source for geese by measuring the growth performance, serum biochemical indices, metabolic enzyme activity, and apparent digestibility at different fiber levels in geese.

Material and Methods

Animal Ethics

The experiment was approved by the Laboratory Animal Ethics Committee of the Shanghai Academy of Agricultural Sciences (number: SYXK (HU), 2015-0007). Methods and ethics complied with the relevant regulations.

Experimental Design, Diets, and Birds

A total of 300 4-week-old Zhedong white geese with similar body weight were purchased from the Zhedong White Goose Research Institute of Xiangshan County and randomly divided into three treatments. CF levels, with rice hull as the main fiber source, were 4.8% (soybean 20.3%, soybean germ 0%, bran 9%, rice hull 5%), 6.1% (soybean 15.4%, soybean germ 4.5%, bran 2.1%, rice hull 10.6%), and 6.9% (soybean 0%, soybean germ 15.4%, bran 0%, rice hull 16.3%) in *Table 1*. Each treatment consisted of 10 repetitions (five male and five female). All the geese had free access to water or feed and were vaccinated in a timely manner. The pre-feeding period was 7 d, and the formal test period was 21 d.

Table 1. *Feed ingredients and analyzed chemical composition of geese diets. (air-dry basis %)*

Ingredients	CF Levels		
	4.8%	6.1%	6.9%
Corn	63.00	64.80	65.70
Soybean	20.30	15.40	0.00
Soybean germ	0.00	4.50	15.40
Bran	9.00	2.10	0.00
Rice hull	5.00	10.60	16.30
Premix[a)]	1.00	1.00	1.00
Stone powder	0.50	0.40	0.40
Calcium Hydrogen Phosphate	0.90	0.90	0.90
Salt	0.30	0.30	0.30
Total	100.00	100.00	100.00
Nutrient levels			
ME(Mcal/kg)[b)]	12.16	12.11	12.04
CP	15.38	15.50	15.26
EE	2.45	2.58	3.51
CF	4.80	6.10	6.90
Ca	0.50	0.45	0.44
TP	0.50	0.48	0.46

[a] *Per kilogram of diets including: multiple vitamin- 50 g, trace elements- 50 g, garlicin- 30 g, lysine- 100 g, methionine- 50 g, salt- 100 g, stone powder- 100 g, Myco-Ad- 100 g, Zeolite powder-420 g;* [b] *Nutrient levels were all calculated values*

Sample Collection

At the end of the experiment, two geese (one male and one female), close to the average weight, were randomly selected from each replicate. Blood samples were collected from the wing veins, and the geese were slaughtered by jugular vein exsanguination. Serum was separated after 30 min and stored at -20°C. For each treatment 10 geese (five male and five female) close to the average weight were randomly selected, and the gizzard (containing chyme), duodenum (containing chyme), and pancreas were separated after execution. Samples were frozen in liquid nitrogen and stored in a refrigerator at -80°C. The

excrement from each replicate was collected for three consecutive days, which was then treated to remove impurities, dried at 65°C with 10% hydrochloric acid, crushed after 24 h, and stored at 4°C for later use.

Parameters Measured

Serum glucose, total cholesterol, high-density lipoprotein, low-density lipoprotein, and blood urea nitrogen were measured using the kits of Shanghai Bogu Biotechnology. The activities of protease, amylase, and lipase in the pancreas, gizzard, and duodenum were determined according to the instruction manual of the kit produced by Nanjing Jiancheng Bioengineering Institute. Serum Insulin (INS), Insulin-like Growth Factor-1 (IGF-1), Growth Hormone, thyroxine, and Thyroid Stimulating Hormone were determined by Nanjing Jiancheng Bioengineering Institute.

Acid-insoluble ash was used as an indicator of apparent nutrient availability. The crude ash content was determined using the burning method (GB/T 6438-2007); the crude protein content was determined using the Kjeldahl method (GB/T 6432-1994); and the crude fat content was determined using the ether extraction method (GB/T6433-2006).

Statistical Analysis

Statistical analyses were performed using one-way ANOVA variance using SPSS statistical software (version 20.0, SPSS, Inc., Chicago, IL, USA). The Duncan method was used for multiple comparisons between groups, and $P>0.05$ (insignificant difference), $P<0.05$ (significant difference) and $P<0.01$ (extremely significant difference) were used as the criteria for judging the difference.

Results

Growth Performance

The results in *Table 2* show that when the CF level in the rice hull diet increased from 4.8% to 6.9%, the final body weight, average daily gain, and feed/gain (F/G) of geese increased; the CF level was 6.9% in final body weight (FBW), average daily gain (ADG), and (F/G), which were significantly higher than 4.8%, but there was no significant difference between the CF levels of 6.1% and 4.8%.

Serum Biochemical Indicators

As shown in *Table 3*, as the level of CF in the diet increased, the concentrations of INS and IGF-1 in the goose serum increased significantly, and the concentration was the

Table 2. *Effects of CF levels on growth performance in geese*

Items	CF Levels		
	4.8%	6.1%	6.9%
Initial body weight, g	2036.60±143.67	2021.08±211.14	2031.40±185.30
Final body weight, g	3447.00±276.81^{b}	3559.50±444.27^{ab}	3866.60±327.26^{a}
Average daily gain, g/d	67.16±6.65^{b}	73.26±7.76^{ab}	87.36±8.01^{a}
F/G	4.17±0.16^{b}	4.82±0.35^{ab}	5.65±0.86^{a}

Different lowercase letters with the same column date meant significant difference (P£ 0.05). Each value represents the mean of 10 replicates

Table 3. *Effects of CF levels on serum biochemical indicators in geese*

Items	CF Levels		
	4.8%	6.1%	6.9%
INS, ng/mL	0.47±0.04^{b}	0.46±0.07^{b}	0.57±0.08^{a}
IGF-1, ng/mL	11.27±3.23^{b}	11.99±3.26^{b}	15.48±3.44^{a}
GH, ng/mL	6.87±1.47	6.29±2.94	6.39±2.09
T4, ng/mL	0.45±0.13	0.45±0.09	0.49±0.18
TSH, ng/mL	1.92±0.92	2.15±0.97	2.05±0.52
GLU, mmol/L	29.03±3.16	28.51±2.42	25.34±2.21
T-CHO, mmol/L	3.36±0.53^{a}	2.35±0.31^{b}	0.23±0.06^{c}
HDL-C, mmol/L	7.79±0.83^{a}	5.93±0.76^{b}	4.23±0.57c
LDL-C, mmol/L	2.44±0.31	1.86±0.28	1.80±0.21
BUN, mmol/L	31.92±3.57	26.78±2.27	28.64±2.50

Different lowercase letters with the same column date meant significant difference (P£0.05). Each value represents the mean of 10 replicates

Table 4. *Effects of CF levels on the Digestive enzyme activity in geese*

Items	CF Levels		
	4.8%	6.1%	6.9%
Tryptic amylase activity, U/mgprot	1092.69±102.11	939.53±78.26	845.25±74.24
Gastric amylase activity, U/mgprot	7.24±0.71	5.87±0.62	5.68±0.63
Intestinal amylase activity, U/mgprot	32.27±2.40^{a}	24.26±2.62^{ab}	13.61±1.16^{b}
Trypsin activity, U/mgprot	93.68±8.63^{b}	103.76±10.08^{a}	105.54±10.27^{a}
Pepsin activity, U/mgprot	26.33±2.17	21.1624±1.67	22.09±2.39
Intestinal protease activity, U/mgprot	0.74±0.07	0.63±0.06	0.54±0.06
Pancreatic lipase activity, U/mgprot	186.67±10.47	171.65±16.94	167.55±11.39
Gastric lipase activity, U/mgprot	142.08±12.64	109.69±10.92	97.03±9.27
Intestinal lipase activity, U/mgprot	9.55±0.84	7.84±0.70	12.08±0.96

Different lowercase letters with the same column date meant significant difference (P£ 0.05). Each value represents the mean of 10 replicates

Table 5. *Effects of CF level on the serum apparent digestibility in geese*

Items	CF Level		
	4.8%	6.1%	6.9%
Crude ash, %	30.54±2.66^{a}	27.99±0.26^{b}	26.61±1.20^{b}
Crude protein, %	16.93±1.17^{b}	18.99±2.32^{ab}	19.59±1.85^{a}
Crude fat, %	47.46±3.51^{a}	39.66±2.85^{b}	38.91±3.69^{b}

Different lowercase letters with the same column date meant significant difference (P£ 0.05). Each value represents the mean of 10 replicates

highest when the CF level was 6.9%, which was significantly higher than that of the other two groups (P<0.05). There was no significant change in the serum concentrations of growth hormone, thyroxine, and thyrotropin (P>0.05). Similarly, as the level of CF increased, the concentrations of total cholesterol and high-density lipoprotein cholesterol decreased, and the differences among the groups were extremely significant (P<0.01)

Digestive Enzyme Activity

The results in *Table 4* show that the decreasing order of amylase activities was pancreatic, intestinal, and gastric. The intestinal amylase activity at a CF level of 6.9% was lower than that at 4.1% (P<0.05); the decreasing order protease activities was pancreatic, gastric, and intestinal. Protease activities at CF levels of 6.9% and 6.1% were higher than that at 4.1% (P<0.05). The decreasing order of lipase activities were pancreatic, gastric, and intestinal.

Nutrient Digestibility

The results in *Table 5* show that the apparent digestibility of crude ash and crude fat decreased significantly at CF levels of 6.9% and 6.1% (P<0.05), whereas the digestibility of crude protein increased (P<0.05).

Discussion

The digestion and utilization of fiber in geese is a process involving gastrointestinal participation, cellulolytic enzymes, and intestinal microorganisms, which mainly include mechanical grinding, chemical digestion, and microbial degradation, amongst other methods [19]. An appropriate source and level of fiber can promote intestinal peristalsis, increase the secretion of digestive enzymes, and improve growth performance [5,20,21]; however, poor fiber quality or high feeding levels can lead to an increase in the composition of anti-nutritional factors and interfere with the absorption of nutrients [22]. The results showed that a CF level of 6.9% significantly increased FBW and ADG, and the decomposition of cellulose in goose intestines mainly depends on cellulase and hemicellulase secreted by intestinal microorganisms. Cellulose was found to increase the abundance of cellulolytic bacteria in the gut [23] and increase the microbiome of hydrolyzed hemicellulose [24].

Nutrient digestibility is closely related to dietary composition and nutrient levels, especially lignin and acid detergent fiber content [25]. In this study, the apparent digestibility of crude ash and crude fat decreased gradually with the increase in rice hull addition to the diet, which may be due to the high acid washing lignin content of rice hull [26,27]. It may also be that the CF content in the diet is high, which causes the goose to eat more feed to maintain nutrition, and the speed of chyme passing through the intestine is accelerated, so that the residence time of the feed in the cecum is too short, which affects the digestion of CF in the large intestine. This may also be the reason for the significant increase in F/G.

IGF-1 can promote cell proliferation, differentiation, and secretion; participate in glucose metabolism and glucose transport in adipose tissue; promote fat and glucose synthesis; and improve glucose utilization [28]. INS is a multifunctional protein hormone secreted by pancreatic β-cells. Many in vitro experiments have shown that INS can stimulate glucose absorption and fat synthesis, and inhibit lipolysis [29]. The results of this study showed that the addition of rice hulls to the diet significantly increased

the concentration of INS and IGF1 in the serum, indicating that the addition of rice hulls was beneficial to the growth of geese and deposition of fat in them.

The mucus barrier, which is composed of gelatinous mucus secreted by intestinal epithelial cells and digestive glands, is an important part of the intestinal mucosal barrier. Along with their role in preventing potential opportunistic pathogens from adhering to the surface of the intestinal lumen, digestive enzymes are important indicators of animal feed intake. Its level of activity directly affects the digestion and absorption of food [30]. Dietary fiber affects the physicochemical properties of chyme, improves intestinal morphology, and stimulates the secretion of digestive enzymes [31]. The results of this study show that the activity of pancreatic amylase is the highest, which is tens to hundreds of times higher than that of intestinal and gastric amylase, and that intestinal alpha-amylase activity decreases with increasing dietary CF levels [32]. Trypsin secreted by the pancreas is an important digestive enzyme that breaks down proteins in the gut and hydrolyzes the proteins between cells to disperse the cells. Under this action, the protein ingested by the goose from the diet is hydrolyzed into small molecular peptides and amino acids [33]. Supplementation with trypsin inhibitors in the diet significantly reduces the digestibility of various amino acids [34]; in contrast, adding exogenous trypsin to the diet increases amino acid digestibility [35]. Therefore, trypsin affects the rate of digestion in poultry [36]. In this study, we found that CF was positively correlated with trypsin.

Rice hull is a fiber source for geese, and CF levels between 6.1% and 6.9% can significantly improve the growth performance of geese, increase IGF-1 and INS serum concentrations, reduce total cholesterol content, and increase trypsin levels.

Availability of Data and Materials

The original data and materials presented in the study are included in the article, further inquiries can be directed to the corresponding author (D-W He).

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Ethical Approval

The experiment was approved by the Laboratory Animal Ethics Committee of the Shanghai Academy of Agricultural Sciences (number: SYXK (HU), 2015-0007). Methods and ethics complied with the relevant regulations.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

Writing-original draft, H-Y Wang; Formal Analysis, G-Q Li and H-Y Wang; Investigation, X. Wang, Y-Z Yang; Conceptualization, S-M Gong, Y. Liu and C. Wang; Project administration, D-Q He; Writing-review, H-Y Wang and D-Q He; Editing, H-Y Wang and D-Q He; Validation, H-Y Wang and D-Q He; Supervision: H-Y Wang and D-Q He; Funding acquisition, H-Y Wang. All authors have read and agreed to the published version of the manuscript.

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RESEARCH ARTICLE

Distribution of Serotypes and Antibiotic Resistance of Avian Pathogenic *Escherichia coli* Strains Isolated from Chickens

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Abstract: Avian pathogenic *Escherichia coli* (APEC) causes systemic or localized infections with different clinical courses such as septicemia, air sac disease, polyserositis and coligranuloma. Colibacillosis causes significant economic losses in the poultry industry due to the need for the control of the disease by causing carcass contamination, decreased feed conversion rate, mortality in poultry. In this study, 99 *E. coli* strains were isolated from different production units in the same integration. Antimicrobial susceptibility tests of all strains were performed and thirty strains with multidrug resistance (MDR) in the different production units were serotyped. The highest resistance was observed against oxytetracycline, erythromycin amoxicillin and doxycycline. Multidrug resistance was observed at a rate of 81.81% (81/99). Among the 30 strains that could be serotyped, O78 and O125 were determined as the most prevalent serogroups with 43.3% (13/30) and 16.6% (5/30) rates, respectively, while O1, O8, O18, O142, O143, O157, O158, O164 and O169 were found as rare serogroups. O78 was determined as a high antibiotic resistant strain in isolated *E. coli* strains and a dominant serotype in the selected strains and this study demonstrated that a correlation can be between breeders and their progeny.

Keywords: *Antibiotic resistance, Chicken, Escherichia coli, Serogroups*

Tavuklardan İzole Edilen Avian Patojenik *Escherichia coli* Suşlarının Antibiyotik Direnci ve Serotip Dağılımı

Öz: Avian patojenik *Escherichia coli* (APEC) koligranuloma, poliserözitis, hava kesesi yangısı, septisemi gibi farklı klinik seyirler izleyerek sistemik ya da lokal enfeksiyonlara sebep olmaktadır. Kolibasillozis kanatlı hayvanlarda karkas kontaminasyonu, yem dönüşümünde azalma, mortaliteye sebep olabildiğinden hastalığın kontrolü, kanatlı hayvan endüstrisinde önemli ekonomik kayba neden olmaktadır. Bu çalışmada aynı entegrasyona ait farklı üretim birimlerindeki kanatlı hayvanlardan 99 *E. coli* suşu izole edildi. Tüm suşların antimikrobiyal duyarlılıkları test edildi ve farklı üretim birimlerindeki çoklu ilaç direncine sahip 30 suş serotiplendirildi. En yüksek direnç oksitetrasiklin, eritromisin, amoksisilin ve doksisiline karşı belirlenmiştir. %81.81 (81/99) oranında çoklu ilaç direnci gözlenmiştir. Serotiplendirilen 30 suş arasında O1, O8, O18, O142, O143, O157, O158, O164 ve O169 ise nadir serogruplar olarak belirlenirken O78 %43.3 (13/30) ve O125 %16.6 (5/30) en yaygın serogruplar olarak saptanmıştır. İzole edilen *E. coli* suşlarında yüksek antibiyotik direnci ve serotiplendirilen *E. coli* suşlarında O78'in baskın serotip olduğu tespit edildi. Ayrıca bu çalışma damızlık ve bu damızlıklara ait soylardan izole edilen *E. coli* suşları arasında bir ilişki olabileceğini de ortaya koymuştur.

Anahtar sözcükler: *Antibiyotik direnci, Tavuk, Escherichia coli, Serogrup*

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Introduction

Escherichia coli is an important member of the gut microbiota of humans and animals. However, some strains are potentially pathogenic and cause both enteric and extraintestinal infections in humans and animals [1,2]. APEC is a subgroup of extraintestinal pathogenic *E. coli* (ExPEC), a pathotype that also includes uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) of human [2-4]. APEC, called avian colibacillosis in chickens, turkeys, and other avian species, causes various clinical syndromes including colisepticemia, coligranuloma (Hjarre's disease), air sac disease (chronic respiratory disease, CRD), swollen-head syndrome, peritonitis, yolk sac infection, polyserositis, pericarditis, perihepatitis, salpingitis and enteritis [5-7]. APEC infections have significant morbidity and mortality in birds of all ages, resulted with serious economic losses in the poultry industry [6,7]. Diseases such as mycoplasmosis, Newcastle disease, infectious bursal disease, coccidiosis or nutritional deficiency etc. predispose all poultry to colibacillosis [5,7]. Reducing the incidence and mortality and successful treatment of avian colibacillosis mainly depends on the use of antibiotics [4,6,8]. However, misuse and overuse of antibiotics have led to the development of antibiotic resistance. Contamination of poultry carcass by antibiotic resistant bacteria may increase the risk of transmission of antibiotic resistance genes to humans [4,8,9]. The World Health Organization (WHO) has published a list of antibiotic-resistant "priority pathogens" of 12 families of bacteria of international concern. *E. coli* has been included in the list of 12 microorganisms of international importance that cause the most prevalent infections in different areas [10].

APECs are characterized by many methods, including serotyping. The most common serotypes associated with infections of APECs isolated from septicemic chickens studied to date have been shown to be O1, O2, O5, O8, O11, O15, O25, O78, and O88. Other serotypes were found less frequently. Their prevalence varies depending on many factors such as the region of origin of the disease, age, species and utility type of the poultry [3,11-16]. There are a few studies regarding to avian *E. coli* and antibiotic resistance in our country [17-19].

The aim of this study is to determine antibiotic resistance in APEC strains isolated from different poultry production units (broiler, breeding, hatchability) and common serotypes in multidrug resistant APEC strains.

Material and Methods

Sampling and Cultural Examination

Avian *E. coli* strains were isolated from internal organs (pericardial fluid, heart blood, liver and spleen) collected from 21 broilers, 35 breeding flocks that were diagnosed with colibacillosis and 33 eggs from the incubator during hatchery were diagnosed one-day chick dead in the same integration in Türkiye. Samples were cultured on MacConkey agar and incubated overnight at 37°C aerobically. Lactose positive, pink colonies were selected and identified as *E. coli* by biochemical tests [6]. All strains were kept at -20°C in Luria-Bertani (LB) medium containing 15% glycerol until tests were performed.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility of all *E. coli* strains was determined using Kirby-Bauer disc diffusion method according to the standards Clinical and Laboratory Standards Institute (CLSI) [20]. Each strain was tested for susceptibility to florfenicol (30 µg), erythromycin (15 µg), doxycycline (30 µg), enrofloxacin (5 µg), sulfamethoxazole/trimethoprim (25 µg), amoxicillin (10 µg), and oxytetracycline (30 µg). *E. coli* ATCC 25922 was used as a quality control strain. The antibiotics tested were selected considering the antibiotics commonly used in the treatment of poultry with colibacillosis. APEC strains showing resistance to ≥3 classes of antibiotic were determined as multidrug resistant strains [21].

O-Serotyping

APEC strains with multidrug resistance (MDR) that were isolated from different production units were serotyped. For this, total of 30 APEC strains were used. To identify the serogroup of the APEC isolates, O-serogroup typing was performed by slide agglutination using all polyvalent and monovalent antisera provided by the manufacturer (Denka Seiken, Co., Ltd., Tokyo, Japan) [22].

Results

In this study, total of 99 APEC strains were isolated from chicken (21 broilers) and different types of husbandries (35 breeding and 43 hatchability). *E. coli* strains demonstrated high resistance to oxytetracycline and erythromycin, amoxicillin, doxycycline, sulfamethoxazole/trimethoprim and less resistance to enrofloxacin and florfenicol at rates of 82.83% (82/99), 75.76% (75/99), 67.68% (67/99), 59.69% (59/99), 38.38% (38/99) and 33.33% (33/99), respectively. Although the most common observed resistant phenotypes were determined against oxytetracycline, amoxicillin and erythromycin in all production units, the highest resistance determined 100% (5/5) and 92.3% (12/13) in broiler *(Table 1)*.

Many of the strains exhibited resistance to ≥3 of the seven antibiotics tested. According to the MDR profiles; 95.23% of strains isolated from broiler, 71.42% from breeding and 83.72% from hatchability. Multidrug resistance patterns of the strains to six antibiotic classes revealed that 18 APEC

Table 1. *Antibiotic resistance according to the production units*

Production Units	Antibiotics						
	Amphenicol	Tetracycline		B-Lactam	Sulfonamide	Macrolide	Fluoroquinolone
	Florfenicol % (n)	Oxytetracycline % (n)	Doxycycline % (n)	Amoxicillin % (n)	Sulfamethoxazole/ Trimethoprim % (n)	Erythromycin % (n)	Enrofloxacin % (n)
Broiler (n = 21)	85.71 (18)	100 (21)	85.71 (18)	95.23 (20)	90.47 (19)	95.23 (20)	90.47 (19)
Breeding (n = 35)	17.14 (6)	74.28 (26)	57.14 (20)	71.42 (25)	57.14 (20)	74.28 (26)	28.57 (10)
Hatchability (n = 43)	20.93 (9)	81.39 (35)	67.44 (29)	69.76 (30)	46.51 (20)	83.72 (36)	20.93 (9)
Total (n = 99)	33.33 (33)	82.83 (82)	67.68 (67)	75.76 (75)	59.69 (59)	82.83 (82)	38.38 (38)

strains were resistant to all of the antibiotics. We detected a rate of 18.18% and APEC strains showed multidrug resistance ≥3 antibiotic class rate of 81.81% *(Table 2)*.

Thirty multidrug resistance *E. coli* strains were selected and serotyped. The most common serogroups were O78 (43.3%) and O125 (16.6%) and the remaining 12 strains were O1 (6.6%), O18 (6.6%), O169 (6.6%), O8 (3.3%), O142 (3.3%), O143 (3.3%), O157 (3.3%), O158 (3.3%) and O164 (3.3%), serogroups *(Table 3, Table 4)*. O78 serogroup was found resistant to oxytetracycline, sulfamethoxazole/ trimethoprim, amoxicillin-doxycycline and erythromycin at the rates of 12/13 (92.3%), 11/13 (84.6%), 10/13 (76.9%), 9/13 (69.2%), respectively, and the O125 serogroup was resistant to erythromycin, oxytetracycline-doxycycline and sulfamethoxazole/trimethoprim, amoxicillin, at the rates of 5/5 (100%), 4/5 (80%), 3/3 (100%), respectively.

Discussion

Increasing antibiotic resistance is an important public health problem and resistance continues to spread due to many factors. Antibiotics are used for therapeutic and prophylactic purposes for broilers as stimulating growth in production. Some antibacterial drugs, such as ampicillin, tetracycline, chloramphenicol, enrofloxacin, neomycin, which are used in the treatment of *E. coli* infections to become resistant by inhibiting the microflora of the

Table 2. *Rate of APEC strains exhibiting multidrug resistance (MDR)*

Production Units	Resistance to ≥3 Antibiotics % (n)	Resistance to ≥4 Antibiotics % (n)	Resistance to ≥5 Antibiotics % (n)	Resistance to ≥6 Antibiotics % (n)	Resistance to ≥7 Antibiotics % (n)
Broiler (n=21)	95.23 (20)	95.23 (20)	95.23 (20)	90.47 (19)	66.66 (14)
Breeding (n=35)	71.42 (25)	60 (21)	48.57 (17)	14.28 (5)	8.57 (3)
Hatchability (n=43)	83.72 (36)	69.76 (30)	39.53 (17)	9.30 (4)	2.32 (1)
Total (n=99)	81.81 (81)	71.71 (71)	54.54 (54)	28.28 (28)	18.18 (18)

Table 3. *Relationship between O serogroups and production units*

Production Units	O Serogroups										
	O1 % (n)	O8 % (n)	O18 % (n)	O78 % (n)	O125 % (n)	O142 % (n)	O143 % (n)	O157 % (n)	O158 % (n)	O164 % (n)	O169 % (n)
Broiler (n =5)	-	-	-	40 (2)	20 (1)	-	-	-	20 (1)	-	20 (1)
Breeding (n =15)	6.6 (1)	-	13.3 (2)	60 (9)	13.3 (2)	-	6.6 (1)	-	-	-	-
Hatchability (n =10)	10 (1)	10 (1)	-	20 (2)	20 (2)	10 (1)	-	10 (1)	-	10 (1)	10 (1)
Total (n = 30)	6.6 (2)	3.3 (1)	6.6 (2)	43.3 (13)	16.6 (5)	3.3 (1)	3.3 (1)	3.3 (1)	3.3 (1)	3.3 (1)	6.6 (2)

Table 4. *Percentages and numbers of APEC strains in O78 (n=13) and O125 (n=5) displaying resistance to tested antibiotics*

O Serogroups	Antibiotics						
	Florfenicol % (n)	Oxytetracycline % (n)	Amoxicillin % (n)	Sulfamethoxazole/ Trimethoprim % (n)	Erythromycin % (n)	Enrofloxacin % (n)	Doxycycline % (n)
O78 (n = 13)	30.76 (4)	92.3 (12)	76.9 (10)	84.6 (11)	69.2 (9)	38.4 (5)	76.9 (10)
O125 (n = 5)	40 (2)	80 (4)	60 (3)	60 (3)	100 (5)	20 (1)	80 (4)

digestive system and these bacteria play an important role in the transfer of resistance genes and cause serious infections that affect the food chain [23,24]. For these reasons, since January 2006, legally importing feed additives containing antibiotics in Türkiye has not been allowed [25].

In this study, a high rate of antibiotic resistance was observed against antibiotics that are regularly used in treatment of poultry diseases in Türkiye. APEC strains regardless of breeding types of poultry demonstrated resistance to oxytetracycline and erythromycin at rate of 82.83%, amoxicillin with rate of 75.76%, doxycycline with rate of 67.68%, sulfamethoxazole/trimethoprim with rate of 59.69% and less resistance to enrofloxacin and florphenicol with rates of 38.38% and 33.33%, respectively. In our study, high tetracycline resistance is similar to previous studies [26] and supporting the findings of other studies conducted in other countries [3,11,13-16,27,28]. Jiang et al.[27] also reported that doxycycline is known as a type of tetracycline, resistance is higher than other antibiotics in China, but in our study tetracycline resistance follows the oxytetracycline. Gomis et al.[29] found as 61%, 65.2%, 65.2% resistance rates to erythromycin, tetracycline and doxycycline, respectively and reported that all isolates were susceptible to enrofloxacin. Al Agamy [30] reported that APEC strains were resistant to tetracycline and doxycycline at rates of 85% and 83%, respectively. Aggad et al.[31] found 45% enrofloxacin resistance and Li et al.[32] reported that resistance to florfenicol, sulfamethoxazole and trimethoprim, enrofloxacin 29%, 100% and 83%, respectively. Although different production units showed significant differences among antibiotic resistance profiles in our study, oxytetracycline resistance was still at the highest level. While the breeder and hatchery strains were significantly lower resistant against to enrofloxacin, sulfamethoxazole-trimethoprim and florfenicol; resistant strain rate was quite high in broiler. In particular, this situation showed that the antibiotics have not been used properly in broiler than other production types.

According to the results of multidrug resistance in APEC strains, 81.81% of APEC strains were found to be resistant to ≥3 antibiotics class, and 18.18% were resistant to all tested antibiotics. Several reports have concluded that MDR is more common than resistance to single antibiotic [33,34].

In the study, serotyping was performed on a total 30 MDR APEC strains and the majority of APEC serotypes obtained from all production units were identified as O78 as in other researches [11,13-16] and the second one is O125. Also, Dahshan et al.[35] reported that O125 serotype was among the most common serotypes in a study of stool and drinking water. Distribution of the remaining 12 strains were among O1, O8, O18, O142, O143, O157, O158, O164 and O169. This result is very important for better understanding the profile of APEC strains circulating nowadays in Türkiye, because no study was performed before on serotyping of APEC strains.

The highest antibiotic resistance in O78 and O125 serogroups were determined against oxytetracycline and erythromycin, respectively. The common APEC serotypes were determined as O78 (43.3%) and O125 (16.6%) and the others showed variability between 6.6% and 3.3%. Notable matters, according to these serotypes and resistance profiles were appeared in same integration, also hatchability. These findings are consistent with other reports where diseases caused by APEC strains in both parent flocks and their progeny and spread from breeders to hatcheries on the same farm via environmental contamination or vertical transmission [36-39]. On the other hand, Da Silveira et al.[40] and Da Silveira et al.[41] reported that opportunistic *E. coli* strains could be pathogenic for one day old chicks crossing the egg barriers during the egg laying process and also they could be potential pathogens via plasmids (transfer of virulence factors) after hatching.

As a result, biochemical and serological characterization and antibiotic sensitivity of APEC strains were determined in the integrated poultry production diagnosed with avian colibacillosis. The results obtained from this study suggest that transmission of APEC agents and also antibiotic resistance genes belong to these agents also from broods to broiler chickens could be possible. Thus, APEC strains should be subjected to the antimicrobial susceptibility test for appropriate antibiotic selection in the treatment of the disease.

Availability of Data and Materials

Data supporting the study findings are available from the corresponding author on reasonable request (N. Karacan Sever).

Ethical Approval

The data for this study were collected from 'dead animals or their tissue', so the study does not require any ethical approval.

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

NKS, OSY and MA planned and designed the study. NKS and OSY performed the experiments; NKS, OSY and MA contributed to the analysis and interpretation of data. NKS drafted the manuscript. All authors read and approved the final manuscript.

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RESEARCH ARTICLE

Serology-Based Approach in the Clinical Evaluation of Neonatal Viral Eye Diseases in Kittens: Calicivirus, Herpesvirus and Panleukopenia Virus

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Abstract: This study aimed to examine the distribution of feline calicivirus (FCV), feline herpesvirus (FHV), and feline panleukopenia virus (FPLV), which cause neonatal feline viral diseases in kittens aged one to three months, using a Dot-ELISA bases antibody test kit. The studied parameters included the animals' sex, clinical signs and the Dot-ELISA test kit values. Twenty kittens had eye lesions and 20 were without eye lesions. Basic ophthalmologic examinations were performed, including pupil, corneal, palpebral and menace response reflexes, direct ophthalmoscopy, and fluorescein staining. The study population consisted of 40 kittens (25 female and 15 male); 3 of them are British shorthaired and the rest is 37 tabby kittens. In half of the 20 kittens with eye lesions, the lesions were bilateral and the most common clinical lesions were conjunctivitis, mucopurulent discharge, and blepharospasm. Other notable clinical findings were iris staphyloma, corneal opacity, symblepharon, and panophthalmitis. A higher rate of seropositive results was determined against Calicivirus in kittens. The severity and appearance of the cases could vary depending on the virus accompanying the lesions. In conclusion, the Feline Calicivirus was the most frequently detected virus in 1 to 3-month-old kittens in this study and the clinical presentation may change according to the accompanying virus titers.

Keywords: *Dot-ELISA, Eye disease, Kitten, Newborn, Viral infection*

Yavru Kedilerde Neonatal Viral Göz Hastalıklarının Klinik Değerlendirmesinde Seroloji Temelli Yaklaşım: Calicivirus, Herpesvirus ve Panleukopenia Virus

Özet: Bu çalışmada, 1-3 aylık yavru kedilerde neonatal kedi viral hastalıklarına neden olan feline calicivirus (FCV), feline herpesvirus (FHV) ve feline panleukopenia virus (FPLV) dağılımlarının Dot-ELISA bazlı antikor testi kullanılarak incelenmesi amaçlanmıştır. İncelenen parametreler hayvanların cinsiyetini, klinik belirtilerini ve Dot-ELISA test kiti değerlerini içeriyordu. Yirmi yavru kedide göz lezyonu vardı ve 20'sinde göz lezyonu yoktu. Pupil, kornea, palpebral ve tehdit tepki refleksleri, direkt oftalmoskopi ve floresein boyama dahil olmak üzere temel oftalmolojik muayeneler yapıldı.Çalışma popülasyonu 40 yavru kediden (25 dişi ve 15 erkek) oluştu. Bunlardan 3'ü kısa tüylü İngiliz, geri kalanı 37 tekir kedi yavrusuydu. Göz lezyonu olan 20 yavru kedinin yarısında lezyonlar bilateraldi ve en sık görülen klinik lezyonlar konjonktivit, mukopurulent akıntı ve blefarospazmdı. Diğer dikkate değer klinik bulgular ise iris stafilomu, korneal opasite, simblefaron ve panoftalmitisdi. Yavru kedilerde Calicivirus'a karşı daha yüksek oranda seropozitif sonuç belirlendi. Olguların şiddeti ve görünümü lezyonlara eşlik eden virüse göre değişebilmektedir. Sonuç olarak, bu çalışmada 1-3 aylık yavru kedilerde Feline Calicivirus en sık saptanan virüs olmuştur ve klinik tablo eşlik eden virüs titrelerine göre değişebilmektedir.

Anahtar sözcükler: *Dot-ELISA, Göz hastalığı, Yavru kedi, Yenidoğan, Viral enfeksiyon*

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Introduction

In kittens, eyes open at about 10-14 days old. Premature opening of the eyes rarely occurs in the kitten, but it can cause corneal drying, keratitis, corneal ulcers and conjunctivitis. More common is the delayed opening of the eyelids which is referred to as ankyloblepharon. Ankyloblepharon is usually caused by mucus accumulation and sometimes due to infection *(ophthalmia neonatorum)* [1]. The main viral infectious agents that cause neonatal eye infections in cats include feline herpesvirus (FHV), feline calicivirus (FCV), and less commonly feline panleukopenia virus (FPLV) [2,3].

FHV is one of the main causes of conjunctivitis in kittens. Primary symptoms seen in kittens include fever, sneezing or coughing, rhinitis, nasal discharge, and conjunctivitis. Transmission of FHV-induced neonatal ophthalmia can occur from mother to kitten or shortly after birth. It occurs in kittens, especially in the 8-12 week age range when maternal antibodies are reduced [4]. The cytopathic virus causes severe ulceration of mucosal surfaces and serosanguineous ocular or nasal discharge. Affecting the substantia propria of the conjunctiva and the stroma of the cornea, symblepharon or adhesion may occur between these tissues. In a viral infection, large amounts of inflammatory debris may accumulate in the conjunctival sac *(conjunctivitis neonatorum)* [2].

Feline calicivirus (FCV) is primarily a viral pathogen of the cat's respiratory tract and can also cause oral ulcers and polyarthritis. FCV can cause severe conjunctivitis in cats but is a less common cause of conjunctivitis than FHV [4]. In a study in cats with ocular surface disease and upper respiratory tract infection, moderate to severe conjunctivitis was seen with conjunctival epithelial erosions in FCV-positive cats [5].

Feline panleukopenia virus (FPLV), a feline Parvoviral pathogen, infects cats mostly up to 1 year of age through intrauterine transmission. Kittens born to infected cats have tremors, ataxia and hypermetric gait, and cerebellar hypoplasia. Ocular symptoms have been reported as retinal dysplasia and degeneration. Conjunctivitis has been reported in two experimentally infected kittens [3,6].

Today, several laboratory methods are used to detect viral antigens or antibodies. Among these, serological methods based on the detection of specific antibodies, which are the host's response to the virus, are the most widely used [3]. Dot-ELISA-based methods are one of the rapid ELISA tests that can be a good option according to previous studies [7,8].

The presented study aimed to detect FCV, FHV and FPLV in kittens presented to our clinic with neonatal ophthalmia symptoms, using a rapid Dot-ELISA test kit and to evaluate them together with clinical symptoms.

Material and Methods

Ethical Statement

The use of the data constituting the study was approved by the Local Ethics Committee (Approval no: 2021/138).

Clinical Examination

The data were obtained from forty kittens divided into 2 groups, 20 patients with various eye complaints and 20 healthy kittens who came for their first examination and vaccinations to the Animal Hospital. All cases underwent basic ophthalmologic examinations, including pupil, corneal, palpebral and menace response reflexes, direct ophthalmoscopy, and fluorescein staining. If deemed necessary in a condition such as corneal edema, hyphema or total symblepharon, vitreous or retinal abnormalities, complementary techniques were performed including ocular ultrasonography and binocular ophthalmoscope.

Diagnostic Analysis

Blood samples were collected from kittens into serum separator tubes (SST). Sera were separated at 1500 g x 10 min after complete clot formation. The ImmunoComb® Feline VacciCheck Antibody Test Kit (Biogal, Kibbutz Galed, Israel), the method principle is Dot-ELISA, is designed to detect serum IgG antibody titers against FCV, FHV and FPLV in kittens. The assay was performed following the manufacturer's instructions. The final step was the development of a grey color tone following sequential washing and the binding of an enzyme-linked anti-cat immunoglobulin G antibody. Positive results were defined as color tones that were equivalent to or darker than the positive control, while negative results were defined as color tones that were paler than the positive control. Besides, the degree of color tones was also evaluated and semi-quantitative titering was done as follows: S0: Negative, S1 or less: Negative; S2: Weak positive, S3 and S4: Positive, S5 or more: Strong positive.

Statistical Analysis

Collected data were organized in Microsoft Office Excel 2010® and the statistical analyses were performed using descriptive statistics with the IBM SPSS Statistics 21® software. The titers of the groups with and without eye lesions were evaluated according to the Wilcoxon test, and the titers between the groups were evaluated with the Mann-Whitney U tests.

Results

The study was performed on 40 kittens, aged 1-3 months (mean±SD 1.9 months). The mean age of 20 kittens with eye lesions was 1.7 months, the mean age of 20 kittens without lesions was 1.9 months, and the average age of all kittens was 1.8 months. Of these 40 kittens, 25 were female and 15 were male.

The most detected virus in this study was FCV. While S3-S4 and above positive results were found in 19 of the kittens with eye lesions, S2-poor positive results were detected in only one kitten *(Table 1)*.

Table 1. *FCV, FHV and FPLV results of each individual kitten*

Kitten Number	Group	FCV	FHV	FPLV
1	Healthy Kitten	S4	S1	S1
2	Kitten with Eye Lesions	S4	S0	S0
3	Kitten with Eye Lesions	S5	S0	S0
4	Kitten with Eye Lesions	S6	S0	S0
5	Healthy Kitten	S4	S2	S2
6	Healthy Kitten	S6	S1	S2
7	Kitten with Eye Lesions	S4	S2	S2
8	Kitten with Eye Lesions	S4	S0	S6
9	Healthy Kitten	S2	S0	S0
10	Healthy Kitten	S5	S0	S2
11	Healthy Kitten	S3	S0	S2
12	Kitten with Eye Lesions	S6	S0	S5
13	Healthy Kitten	S4	S0	S2
14	Kitten with Eye Lesions	S4	S0	S3
15	Healthy Kitten	S5	S0	S3
16	Kitten with Eye Lesions	S6	S2	S0
17	Healthy Kitten	S2	S0	S5
18	Healthy Kitten	S0	S4	S0
19	Kitten with Eye Lesions	S4	S0	S0
20	Healthy Kitten	S0	S4	S0
21	Kitten with Eye Lesions	S4	S2	S2
22	Kitten with Eye Lesions	S4	S0	S1
23	Kitten with Eye Lesions	S4	S0	S2
24	Kitten with Eye Lesions	S4	S0	S3
25	Healthy Kitten	S4	S1	S0
26	Kitten with Eye Lesions	S2	S5	S1
27	Healthy Kitten	S6	S1	S1
28	Healthy Kitten	S4	S1	S0
29	Kitten with Eye Lesions	S4	S1	S1
30	Kitten with Eye Lesions	S4	S0	S0
31	Kitten with Eye Lesions	S4	S0	S1
32	Healthy Kitten	S1	S0	S0
33	Kitten with Eye Lesions	S4	S0	S3
34	Healthy Kitten	S1	S0	S0
35	Healthy Kitten	S3	S2	S1
36	Kitten with Eye Lesions	S4	S2	S2
37	Kitten with Eye Lesions	S3	S4	S2
38	Healthy Kitten	S2	S1	S1
39	Healthy Kitten	S0	S0	S2
40	Healthy Kitten	S0	S0	S1

In 20 kittens without eye lesions, also FCV was the most detected virus S3-S4 and above positive results in 11, weakly positive results in 3 kittens, while 6 kittens had S1 or less negative results. In kittens without eye lesions, FPLV was found to have positive results of S3-S4 and above in 2 kittens, S2 weakly positive results in 6 kittens, S1 and six negative results in 12 kittens. In FHV, 2 kittens had S3-S4 and above results and 2 with S2 weakly positive results whereas 16 kittens were determined to have S1 and six negative results. However, no significant differences were determined in the intragroup virus titers assessment or the intergroup virus titers assessment of kittens with and without eye lesions *(Fig. 1)*.

Fifteen out of 20 cases with ocular complaints had complaints of not opening the eyelids or being closed due to the discharge. On the other hand, general clinical examination findings included conjunctivitis in 13 kittens, mucopurulent discharge in 7 kittens, corneal opacity in 7 kittens, iris staphyloma in 3 kittens, serous discharge in 2 kittens, panophthalmitis in 2 kittens, keratitis in 2 kittens, corneal edema in 1 kitten and microphthalmia was detected in 1 kitten. Here, conjunctivitis was the most detected lesion with an incidence of 13 kittens *(Fig. 2)*. In 5 kittens, conjunctivitis was observed to be bilateral. In 12 kittens, FCV was the most detected virus, with a positive result of S3-S4 and above. FHV S4 and S5 positive results were determined in 2 cases. FCV was detected in only four cases, 3 of these kittens were siblings *(Fig. 3)*. Again, in one of the conjunctivitis cases, FPLV S5 positive result was obtained in the kitten.

Mucopurulent eye discharge seen in 10 kittens was the second most common clinical sign *(Fig. 4)*. While FCV, S4 and above were positive in all kittens, only FCV was detected in 4 of them. FPLV S3 positive results were determined in 2 of the kittens with conjunctivitis, while S2 and below results were obtained in the other two. On the other hand, serous eye discharge was observed in only 2 kittens and these kittens had FHV-positive results of S4 and above *(Fig. 5)*.

The other most detected lesion was corneal opacity in 7 kittens *(Fig. 2)*. While PCV was positive in these cats, S3-S4 and above in 5 kittens, only FCV S4 was positive in one kitten. Corneal opacity was observed bilaterally in 2 kittens, and FCV S4 was positive in these cats. The other virus-causing corneal opacity was FHV with S4 and S5 positive results *(Fig. 1)*.

Iris staphyloma was the other clinical sign, with FCV-positive results of S4 and above in 3 kittens. In one of these kittens, the case was observed bilaterally with an S6 positive result. The clinical findings observed in kittens with FCV-positive results of S4 and above were symblepharon in 2 kittens, panophthalmitis in 2 kittens, keratitis in 2 kittens,

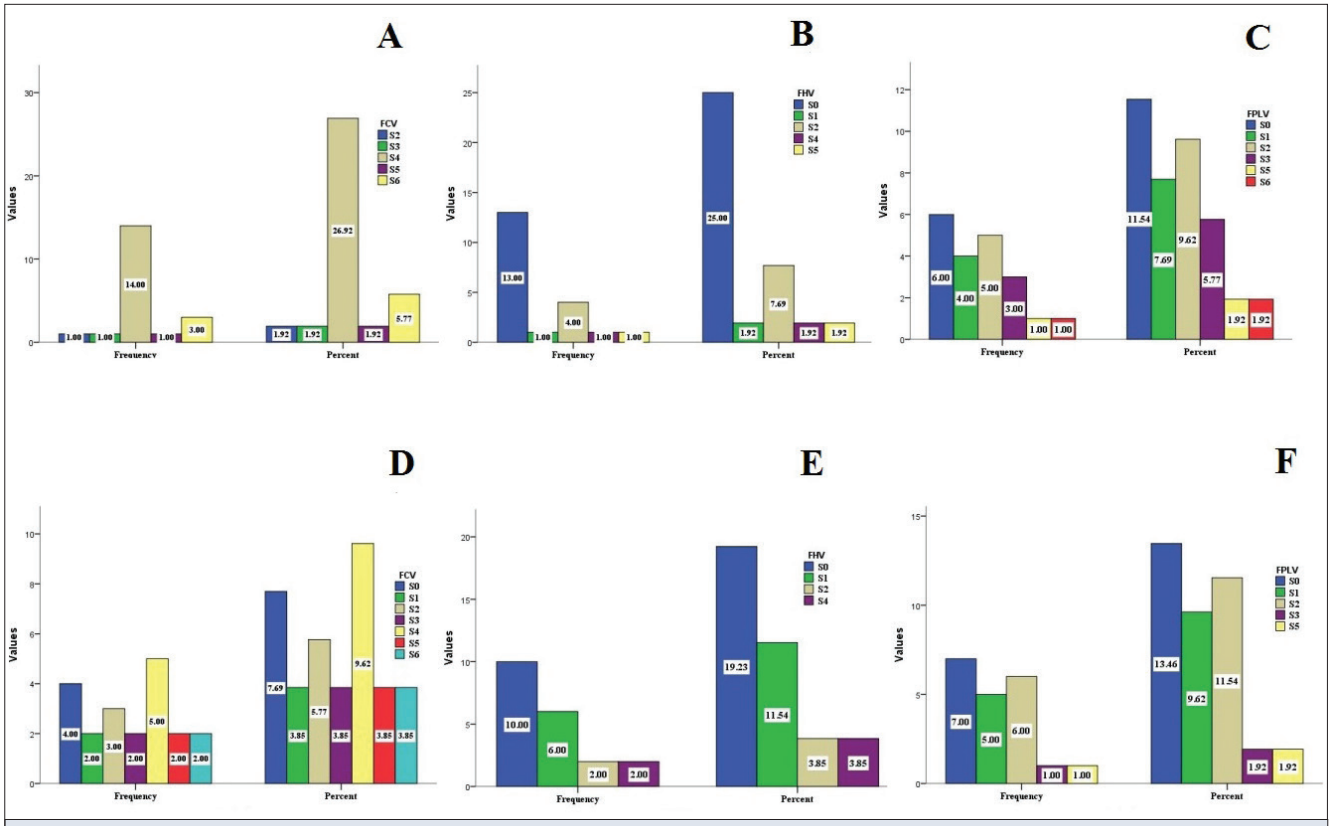

Fig 1. Titers distribution of FCV (A), FHV (B), FPLV (C) in kittens with eye lesions and FCV (D), FHV (E), FPLV (F) in kittens without lesions. FCV was the most detected virus with S4 positive titer in both kittens with eye lesions (14/26.92%) and kittens without lesions (5/9.62%)

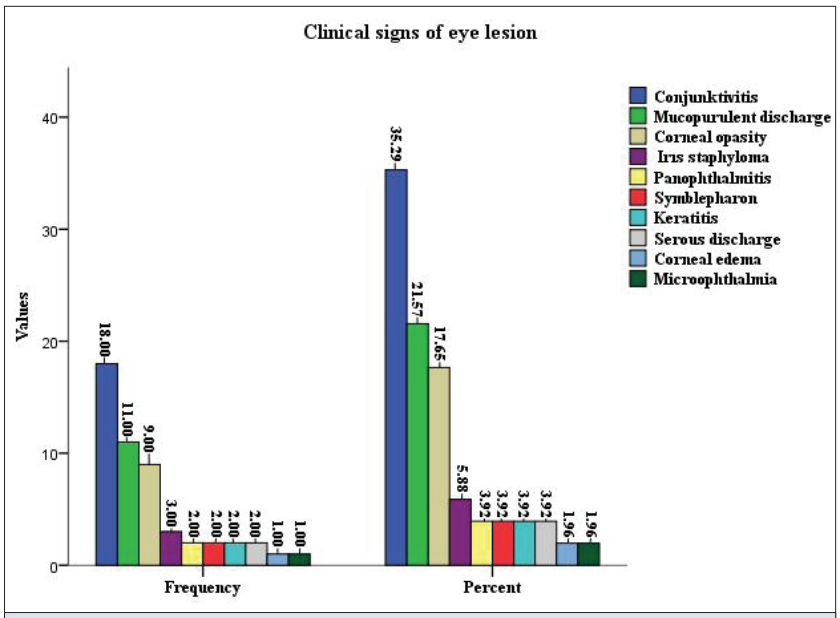

Fig 2. Percentage and frequency of clinical signs. The most common clinical symptom detected in kittens with eye lesions was conjunctivitis (18/35.29%), and the lesion was observed unilaterally in 13 kittens and bilaterally in 5 kittens

corneal edema in 1 kitten, and microphthalmia caused by corneal perforation in 1 kitten *(Fig. 2)*. In one of the keratitis and panophthalmitis cases, FPLV accompanied FCV with a positive result of S5 and above.

The bilateral nasal discharge associated with respiratory system lesions was detected in 15 of the kittens with eye lesions, except for one kitten with unilateral discharge. Fluorescein staining positive results were obtained from

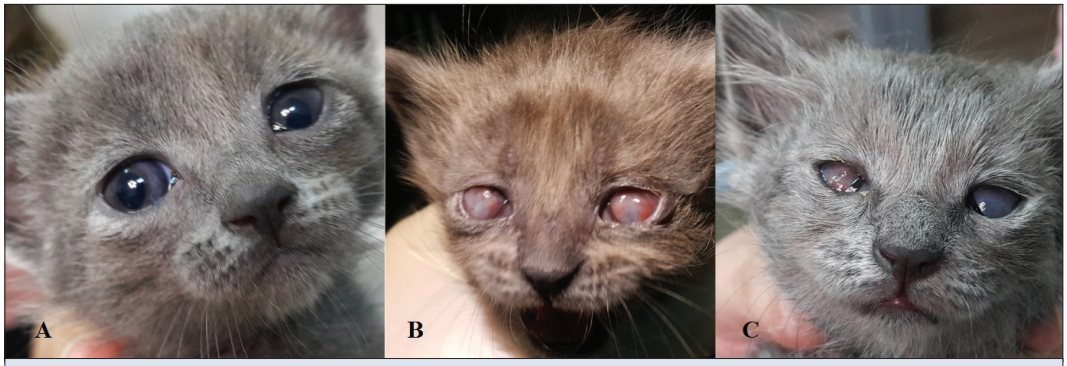

Fig 3. Image of variable lesions, (slight corneal edema (A), severe bilateral keratitis, corneal vascularization and symblepharon (B), mild corneal edema in left eye and partial symblepharon, keratitis and iris stahyloma in right eye (C)) in 3 FCV high positive sibling kittens

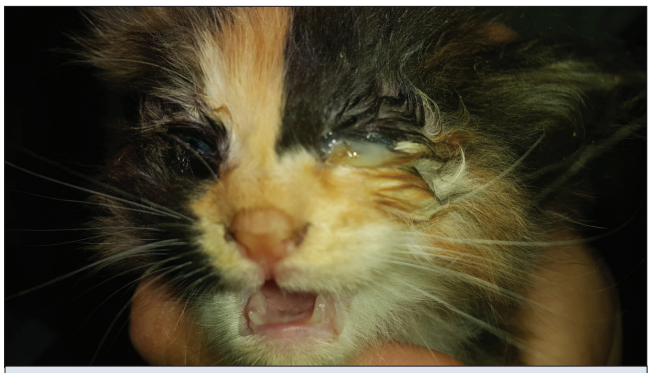

Fig 4. Bilateral ankyloblepheron and mucopurulent ocular discharge in a kitten with FCV

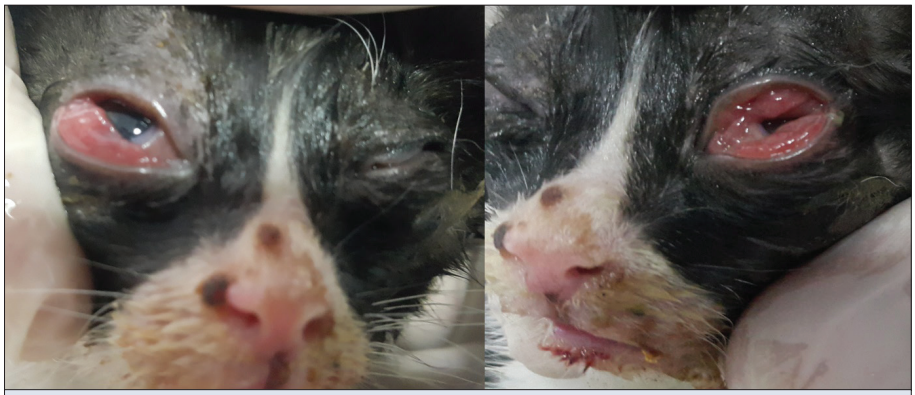

Fig 5. Bilateral blepharitis, serous eye discharge, severe conjunctival swelling (edema, chemosis), nictitating membrane protrusion and bilateral nasal discharge, in an FHV high-positive kitten

only 2 cats. With FCV S4 strong positive result, moderate gingivitis was detected in 5 eyes lesioned kittens, 4 of which were siblings.

Discussion

FHV and FCV are among the most common causes of both upper respiratory tract infections and eye diseases in cats, and FPLV is less effective [9,10]. Particularly, agents transmitted during the intrauterine or postnatal period cause neonatal eye diseases in kittens [3]. In recent years, many epidemiological and prevalence studies have been carried out on these ocular disease agents [3,11-16]. In this study, we aimed to evaluate the serology and clinical findings of FHV, FCV and FPLV together and to interpret how the results to be obtained can be used in the evaluation of clinical cases.

Although the Calicivirus is a well-known viral agent that causes upper respiratory tract infection, gingivostomatitis,

and lameness in cats all over the world, it is also an important ocular disease agent in this species [5,17]. In this study, FCV S3 and above positive titer results were determined in kittens with eye lesions, except for only 1 kitten, in which FCV S2 was weakly positive and FHV S5 strongly positive results were detected. Besides, FCV S4 strongly positive, FHV and FPVL S0 negative titer results were determined in 5 of 20 kittens, while FHV and FPVL S2 weakly positive or below negative titer results were determined in 7 kittens. However, S4's strong positive and above result of FHV in 2 kittens and S3 strong positive result and above FPLV in 4 kittens were determined. As also stated in studies [5,17] above, FCV plays an active and dominant role as a viral eye disease agent, despite local differences.

Kittens receive varying levels of maternal antibodies via colostrum in the first hours after birth, and the amount of antibodies consumed with colostrum varies with the number of kittens, frequency of lactation, and antibody titer contained [18]. Besides, individual antibody levels can vary between siblings, and while some kittens will show no clinical signs and infection, others may develop an infection, which may even be fatal. High positive titers in kittens with clinical signs may indicate an active response, while low positive titers from passively acquired antibodies may reflect maternal antibodies and decrease over time [19]. Most of the kittens included in the study were street-owned or strayed kittens, so the level of maternal antibodies is unknown. It is thought that in kittens without clinical signs, the lack of vaccination, and the high positive titers may be due to the individual immune difference in the maternal antibody or another active asymptomatic infection. Instead, low positive or negative antibody titers in kittens with and without clinical signs may be associated with decreasing maternal antibody levels over time or not being exposed to the viral agent.

The authors state that other disease factors may play a role as cofactors in the changing clinical presentation [5,20]. Conjunctivitis (edema and hyperemia), corneal opacity, iris staphyloma, panophthalmitis, symblepharon, keratitis, corneal edema and microphthalmia were most common in the study *(Fig. 2)*. Except for two kittens with only serous discharge, most kittens had mucopurulent discharge and FCV was detected in each kitten. The determination of S3-S4 as the most detected value in the FCV titer shows that the calicivirus is dominant in the clinical appearance. In this study, in addition to the common symptoms seen in 4 sibling cats, changes in the severity of the clinical appearance, both eye and systemic, were determined. Likewise, in terms of systemic effects, nasal discharge and mouth lesions also differ between 4 siblings. While S2 low positive titers of FHV and FPLV were detected in one of the sibling kittens, FVH S0 and FPLV S1-2 and 3 titers were detected in the other 3 sibling kittens. Considering the titers in these patients, it was thought that variable titer co-factor viruses might be effective in varying clinical manifestations.

In this study, the antibody titers of FCV, FHV and FPLV in neonatal cats were determined with the Dot-ELISA kit and the clinical appearance and prevalence of eye lesions were evaluated. According to reports, FHV is the most prevalent etiology of ocular lesions in kittens [3,20-22]. In the presented study, however, the most common antibodies against FCV were detected. As stated before other factors may have effects on the clinical appearance of feline viral ocular diseases [5,20]. To determine and eliminate these factors a more comprehensive study needs to be performed on this subject. Although the Dot-ELISA test kit data gave faster results in detecting the disease compared to other test methods, i.e. Virus Neutralization or Hemagglutination Inhibition [7] and confirmed the clinical appearance, Our findings show that the calicivirus may cause neonatal ophthalmia more commonly than the herpes virus, and clinical examination findings should be more important and prioritized for clinicians in the evaluation and treatment of neonatal ophthalmic patients.

Availability of Data and Materials

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

Financial Support

This research did not receive any financial support.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Statement

The use of the data constituting the study was approved by the Local Ethics Committee (Approval no: 2021/138).

Author Contributions

ÇG: conceptualization, methodology, investigation, writing-original draft. SS: conceptualization, methodology, writing-review and editing. FEÖ: methodology, supervision, writing-review and editing.

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Case Report

Assessment of Ocular Lesions in a Persian Cat Concurrently Infected with *Chlamydia felis*, Herpesvirus and Coronavirus

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Abstract: In this case report, *Chlamydia felis*, herpesvirus and coronavirus correlations were revealed in a Persian cat with some complaints related to the eye. Upon ophthalmological examination, there was a dark brown lesion on the right corneal surface, and some clinical signs observed in the left eye resembling limbal insufficiency. As a result of ELISA test in blood, the FCoV and chlamydia antibody titers were positive at the S4 level. Chlamydia infection was confirmed by real-time PCR analysis in blood and conjunctival samples. Tear samples were found positive upon the herpesvirus antigen test. The lesions regressed after treatment, and the test results for chlamydia were negative following the 7-week treatment period.

Keywords: *Feline chlamydia, Conjunctivitis, Limbus, Sequester*

Chlamydia felis, Herpesvirus ve Koronavirus Enfeksiyonunun Eşzamanlı Seyrettiği Bir İran Kedisinde Oküler Lezyonların Değerlendirilmesi

Özet: Bu olgu sunumunda göze ilişkin şikayetleri olan bir İran kedisinde *Chlamydia felis*, herpesvirus ve koronavirüs korelasyonu ortaya konuldu. Oftalmolojik muayenede, sağ gözün kornea merkezinde koyu kahverengi bir lezyon, sol gözde limbal yetmezlik benzeri klinik bulguların şekillenmiş olduğu görüldü. Kan ELISA sonucuna göre FCoV ve klamidya antikor titresi S4 seviyesinde pozitif çıktı. Kan ve konjunktival svap örneklerinde real-time PCR ile klamidya enfeksiyonu doğrulandı. Herpesvirüs antijen testi sonucunda gözyaşı örnekleri pozitif bulundu. Uygulanan 7 haftalık tedavi sonrasında lezyonların gerilediği ve klamidya test sonucunun negatife döndüğü görüldü.

Anahtar sözcükler: *Kedi klamidyası, Konjunktivitis, Limbus, Sekester*

Introduction

Chlamydia felis is an intracellular bacterium with zoonotic and immunosuppressive potential that affects various species. It is associated with severe respiratory system infections and has a special affinity to the conjunctiva in felines [1]. Chlamydia produces the foregoing effect mostly by the contribution of herpesvirus. It was reported that herpesvirus not only caused conjunctivitis, but also severe keratitis. The virus especially invades the corneal epithelial cells, causing severe cytolysis followed by persistent corneal ulcers. Relevant studies suggested that high concentrations of herpesvirus were present in corneal necrosis [2].

Feline coronavirus (FCoV) is a highly contagious viral agent that causes infection in domestic and wild cats worldwide. Approximately 20-60% of domestic cats were positive for FCoV, while that rate reached up to 90% in animal shelters or multi-cat houses. FCoV is transmitted via faecal-oral route binds to aminopeptidase N (APN) receptors located in the intestinal mucosa, infecting enterocytes and then spreading from the intestine through monocyte-associated viremia. It can also replicate in

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the monocytes/macrophages of healthy cats [3,4] and is transported to target tissues, including the peritoneum, pleura and kidneys. It can also cause uveitis after the deterioration of the blood-eye barrier [5,6].

In this case report, concurrent *C. felis*, herpes, coronavirus ocular infection in a cat living alone in a home environment was pointed out, and their correlation was revealed.

Case History

A one-year-old, male, Persian cat was brought to the ophthalmology clinic with eye complaints. An "informed consent form", involving the whole process was obtained in the relevant case. According to the anamnesis, respiratory and ocular symptoms (i.e. severe eye itching, blepharospasm, conjunctivitis, and dark brown discharge) started subsequent to the vaccination at age of 3 months and thereafter recovered upon treatment. Similar symptoms reoccurred in the right eye at age of 9 months. In addition, there was a dark brown lesion, which was diagnosed as sequestrate approximately 2-3 mm in diameter, in the centre of the right cornea *(Fig. 1)*. In addition, conjunctivalization resembling limbal insufficiency findings were observed on cornea of the left eye *(Fig. 2)*. Slit lamp biomicroscopy before and after 2% sodium fluorescein dye was used to diagnose limbal defects. Findings of slit lamp examination under white light included corneal conjunctivalization and superficial vascularization. Examination under cobalt blue illumination manifested the presence of abnormal cells on the corneal surface.

Since the dryness of the corneal surface was noted in the examination, hyaluronic acid and artificial tears were applied to each eye. By the 10th day of treatment, brown eye discharge, blepharospasm and conjunctivitis were decreased. Fluorescein test indicated the presence of an ulcerative area around the brown corneal lesion. Tobramycin and acetylcysteine eye drops were added to the treatment. Viral and bacterial tests of the blood and tear samples were performed due to the chronicity and persistent recurrence of the condition. As a result of the ELISA test in blood, the FCoV and chlamydia antibody titers were positive at S4 level. Chlamydia infection was confirmed by positive real-time PCR analysis in blood and conjunctival samples. Tear samples tested positive upon the herpesvirus antigen test.

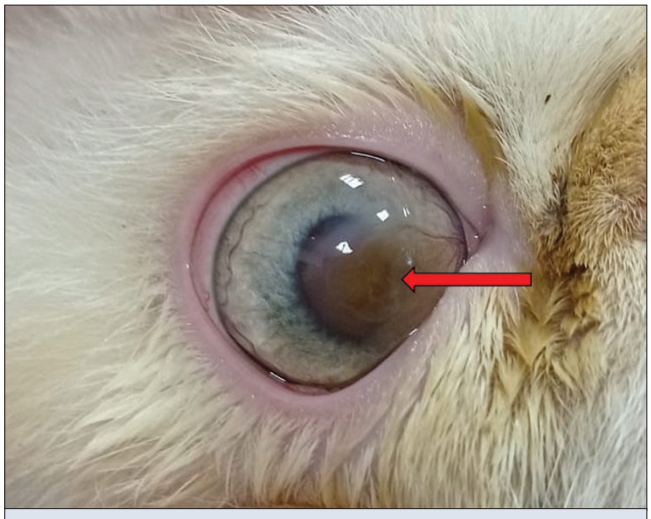

Fig 1. Corneal sequestra *(arrow)* and superficial vascularization around the lesion in the center of the right cornea

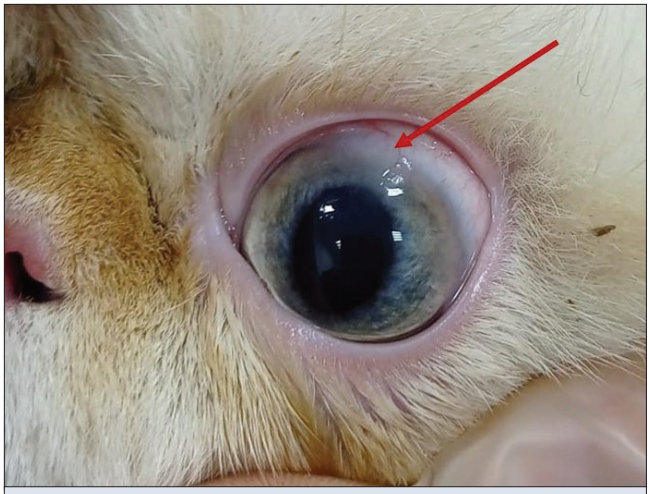

Fig 2. Conjunctivalization of the left cornea *(arrow)*

Following the test results, doxycycline was prescribed (10 mg/kg, po) for a 21-day therapy. A marked demarcation area around the corneal lesion was revealed and the symptoms have regressed. The animal underwent a real-time PCR test after 21 days with an aim to follow up the chlamydia infection and treatment, and the test result was still positive. Doxycycline treatment was continued for another 4 weeks, then the animal tested as chlamydia negative. Concurrently, the FCoV antibody test indicated a decrease in antibody titer to S1 level after the chlamydia treatment. Food supplements (Vetomune, Vet Expert) intended for viral diseases and changes in home layout to minimize stress were proposed. While no special treatment was preferred for herpesvirus, it was thought that the aforementioned applications would provide viral control.

Discussion

Chlamydia felis has a relatively higher host specificity in comparison with other *Chlamydiaceae* species. Although it was reported as cat-specific for many years, now it has been also detected in the canines as well as in a Eurasian lynx with conjunctivitis [7]. *C. felis* can cause keratoconjunctivitis or follicular conjunctivitis in males with the potential risk for people with close contact with infected animals [8].

Chlamydia infection is highly prevalent among the felines living in closed and crowded spaces under neglected conditions, among pedigreed cats subject to continuous reproduction and among stray cats with conjunctivitis [9,10]. As the present case was an indoor cat with no connection with exteriors, living alone in a house under favorable care conditions, it may have inherited *C. felis* from the mother. Such that the occurrence of the first ophthalmological symptoms upon administration of the combination vaccine may be indicative of the fact that *C. felis* infection became opportunistic as a result of the alteration in the immune system due to vaccination.

Serological research demonstrated that 10% or above of the pets without vaccination also had antibodies. Cats aging less than 9 months is the most affected age group as regards to *C. felis*. [10]. The infection history of the presented case is consistent with those reports.

There are many types of chlamydia, which primarily target mucosal tissues. *C. felis* is the most common among these species, and its primary target is conjunctiva. Severe ocular discharge, hyperemia in the third eyelid, chemosis, and blepharospasm are typical in cases of *C. felis* ocular infection. As a result of the fact that *C. felis* did not show affinity to the cornea, the direct corneal symptoms, especially including keratitis and corneal ulcer, were not associated with *C. felis* infections [1-7]. Relevant studies did not suggest conclusive evidence that *C. felis* was a primary pathogen or a source of secondary infection in cats with corneal sequestrate [11]. The PCR examinations on sequestrate samples and conjunctival cells did not provide any evidence of bacterial antigen, while viral DNAs and especially the herpesvirus DNA were abundant [12]. In the present case, there were remarkable lesions, which could be associated with herpesvirus in the cornea of the right eye.

There are a number of feline upper respiratory tract infections with ocular manifestations. Therefore, the viral and bacterial diseases should always be included in the differential diagnosis list in patients presented with the above-mentioned symptoms. Laboratory tests are essential for the detection of infectious agents during the differential diagnosis. The affinity of the pathogens to the ocular and adnexal tissues allows for the chosen tests to be diagnostically decisive. PCR techniques are the method of choice for the diagnosis of many infections since they are more sensitive compared to the isolation techniques [10].

Feline chlamydia infection can well respond to antibiotic treatment. Doxycycline is the treatment of choice for *C. felis* infection [13] and is most frequently used at a daily dose of 10 mg/kg orally, but 5 mg/kg oral twice daily can be used in cases of vomiting with single-day dosing. Relevant studies suggested that treatment should be continued for a 4-week time period to ensure the elimination of targeted organism. It was recommended to continue the treatment for another two weeks upon improvement of the clinical symptoms as recurrence might occur occasionally after early discontinuation of treatment [14]. In the present case, while chlamydia was totally eliminated the level of coronavirus antibody also decreased (S1), which indicates that treatment for a single factor in concurrent infections may also be efficient on other factors.

In conclusion, *C. felis*, herpesvirus, and coronavirus infections are predominantly seen in poor care conditions, especially in multi-cat environments, and therefore the present case is remarkable for detecting *C. felis*, herpesvirus, and coronavirus infections in a fully vaccinated cat under good care conditions, living as a single individual at home. It was suggested that while ophthalmological symptoms were associated with active concurrent *C. felis*, herpesvirus and coronavirus infections, coronavirus carriage might also have a suppressive effect on immunity, increasing the resistance of *C. felis* and herpesvirus to treatment.

Availability of Data and Materials

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

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Competing Interest

The author declared that there is no conflict of interest.

Author's Contributions

The authors have equally contributed to the preparation of this manuscript

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ETHICAL PRINCIPLES AND PUBLICATION POLICY

Kafkas Universitesi Veteriner Fakultesi Dergisi follows and implements internationally accepted ethical standards to provide the necessary support to original scientific ideas and to publish high quality, reliable scientific articles in this direction. The journal's **publication policy** and **ethical principles** include the ethical standards of conduct that should be followed by author(s), journal editor(s), associate editors, subject editors, reviewers, and publishers who are the participants of this action.

The ethical statement of Kafkas Universitesi Veteriner Fakultesi Dergisi is based on the principles indicated in the "COPE Code of Conduct and Best Practice Guidelines for Journal Editors" *(http://publicationethics.org/files/Code_of_conduct_for_journal_editors_Mar11.pdf)* and "COPE Best Practice Guidelines for Journal Editors" *(http://publicationethics.org/files/u2/Best_Practice.pdf)*.

GENERAL ETHICAL PRINCIPLES

• Objectivity and Independence

Editor-in-chief, editors, associate editors, and referees conduct the evaluation process of the manuscript sent to the journal objectively and in coordination within the framework of ethical principles. Editorial decisions are independent, and internal or external factors cannot influence these decisions. In accordance with the principle of impartiality, academics working in our institution are not deemed eligible to work as a section editor in Kafkas Universitesi Veteriner Fakultesi Dergisi, in order not to be effective in the evaluation of articles due to conflict of interest.

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The content of the articles and the personal information of the authors such as name, e-mail address, and telephone numbers that are sent to Kafkas Universitesi Veteriner Fakultesi Dergisi are used only for the scientific purposes of the journal and not for other purposes, and cannot be shared with third parties. Article evaluation processes are also carried out confidentially.

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The corresponding author of the article should declare the contributions of the authors to the work under the title of "Author contributions". The corresponding author is primarily responsible for the problems that may arise in this regard.

In multidisciplinary studies, 2 authors who are from different disciplines can be "equivalent first name authors" and up to most 3 authors who are also from different disciplines can be "equivalent second name authors".

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The authors should declare that the article they presented contained the original research results, that the study data were analyzed correctly, and that they were prepared for publication using adequate and appropriate references, in the "cover letter" section of the on-line system at the submission stage. Using expressions such as "it is the first study done", "there has been no previous study on this subject" and "there is a limited number of studies" to add originality and importance to the article is not acceptable and may cause prevention of the scientific evaluation of the article by the editor.

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Some authors may tend to divide study data into two or more articles and publish the results in different journals also having different authors names and orders. In principal, Kafkas Universitesi Veteriner Fakultesi Dergisi is against multi-part publication. When necessary, the ethical committee approval information of the study, project information, congress presentations, etc. are checked and such situations that will create an ethical problem are identified and reported to the authors.

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If any ethical problem is detected about the article that cannot be compensated and cannot be eliminated with erratum after the article is published, the editor-in-chief and associate editors prepare a justification about the article and apply the retraction procedure to the article. The text file on the web page of a retracted article is blocked and the reason for retraction is added to the system as a file, ensuring that it is constantly in the archive.

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ARTICLE EVALUATION AND PUBLICATION PROCESS

• Initial Evaluation Process

Articles submitted to Kafkas Universitesi Veteriner Fakultesi Dergisi are primarily evaluated by the editors and associate editors. At this stage, articles not having suitable scope and aims, with low original research value, containing scientific and ethically important errors, having low potential to contribute to science and the journal, and having poor language and narration are rejected by the editor without peer-review process. Initial evaluation process takes up to most 2 weeks.

• Preliminary Evaluation Process

Articles that are deemed appropriate for editorial evaluation are sent to the subject editor related to the category of articles to be examined in terms of scientific competence and to the statistics editor for evaluation in terms of statistical methods. The suject editors examine the article in all aspects and report their decisions (rejection, revision or peer-review) to the chief editor. This stage takes about 1 month.

• **Peer-review Process**

Peer-review process is applied to the articles that have completed preliminary evaluation process. Suggestions of subject editors are primarily considered in referee assignment. In addition, reviews can be requested from the referees registered in the journal's referee pool. At least 2 referees are assigned for peer-review. Opinion of more referees can be required depending on the evaluation process. At this stage, referees send their decision (reject, revision or accept) about the article to the editor-in-chief. If the rejection decision given by a referee reflects sufficient examination and evidence-based negativities or ethical problems about the scientific content and accuracy of the article, this decision is checked by the editor-in-chief and associate editors and submitted to the authors regardless of the other referees' decisions. The time given to referees to evaluate an article is ~4 weeks.

• **Publication Process of an Article**

Total evaluation period of an article, which is completed in the peer-review phase after completing the initial and preliminary evaluation process, takes 4-6 months. The articles that have completed the subject editorial and peer-review evaluation stages and accepted by the editorial are sent to the corresponding author for final checks and necessary final additions. After the acceptance, the article designed in the publication format of the journal is given an DOI number and published immediately on the Article in Press page. When it is time to publish the periodic edition of the journal, a selection is made from the articles kept on the Article in Press page, taking into account the submission date. The time it takes for the article to be published by taking the page number is 6-12 months.

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The publisher (Dean of the Faculty of Veterinary Medicine of Kafkas University) contributes to the execution of the journal's routine processes such as printing, archiving, and mailing, in line with requests from the editor.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and assistants in the article evaluation process and decisions.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and associate editors in the article evaluation process and decisions.

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Kafkas Universitesi Veteriner Fakultesi Dergisi follows internationally accepted principles and criteria and takes the necessary decisions to apply in the journal.

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The editor-in-chief/editors/associate editors make every effort to ensure that the journal's subject editors and referee pool have international qualifications. Likewise, it makes the necessary attempts to strengthen the author's profile.

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The editor-in-chief conducts the evaluation/revision process between the authors and subject editors and referees, and ensures that it is completed within the prescribed time.

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The editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi and the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) keep all the articles (electronic and printed) published in the journal in their archives. All articles and their attachment files sent to the journal are kept securely in the archive. In light of the technological developments, the editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi regularly performs electronic processes for the development and updating of materials in digital environment and

presents them to its readers on condition of keeping in safe the original documents and information regarding the articles.

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Subject editors do reviews and evaluations in accordance with the main publication goals and policies of the journal and in line with the criteria that will contribute to the development of the journal.

Author information is kept confidential in articles sent to the subject editor for preliminary evaluation by the editor.

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If a referee has an interest relationship with the author(s) on one or more issues, he/she must report the situation to the editor and ask his/her to withdraw from the referee position. The same is also applicable when the authors illegally obtain information about the referees of the article and try to influence them.

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Referee(s) may request revision many times for the article they evaluated.

The content of the referee reports is checked and evaluated by editor-in-chief/editors/associate editors. The final decision belongs to the editorial.

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It is not tolerable for the author (s) to send an article, which has been already sent to another journal, to Kafkas Universitesi Veteriner Fakultesi Dergisi within the scope of "which accepts" or "which publishes first" approach. If this is detected, the article is rejected at any stage of the evaluation. As a possible result of these actions, in the process following the previous acceptance of the article sent to another journal, the withdrawal request with this excuse that the authors submit for this article, the evaluation process of which is going on in our journal, is evaluated by the editors and associate editors of the journal and disciplinary action on the grounds of ethical violations about those responsible is started. This unethical action is also informed to the journal editor (if known) who accepted the article.

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Authors should choose the references they use during the writing of the article in accordance with the ethical principles and cite them according to the rules.

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