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Review Article

A Systematic Review of the Neglected Parasite *Cyclospora cayetanensis* in Türkiye from 2004 to 2023

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Abstract

Cyclospora cayetanensis is a parasitic pathogen causing gastroenteritis, transmitted through contaminated food and water, and is prevalent in tropical and subtropical regions. Since *C. cayetanensis* requires different methods like acid-fast staining for detection, studies on its prevalence are insufficient. This study aimed to determine the prevalence of *C. cayetanensis* in Türkiye over the last two decades. A comprehensive literature search was conducted in databases Google Scholar, PubMed, Scopus, Mendeley, Web of Science, YOKtez, TR Dizin, and Türk MEDLINE Index for relevant English and Turkish publications from 2004 to 2023. Out of 184 identified articles, 47 met the inclusion criteria, of these, 41 were patient samples and 6 on water samples. *C. cayetanensis* prevalence averaged 32.7% in water samples and 2.71% in patient samples. *Cyclospora* was most frequently detected in the Aegean Region and Izmir province. The most frequent diagnostic method in the studies was the Kinyoun Acid-Fast Staining method (91.4%). Studies conducted in Türkiye have been limited to regions where expert parasitologists are available. High rates of positivity were detected in water samples. To accurately determine the prevalence in Türkiye, laboratory conditions must be provided to detect *C. cayetanensis* in every province.

Keywords: *Cyclospora cayetanensis,* Türkiye, Systematic reviews, Neglected diseases

Introduction

Cyclospora cayetanensis is a coccidian parasite that causes gastrointestinal disorders and prolonged diarrhea in humans. It causes serious diarrhea, especially in immunocompromised individuals, the elderly, and children. Furthermore, *C. cayetanensis* holds significant epidemiological importance in food and waterborne diarrhea outbreaks and endemic diseases such as traveller's diarrhea [1,2].

Worldwide, approximately 780 million people do not have access to safe drinking water and 2.5 billion people do not have access to improved sanitation. Parasitic infections that develop due to this condition cause many diseases such as diarrhea, malnutrition, and growth retardation [3]. According to World Health Organization (WHO) 2019 data, diarrhea ranks second among preventable and treatable diseases among deaths in children under five years of age. Diarrhea affects approximately 1.7 billion children globally each year and causes the death of approximately 370,000 children under the age of five [4,5]. A systematic review including 195 countries reported that coccidian protozoa play a significant role in the etiology of fatal diarrhea [6].

Among coccidian parasites, *C. cayetanensis* is not well known and is often neglected. The reasons for this situation may include insufficient awareness of the disease by clinicians, the lack of appropriate diagnostic methods applied in laboratories, and the absence of parasitological evaluation conducted by expert personnel [7]. Diagnosis of *C. cayetanensis* is conducted using acid-fast staining. Since *C. cayetanensis* cannot be detected by the native-Lugol method and there are no commercial serological kits that can be used in its diagnosis, data regarding its prevalence and incidence in risk groups do not reflect reality [8].

Incorrect and incomplete diagnosis also delays treatment. Trimethoprim/sulfamethoxazole (TMP/SMX) are the most commonly preferred drugs for treating cyclosporiasis. There is currently no effective alternative treatment

protocol defined for patients who do not respond to standard treatment or who are allergic to sulfa drugs. Due to differences in treatment protocols compared with other parasites and causative agents of diarrhea, especially in immunosuppressed individuals, prolonged diarrhea that does not heal related to cyclosporiasis has been observed [9].

This manuscript was prepared according to the guidelines outlined by the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) [10].

This article aims to investigate the prevalence of *C. cayetanensis*, a neglected parasite, in Türkiye in the last twenty years and to reveal the factors affecting its prevalence in a systematic manner.

Material and Methods

Search Strategy

For this systematic review, different combinations of the name of the parasite and the terms "Türkiye" were searched together in six international and three national databases in both English and Turkish. In the screening, electronic searches were made using the keywords "*Cyclospora cayetanensis - C. cayetanensis - Cyclospora - Cyclospora sp*., cyclosporiasis" and "Turkey", "*Cyclospora cayetanensis - C. cayetanensis - Cyclospora - Cyclospora sp*., siklosporiyaz" and "Türkiye".

Searching platforms for the present study included the following: Google Scholar, PubMed, Scopus, Mendeley, Web of Science, YOKtez *(https://tez.yok.gov.tr/UlusalTezMerkezi/),* TR Dizin *(https://trdizin.gov.tr)* and Türk MEDLINE.

YOKtez is the official website of the Turkish Higher Education Council, where master's, doctoral, and medical specialty theses are published. TR Dizin was created by the National Academic Network and Data Center (Ulakbim) of the Scientific and Technological Research Council of Türkiye (TÜBİTAK). It consists of journals on the basic subjects of health sciences, veterinary medicine, science, dentistry, pharmacy, engineering, and social and human sciences. TürkMedline is a database that collects articles in scientific and periodical health sciences journals published in Türkiye.

Study Selection, Inclusion, and Exclusion Criteria

This research includes cross-sectional studies that can reveal the prevalence of *C. cayetanensis* according to regions and years in Türkiye. The study included original articles, case reports, and theses published between January 2004 and December 2023, which could reveal the prevalence of *C. cayetanensis* in Türkiye. Articles that did not have epidemiological and statistical data, reviews, drug studies, unpublished data, and studies that did not contain suitable and reliable data were excluded from this review.

The studies obtained because of the screening were uploaded to the EndNote program and then screened for duplicate publications in different journals. In addition, the publications were transferred to Excel and re-examined to check for duplicates *(Fig. 1)*.

Study Selection

The selection of articles to be included in the research consists of two stages:

1. Scanning of titles and abstracts.

2. Scanning of the full texts.

Two independent reviewers worked at both stages of determining the article to be included. The inclusion and exclusion criteria for the study were determined according to the answers to the questions given below. Articles that received the approval of both referees were included in this study.

The following questions were used for title/abstract screening:

1) Is there a study that reveals the prevalence of *C. cayetanensis* in Türkiye according to the title/abstract?

2) Is this study an original paper, thesis, or case report?

If the scanned sections met the inclusion criteria, the full text of the article was obtained and reviewed.

The second stage of screening involved a full-text review using questions that required a 'Yes' or 'No' response. Any article that received a "No" response to any question was excluded from the review. All publications that were not excluded at this level were included in the systematic review.

The following questions were used in the second stage of screening:

1) Does the full text provide information on the prevalence of *Cyclospora* in Türkiye?

2) Is the region/city where the study in the full text was conducted clearly?

3) Are the methods used for data collection appropriate?

4) Are the data in the full text consistent with each other?

5) Are full-text findings appropriate for statistical data?

Data Items: Study-Level Data

The data from the studies published in the specified databases were examined comprehensively. For each article that met the inclusion criteria, the following data were collected: reference, study design, study location, methods applied, publication year, patient population, number of patients, and outcome information.

Statistical Analysis

The information extracted from the studies was entered

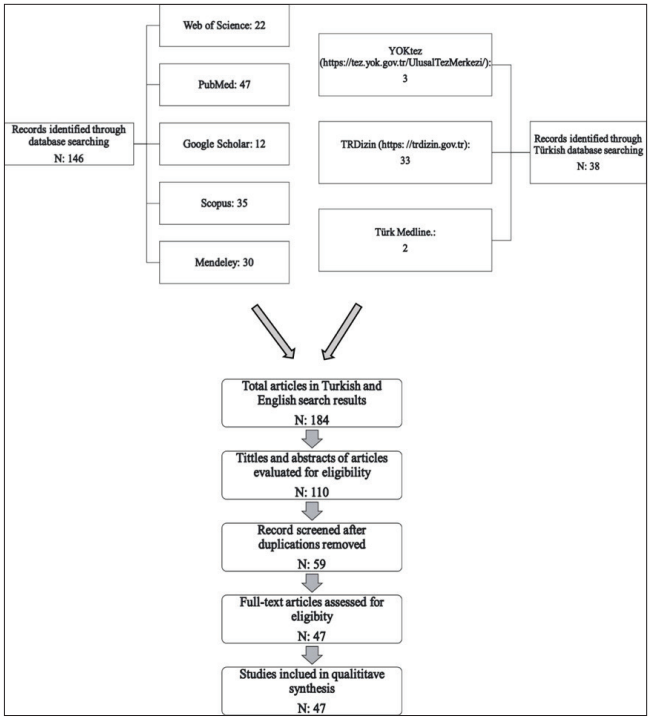

Fig 1. Study search process

into the CMA (Comprehensive Meta-analysis V3) software. The heterogeneity among the studies were assessed using the I^2 test, and the results were analyzed based on the heterogeneity determined by the random effects model. The presence of publication bias was investigated using the Egger test and Funnel plot. A meta-regression analysis was conducted to explore the factors contributing to the observed heterogeneity among the included studies.

Results

Among the 184 studies retrieved from databases covering 2004 to 2023 (20 years), 47 met the eligibility criteria for inclusion in this systematic review. Of the 184 articles obtained, 74 were excluded from the study because they were related to various scientific fields other than medicine. 51 studies were excluded because they were duplicates across various search engines and 12 studies were excluded because they did not provide epidemiological data such as drug resistance and sequence analysis. Data revealing the summaries of the studies obtained through this review are given in *Table 1*.

In the systematic review of 28 studies involving a total of 54.,043 human, the I^2 heterogeneity test showed high heterogeneity (I^2: 97.2). Based on this, the random effects method was used to analyze the results. The meta-analysis indicated a prevalence of *C. cayetanensis* in 2.3% (95% CI: 1.6-3.4) of human in Türkiye *(Fig. 2)*. Furthermore, the presence of publication bias in the studies was assessed using the Egger test, indicating such bias's existence (P-value = 0.0003). The funnel plot of the distribution

Table 1. *The main characteristics of 46 studies included in the present review*

Study Type	Sample Type	City	Diagnostic Method	Year	Total Samples Size (n)	Positive Samples (n)	Prevalence (%)	Reference
OR	H	Erzurum	MAF	2004	4322	1	0.02	[11]
OR	H	İzmir	Con, MAF, Tric	2006	4986	23	0.46	[12]
OR	H	İzmir	Con, KAF	2007	3925	75	1.91	[13]
OR	H	İzmir	KAF	2007	191	9	4.71	[14]
OR	H	İzmir	MAF	2007	554	11	1.99	[15]
OR	H	Kars	Con, MAF	2008	138	1	0.72	[16]
OR	H	İstanbul	MAF, PCR, Flor	2010	1876	20	1.07	[17]
OR	H	Diyarbakır	KAF	2012	75	13	17.33	[18]
OR	H	Van	Con	2012	6267	7	0.11	[19]
OR	H	Eskişehir	Con, MAF	2012	225	1	0.44	[20]
OR	H	İzmir	Con, MAF, Tric	2012	5073	187	3.69	[21]
OR	H	İzmir	Con, KAF	2012	873	27	3.09	[22]
OR	H	İstanbul	MAF, PCR	2013	91	2	2.20	[23]
OR	H	İzmir	PCR	2014	53	14	26.42	[24]
OR	H	Van	Con, MAF, Tric	2015	5985	7	0.12	[25]
OR	H	Malatya	KAF, Flor	2015	2281	129	5.66	[26]
OR	H	İstanbul	KAF, MAF, PCR	2017	115	3	2.61	[27]
OR	H	Van	Con, KAF	2018	150	8	5.33	[28]

Table 1. *The main characteristics of 46 studies included in the present review (continued)*

Study Type	Sample Type	City	Diagnostic Method	Year	Total Samples Size (n)	Positive Samples (n)	Prevalence (%)	Reference
OR	H	İzmir	Con, KAF	2018	65	2	3.08	[29]
OR	H	Van	KAF	2018	150	4	2.67	[30]
OR	H	İzmir	Con, MAF, Tric	2019	10.726	828	7.72	[31]
OR	H	İzmir	PCR	2020	62	6	9.68	[32]
OR	H	Van	MAF, PCR	2021	200	24	12.00	[33]
OR	H	Malatya	Con KAF	2021	1057	52	4.92	[34]
OR	H	İzmir	KAF	2022	529	21	3.97	[35]
OR	H	Diyarbakır	KAF, Tric	2022	3624	15	0.41	[36]
OR	H	Aksaray	PCR	2023	232	8	3.45	[37]
OR	H	İzmir	Con, KAF, Tric	2023	4518	51	1.13	[38]
OR	W	Ankara	KAF	2008	173	47	27.17	[39]
OR	W	Giresun	KAF, Tric	2016	300	112	37.33	[40]
OR	W	Ordu	KAF, Tric	2017	228	56	24.56	[41]
OR	W	Samsun	KAF, Tric	2017	144	96	66.67	[42]
OR	W	Denizli	KAF, Tric	2022	84	5	5.95	[43]
OR	W	Iğdır	MAF, PCR	2023	69	11	15.94	[44]
CR	H	Kayseri	MAF	2004	6	6	100.00	[45]
CR	H	İzmir	Con, KAF, Flor	2006	1	1	100.00	[46]
CR	H	Ankara	MAF	2006	1	1	100.00	[47]
CR	H	Ankara	MAF	2006	5	5	100.00	[48]
CR	H	Malatya	Con, MAF	2008	1	1	100.00	[49]
CR	H	Ankara	Con	2009	1	1	100.00	[50]
CR	H	Kayseri	Con, KAF	2009	3	3	100.00	[51]
CR	H	Eskişehir	MAF	2010	1	1	100.00	[52]
CR	H	İzmir	MAF, Tric	2010	1	1	100.00	[53]
CR	H	Diyarbakır	KAF	2011	2	2	100.00	[54]
CR	H	Malatya	KAF	2012	1	1	100.00	[55]
CR	H	Van	EZN	2012	2	2	100.00	[56]
CR	H	Van	Con, MAF	2016	7	7	100.00	[57]

MAF: *Modified acid fast,* ***KAF:*** *Kinyoun acid fast,* ***EZN:*** *Erlich Ziehl Neelson,* ***Con:*** *Concentration method,* ***Tric:*** *Trichrome strain,* ***Flor:*** *Fluorescence method,* ***OR:*** *Original research,* ***CR:*** *Case Report,* ***H:*** *Human,* ***W:*** *Water*

bias in the reviewed studies is presented in *Fig. 3*. Meta-regression of the effects of sample size and year on the prevalence of *C. cayetanensis* in human is presented in *Fig. 4* and *Fig. 5*.

Of the articles, 41 were studied from patient stool samples, while 6 were water samples *(Table 1)*. Of the six water samples, five were surface freshwater and one was wastewater sample. In the original studies, the highest positivity rate was observed in the water sample (66.67%) and the lowest positivity rate was observed in a retrospective study on patients (0.02%). The average prevalence of *C. cayetanensis* in water samples was 32.7%.

In the studies, at least one of the native Lugol, sedimentation, acid-fast staining, trichrome staining, and molecular methods were used to detect the parasite. The distribution of the methods used is given in *Table 2*. When the studies were examined, it was found that the acid-fast staining method was used in 43 out of 47 studies (Different modifications of the acid-fast staining method were evaluated together. Two acid-fast staining methods were used in one study).

The most common complaints in patients with *C. cayetanensis* are prolonged diarrhea, abdominal pain, and vomitin. Forest plot of the prevalence of *C. cayetanensis* among human in Türkiye based on the random-effects

Table 2. *The distribution of methods used for the diagnosis of C. cayetanensis*

Methods	Number of Studies	Percent
Concentration	18	38.30%
KAF	22	46.81%
MAF	21	44.68%
EZN	1	2.13%
Tricrome	11	23.40%
PCR	8	17.02%
Floresan	3	6.38%

KAF: *Kinyoun acid fast,* ***MAF:*** *Modified acid fast,* ***EZN:*** *Erlich Ziehl Neelson*

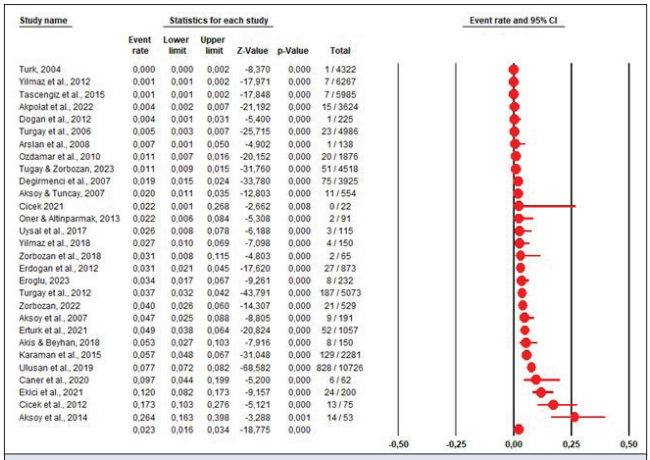

Fig 2. Forest plot of the prevalence of *C. cayetanensis* among human in Türkiye based on the random-effects method. CI: confidence interval

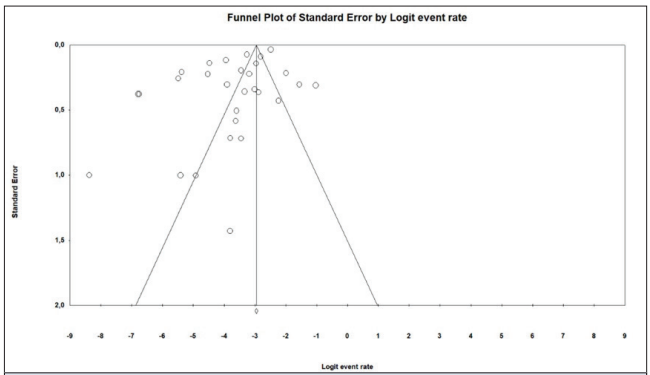

Fig 3. Funnel plot of the distribution bias in the reviewed studies

method by years, region and city are presented in *Fig. 6*, *Fig. 7*, and *Fig. 8*. Prevalence rates of *C. cayetanensis* by province in Türkiye is presented in *Fig. 9*. When the incidence of *Cyclospora* was examined over the years, the lowest positivity rate was observed in 2004 (0.05%), while the highest positivity rate was observed in 2011 (15.72%) *(Table 1)*.

In 36 out of 47 studies, no information was found regarding seasonal transmission, while in 11 studies, it was reported

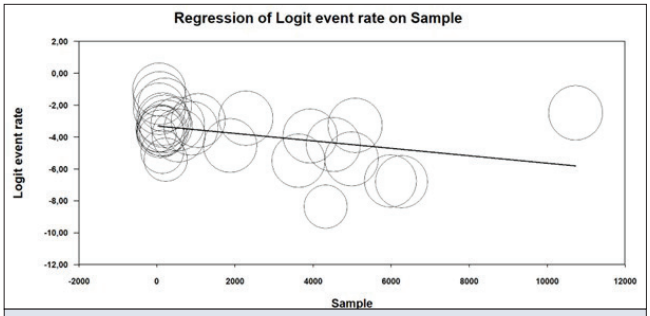

Fig 4. Meta-regression of the effect of sample size on the prevalence of *C. cayetanensis* in human

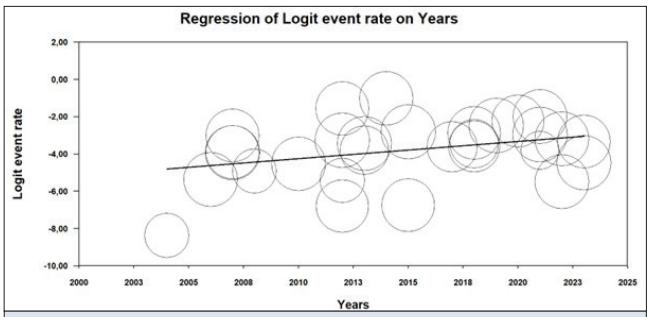

Fig 5. Meta-regression of the impact of the year of conducting studies on the majority of *C. cayetanensis* in human

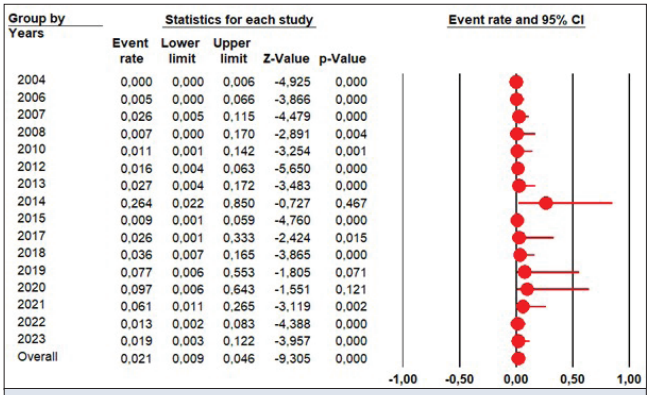

Fig 6. Forest plot of the prevalence of *C. cayetanensis* among human in Türkiye based on the random-effects method by years

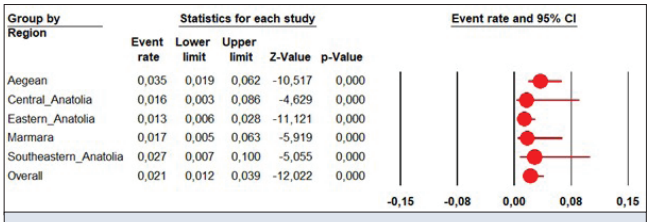

Fig 7. Forest plot of the prevalence of *C. cayetanensis* among human in Türkiye based on the random-effects method by region

that diseases are most commonly seen in the spring and summer.

It was determined that studies on *C. cayetanensis* were carried out in six of Türkiye's seven regions and 17 of 81 provinces. In Türkiye, the highest number of studies among regions was conducted in the Aegean region

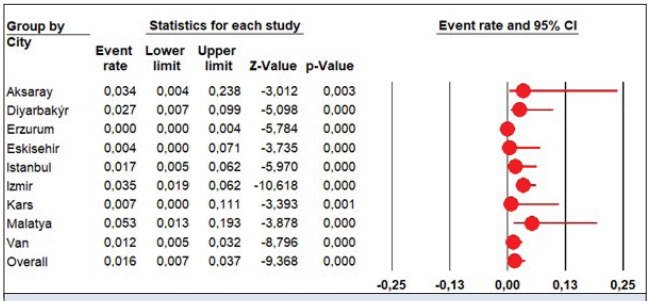

Fig 8. Forest plot of the prevalence of *C. cayetanensis* among human in Turkey based on the random-effects method by city

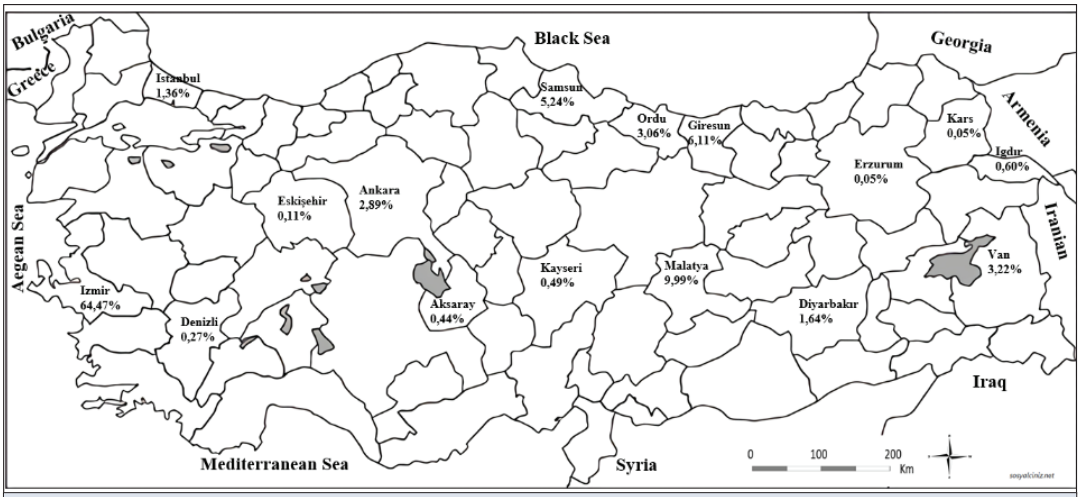

Fig 9. Prevalence rates of *C. cayetanensis* by province in Türkiye

(13/29), and among provinces Izmir (13/29) *(Table 1)*.

C. cayetanensis was detected in water samples from four regions and six provinces of Türkiye *(Table 1)*. Among the studies on *Cyclospora*, the highest positivity rate was observed in water samples (average 32.77%).

When all studies were evaluated together, *C. cayetanensis* was detected most frequently in İzmir (64.47%), Malatya (9.99%), and Giresun (6.11%) *(Fig. 9)*.

Discussion

This systematic review provides comprehensive data on the prevalence of *C. cayetanensis* in Türkiye. Of the 47 articles examined in the study, 41 belonged to human samples and 6 to water samples *(Table 1)*. The average prevalence of studies conducted in Türkiye has been determined to be 3.21%. The global prevalence of *C. cayetanensis* was reported to be 3.55% in a 2023 study and 3.4% in a 2024 meta-analysis examining *C. cayetanensis* infection worldwide. The results of this study are consistent with the global average [58-60]. The prevalence of *C. cayetanensis* varies by country's development status, with rates of 7.6% (83/921) in low-income countries, 4.8% (3.280/48.852) in lower-middle-income countries, 2.9% (2.194/99.419) in upper-middle-income countries, and 0.4% (79/17.419) in high-income countries [60] Since cyclosporiasis is an infection transmitted through food and water, it is more common in developing countries with low hygiene standards. The prevalence rates in Türkiye fall between those reported for lower-middle-income and upper-middle-income countries.

The average positivity rate was found to be 2.75% in human samples and 32.77% in water samples, with a significantly higher rate observed in water samples. The high positivity rate in water samples in Türkiye can be attributed to the following factors. Water is an important transmission route for coccidial parasites and many studies have determined that both sporadic and endemic cases are caused by polluted water. Therefore, the detection of coccidian parasites has become the primary goal in studies conducted [1,2]. Studies on water samples in Türkiye were conducted by experts in the field, in the seasons when the parasite is most prevalent, using diagnostic methods such as the acid-fast staining technique, which is effective for detecting coccidian parasites. In contrast, studies on human samples were predominantly retrospective, covering the entire year and involving large datasets. Therefore, depending on the seasonality, variations in laboratory conditions, and the expertise of the working personnel, not all cases may have been detected in studies

conducted with human samples. Positivity rates in water samples were 27.17% in municipal wastewater and 33.94% in surface freshwater. In a meta-analysis investigating the global prevalence of *C. cayetanensis* in water, the prevalence rate was reported as 6.61% in freshwater and 4.66% in municipal wastewater suggesting that the rates in Türkiye are substantially higher [2]. However, only one study on wastewater and five on surface freshwater have been conducted in Türkiye, making the available data insufficient for reliable comparisons with global rates or robust interpretations.

When studies conducted in Türkiye are examined, the most frequently used diagnostic method for detecting *C. cayetanensis* is the acid-fast staining method (43 out of 47 studies) *(Table 2)*. Studies have shown that PCR is the most reliable method in the diagnosis of *C. cayetanensis* [27,61]. However, when applicability, sensitivity, and cost balance are evaluated together, the most practical method is the acid-fast method.

The most common complaints in people with cyclosporiasis in Türkiye are prolonged diarrhea, abdominal pain and vomiting. Studies indicate that *C. cayetanensis* is more frequently observed in immunosuppressed individuals and causes serious complications [1]. In Türkiye, 8 studies were conducted on immunosuppressed individuals with cyclosporiasis. In cases of prolonged diarrhea and abdominal pain where the source cannot be identified, *C. cayetanensis* should be considered as a potential causative agent.

The lowest positivity rate of *Cyclospora* by year was observed in 2004 (0.05%), while the highest positivity rate was observed in 2011 (15.72%). The most important factor affecting the incidence by year is whether the study is original research or retrospective. In the years when research studies were conducted using tests that could identify *Cyclospora*, the positivity rate increased, while in the years when retrospective studies were conducted, it decreased.

Cyclosporiasis is most frequently seen in summer and spring months in Türkiye. *C. cayetanensis* is a waterborne parasite. During these seasons, increased human contact with untreated water sources and soil, along with higher consumption of fruits and vegetables grown in wetlands, such as raspberries and blackberries, contributes to a rise in parasite incidence.

C. cayetanensis has been detected in 6 regions and 17 provinces in Türkiye *(Table 1)* *(Fig. 9)*. It has been detected most frequently in the Aegean Region among the regions and in Izmir among the provinces. Cyclosporiasis is more commonly seen in underdeveloped rural areas where hygiene is low [5]. However, the Aegean Region and Izmir are among the most developed areas of Türkiye. There is an inconsistency between the results obtained and this situation. The reason for the apparent contradiction between the studies in Türkiye and the literature data can be explained as follows. *C. cayetanensis* cannot be identified by the native-Lugol method and it is a parasite without a commercial diagnostic kit such as cassette tests. To identify this parasite, specific diagnostic methods such as acid-fast staining methods and expert technical personnel in the field of parasitology are required. Therefore, it is very difficult to detect *C. cayetanensis* in routine microbiology laboratories. When the studies were examined, it was observed that all hospitals in regions where *C. cayetanensis* was diagnosed have either a Parasitology Department or a parasitology specialist available.

C. cayetanensis was detected in water samples from four regions and six provinces of Türkiye, and the highest positivity rate among all studies was observed in water samples (average 32.77%) *(Table 1)*. The main reason is that these studies were performed by experts familiar with *C. cayetanensis* and the acid-fast method was used to diagnose coccidian protozoa, an important cause of waterborne diarrhoea [62]. There was no correlation between the locations of these studies and the locations of human *Cyclospora* infection. The studies were designed only to identify waterborne parasitic agents, and *Cyclospora* was the most frequently identified parasitic agent after Cryptosporidium. If similar studies are conducted in other regions and provinces in Türkiye, *C. cayetanensis* will likely be detected in these regions as well.

When all studies were evaluated together, *C. cayetanensis* was detected most frequently in İzmir, Malatya, and Giresun *(Fig. 1)*. The reason for the high prevalence of *C. cayetanensis* in these provinces is, as mentioned above, the existence of expert parasitologists and laboratory conditions that can detect this agent. The prevalence of *C. cayetanensis* in humans in retrospective studies in Izmir was higher than in the original studies in other provinces [31]. The most likely reason for this is that this province, which has the oldest Parasitology Department in Türkiye, has a well-established system of examining all samples received in the laboratory for coccidian parasites.

Conclusion

This systematic review is important as it is the first study to reveal the general prevalence of *C. cayetanensis*, an important but neglected public health problem, in Türkiye. *C. cayetanensis* is a parasite that causes prolonged diarrhea and severe symptoms, especially in immunocompromised individuals. Due to the limited number of studies conducted in Türkiye, the available data cannot accurately reveal the prevalence of this parasite in the country. All of the studies were conducted by specialized parasitologists. Studies with human specimens are mostly retrospective

or include *Cyclospora* agents seen while investigating intestinal protozoa. Limited research has been conducted with water samples, but these studies have observed high positivity rates. Considering these findings, *Cyclospora* is likely to be detected in studies conducted in other regions and provinces of Türkiye. As a result, more accurate epidemiological data can be obtained if clinicians pay more attention to this parasite and if specialized personnel and adequate laboratory conditions are provided in the field of parasitology.

Declarations

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RESEARCH ARTICLE

Identification and Expression of the Target Gene SLC24A2 of oar-miR-377 and Its Novel SNPs Effects on Wool Traits in Sheep

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Abstract

Hair follicle development is closely associated with wool traits. Current studies reveal the crucial role of microRNAs in regulating the specific gene expression by binding to target mRNA involution in hair follicle growth and development, thereby regulating the wool traits. Our previous miRNA sequencing showed that oar-miR-377 has special expression in secondary hair follicle development and SLC24A2 may be a new candidate target using bioinformatics analysis. In this study, the regulatory relationship of oar-miR-377 and its specific target gene SLC24A2 was determined in sheep fibroblasts by dual-luciferase reporter assay, RT-qPCR, and western blot. The variation of oar-miR-377 precursors was detected using PCR and Sanger sequencing, and the association between polymorphisms of oar-miR-377 and wool quality traits was analyzed in Chinese Merino. The result showed that SLC24A2 was a target gene of oar-miR-377. A SNP (276T>C) of oar-miR-377 upstream sequences was identified and extremely significant associated with the coefficient of variation of wool fiber diameter in Chinese Merino sheep (P<0.01). These results suggest that oar-miR-377 promotes secondary hair follicle development by downregulation of SLC24A2 gene expression, and its SNP might be a useful marker for wool quality.

Keywords: oar-miR-377, SLC24A2, SNP, Wool traits, Sheep

INTRODUCTION

Wool is a healthy natural product from sheep, the economic traits of wool include fiber length, fiber diameter, fiber diameter standard deviation, coefficient of variation of wool fiber diameter, fiber crimp, fiber density, and so on. Wool quality is determined by the development of hair follicles. Hair follicles (HFs) are complex and composed of 8 unique cell populations that are derived from the ectoderm and mesoderm, comprised of primary hair follicles and secondary hair follicles [1,2], the secondary hair follicles are a key factor in determining wool quality [3]. In mammals, hair follicles are crucial for temperature regulation, physical protection, sweat and sebum dispersion, sensory and tactile functions, and social interactions [4]. The number of dermal papilla cells and the size of the hair placode are associated with the diameter, crimp, and density of wool fibers, which are of high economic value in the sheep industry [5-7]. Many reports have indicated that several genes may be involved in hair follicle development, such as Wnt10a [8], Sox9 [9], and BMP4 [10]. Additionally, some signaling pathways, such as BMP [11], Eda [12], Shh [13], and TGF-β [14] signaling were revealed to promote or suppress the process of hair follicle development. In animal husbandry, elucidating the genetic mechanisms of the development of hair follicles and wool-related traits is important to improve sheep breeding.

MicroRNAs are non-coding RNAs that include 22nt nucleotide and are widely present in animals and plants, which can negatively regulate gene expression by base pairing of 5-end with the 3 untranslated regions (3'UTRs) of target mRNAs [15,16]. Research has found that microRNAs are expressed in a variety of different hair follicle cells, such as hair follicle stem cells, matrix cells,

outer root sheath cells, and inner root sheath cells, and lots of miRNAs are verified to be specifically expressed in hair follicle cells [17]. Both MiR-203 [18], MiR-206 [19], miR-125b [20], and miR-205 [21] are expressed in hair follicle stem cells. Several SNPs in miRNA genes have been proven to be associated with human diseases by affecting the miRNA-mediated regulatory function. The SNP of mature miR-125a sequences can reduce miR-125a expression in cancer [22]. The SLC24A2 gene is a cation exchanger and serves as the second member of the solute carrier 24 family [23,24]. Although there is no direct evidence that SLC24A2 is related to skin hair follicles, SLC24A5 has been confirmed to be related to animal hair color [25]. RT-PCR results showed that the SLC24A5 gene was highly expressed in skin and eyes and low expressed in other tissues, while the expression level in mouse melanoma was more than 100 times higher than that in normal skin and eyes [26,27].

Our previous research showed that SLC24A2 is a key molecule in hair follicle development signal transduction which also is the target gene of the oar-miR-377 by microRNA-sequencing analysis. However, the potential relationship between SLC24A2 and oar-miR-377, and its SNP effects on wool traits has not been illustrated. In this study, the target binding relationship of SLC24A2 and oar-miR-377 was investigated at cellular levels, and functional mechanisms of SNP of oar-miR-377 were elucidated in sheep populations levels of Chinese Merino (Xinjiang Junken type). The study may provide a basis for the genetic mechanism of wool traits and fine wool sheep breeding.

Materials and Methods

Ethical Statement

This study was approved by the Experimental Animal Care and Use Committee of Xinjiang Academy of Agricultural and Reclamation Sciences (Approval no: XJNKKXY-2020-34).

Experimental Animals and Sample Collection

A total of 265 female Chinese Merino sheep (Xinjiang Junken type) were selected from the sheep breeding farm of the Xinjiang Academy of Agricultural and Reclamation Science. The flock included 55 one-year-old sheep, 110 two-year-old sheep and 100 three-year-old sheep. All the ewes were sourced from a single flock and fed under the same conditions. The peripheral blood was collected from the jugular vein and placed in anticoagulant tubes containing EDTA (1 mg/mL) for genomic DNA extraction. The wool samples were collected to detect the wool quality from 265 Chinese Merino. The ear tissue from three-month-old lamb was collected for cell culture.

Cell Culture of Sheep Skin Fibroblasts

Ear tissue (10x10 mm) was sterilized with 75% alcohol and collected from 3-month-old healthy Merino lambs. After sterilized with 75% alcohol and washed by 1xPBS (pH 7.2) containing penicillin and streptomycin double antibody (1:100), the skin was minced pieces and put into 90 mm dishes containing 10 mL of Dulbecco's Modified Eagle's Medium (DMEM, The Gibco Company) supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin). The cells were placed in Galaxy® 48R CO_2 Incubator from Eppendorf at 37°C and 5% CO_2, the culture medium was changed every 3 days and the growth status of cells was observed. When cell confluency reached 80%, the cells were detached using trypsin-EDTA (0.10% trypsin and 0.02% EDTA, The Gibco Company) for 5 min at 37°C, followed by trypsin digestion method for culture in the next passage.

Prediction of Target Genes and Construction of Recombinant Plasmids

Using Bibiserv *(https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/)* online website to predict oar-miR-377 and sheep SLC24A2 gene 3'-UTR region binding sites. The primers of wild carriers were designed according to the binding site sequence of miRNA and target SLC24A2 gene searched on the NCBI website by Primer5.0 software *(Table 1)*. The upstream primer was introduced to the Not I restriction site, and the downstream primer was introduced to the Xho I restriction site, the primers were synthesized by Sangon Biotech (Shanghai, China). Total RNA was extracted from sheep skin samples using Trizol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized using the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (Beijing Transgene Biotech Co. Ltd., Beijing, China). SLC24A2 gene 3'-UTR sequence containing microRNA binding sites was amplificated by RT-PCR, and the reactions were incubated in a 96-well plate at 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, a final extension of 72°C, PCR products were purified using gel recovery kit, and ligated to pEASY - T1

Table 1. *Sequences of primers*

Primer Names	Primer Sequence	Length (bp)
SLC24A2-F	ATAAGAATGCGGCCGCgccaccatggTGGAAGCGCCTCACAA	589
SLC24A2-R	CCGCTCGAGcgccaccatggCTCTGACCAGCAAGGAGTA	

The lowercase letters indicate NotI and XhoI restriction sites

Table 2. Experimental groups	
Groups	**Experiment Project**
A	pCHECK-W and mimic
B	pCHECK-W and mimic negative control
C	pCHECK-M and mimic
D	pCHECK-M and mimic negative control
E	psiCHECK-2 and mimic negative control

Simple vector, then cloned into a psiCHECK-2 vector and constructed wild-type plasmid (pCHECK-W, *Table 2)*. The mutation sequence of SLC24A2 gene 3'-UTR region binding sites was synthesized in Jikai Gene Company (Shanghai, China), and ligated to psiCHECK-2 vector constructed mutation-type plasmid (pCHECK-M, *Table 2)*. The reconstructed plasmids were sequenced in Sangon Biotech (Shanghai, China). Mimics of oar-miR-377 were designed and synthesized by Jikai Gene Company (Shanghai, China).

Cell Transfection and Dual Luciferase Activity Assay

The skin fibroblasts were digested after the cell density reached approximately 85% confluence and transfected with oar-miR-377 mimic, oar-miR-377 NC (negative control), psiCHECK-2, pCHECK-W and pCHECK-M plasmid using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Experimental groups were divided into 5 groups *(Table 2)*. Each group had three replicates. At 48 h of transfection, the luciferase activity was detected by the Dual-Luciferase Reporter Assay system (Promega) according to the manufacturer's instructions. The luciferase activity of the firefly and renal luciferase was detected using a microplate reader (Thermo Scientific varioskan flash, MA, USA). The firefly and *Renilla* luciferase enzyme activities were measured for each biological sample. The firefly luciferase enzyme activity was normalized to the *Renilla* luciferase enzyme activity. Mean±SD of the relative luciferase activity data were calculated by repeated three times independently.

Western Blotting

The cell proteins were extracted using a whole protein extraction kit (Applygen Technologies, Beijing, China). Protein concentrations in cell lysates were determined spectrophotometrically using the NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE), and adjusted to the same concentration. Heat-denatured protein samples (25 μg per lane) were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) on duplicate gels and transferred to nitrocellulose membrane (Boster, Wuhan, China). The membranes were incubated for 60 min in 10% nonfat dry milk to block nonspecific binding, followed by incubation for 12 h at 4°C with a primary rabbit monoclonal antibody against oar-miR-377 (Boster, Wuhan, China), which was diluted 1:1000 in tris-buffered saline Tween-20 (TBST). The membrane was then washed 2 times for 10 min in TBST, 1 time for 10 min in TBS, and incubated for 1 h at room temperature with a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP) (Boster, Wuhan, China) diluted 1:2500 in TBST. The membrane was washed 2 times for 10 min in TBST and 1 time for 10 min in TBS, and the bound antibody was detected colorimetrically using a DAB detection kit (Boster, Wuhan, China) according to the manufacturer's instructions. The intensity of signals for SLC24A2 was quantified using Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). The grey values of bands were measured using ImageJ software (v1.48, NIH, Bethesda, MD). The relative intensity ratio of SLC24A2 and GAPDH was calculated based on grey values. Mean±SD of the grey value was calculated by repeated three times independently.

RT-qPCR

Sheep skin fibroblasts were cultured after transfection, and their mRNA levels were detected by qPCR after 48 h culture. Total RNA was extracted according to the manufacturer's instructions using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA). The RNA quality was detected using electrophoresis on 1% agarose gel in 1x TAE buffer. The purity and concentration of RNA were detected using Nanodrop 2000. Gene sequences were obtained from the NCBI gene bank, primers of SLC24A2 were designed by Primer 5, and miRNA primers of oar-miR-377 were designed by miR primer 2 *(Table 1)*. The 20 μL PCR reaction mixture contained 10 μL Platinum SYBR GreenImaster, 1 μL forward primer (10 μM), 1 μL reverse qPCR primer (10 μM), 6 μL ddH_2O, 2 μL DNA template. The reactions were incubated in a 96-well plate at 95°C for 5 min, followed by 45 cycles at 95°C for 10 sec, 60°C for 20 sec, and 72°C for 20 sec. All RT-qPCR experiments were performed at least in triplicate, and relative mRNA quantification was performed using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method. The ΔCt values for the SLC24A2 gene were calculated using the Ct values [Ct (test) – Ct (reference)]. Mean±SD of the $2^{-\Delta\Delta Ct}$ data were calculated.

Measurement of Wool Traits

The wool fiber diameter, fiber diameter standard deviation and coefficient of variation of wool fiber diameter were automatically measured from 265 Chinese Merino (Xinjiang Junken type) according to the guidelines of the China Fiber Inspection Bureau, International Wool Trade Organization standards IWTO-TM47 and 57 using OFDA 2000 instrument (Cottle DJ, 2010).

Table 3. *Primers sequences for detection SNPs of oar-miR-377*

Primer Name	Sequences	Annealing Temperature (°C)	Product Length	Chromosome Position/bp
miR-377-1	CCTTGGGAGGACCTTGCT	60	485	chr18:64558969-64559453
	AGAAGCCATCCCAAGCAG			
miR-377-2	CTCTCTGTTCAATCGCAGCTC	60	460	chr18:64558923-64559382
	AATTCACCAAAGGCAACCTC			

Preparation of the Genomic DNA

The genomic DNA was extracted using a Tiangen blood genomic DNA extract kit following the manufacturer's protocol, the concentration and purity of genome DNA were detected by using 1.5% gel electrophoresis.

PCR Amplification and SNP Detection of oar-miR-377

The mature sequence of oar-miR-377 was obtained according to miRBase *(http://www.mirbase.org/)*. 700bp of oar-miR-377 upstream and downstream flanking sequences were searched by the UCSC Genome Browser *(http://genome.ucsc.edu/)*. The primers were designed using Primer 5.0 software, and synthesized by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China) *(Table 3)*.

The mature sequence of oar-miR-377 was individually amplified using miR-377-1 primers and miR-377-2 primers. The PCR reactions were performed by mixing 2.5 µL of genome DNA, 25 µL of 2 x EasyTaq PCR Supermix, 1 µL (10 µM) each of the upstream primers and downstream primers, 20.5 µL of DDW, in a final volume of 50 µL. The cycling was performed on a thermocycler (A200, Longgene Scientific Instrument Company, Hangzhou, China), by the following program: 94°C for 5 min, 35 cycles of 94°C for 30 sec, annealing at 60°C for 30 sec, and 72°C for 30 sec, a final extension of 72°C for 5 min. The PCR products were detected using 1.5% agarose gel electrophoresis and sequenced by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China), The SNP was identified, and the location was determined by Blast against miRBase database (v19, *http://microrna.sanger.ac.uk)*.

Statistical Analysis

Genotype frequencies, allele frequencies, and Hardy-Weinberg equilibriums tests were performed using the Popgene 32 software. For the tested population, the statistical models associated between different genotypes with average wool fiber diameter, fiber diameter standard deviation, and coefficient of variation of wool fiber diameter were: $Y_{ij}=\mu+G_i+A_j+G_ixA_j+e_{ij}$, which Y_{ij} was the phenotypic value of the wool traits, µ was overall population mean, Gi was the fixed effect of genotype, A_j was the fixed effect of age, G_ixA_j was genotype x age interaction, e_{ij} was the random residual.

Data were subjected to the GLM procedures of John's Macintosh Program (JMP version 16.0.0, SAS Institute Inc.), which was used to examine the correlations between genotypes and continuous traits, and to evaluate the least squares means. For all the data, $P<0.05$ was significant, and $P<0.01$ was highly significant.

Results

Culture of Sheep Skin Fibroblasts

Primary sheep fibroblast cells migrated from tissue pieces 5-12 days after explanting. Then, cells continued to proliferate and were passaged when reached 90% confluences. The cells were morphologically consistent with the fibroblast phenotype.

Prediction of miRNA Binding Sites with Target Genes

The secondary structure of oar-miR-377 was obtained by sequencing results, which had two typical stem-loop structures. The 3'-UTR sequence of the SLC24A2 gene was obtained on the NCBI website, and the binding site sequence of the mature sequence of oar-miR-377 was matched with the 3'-UTR of the SLC24A2 gene by Bibiserv software. The mfe was -26.1 keal/mol.

Construction of psiCHECK-2 Wild-type Vector and Mutant Vector

The binding site sequence of the SLC24A2 gene was obtained by PCR amplification. The 589bp PCR production appeared by 1.5% agarose gel electrophoresis. The target gene products were incorporated into the psiCHECK-2 vector using a T4 DNA ligase at 4°C overnight. The sequences of constructed pCHECK-W and pCHECK-M were confirmed by standard BLAST alignment analysis and showed 100% and 97% Identities with Ovis aries solute carrier family 24 member 2 (SLC24A2) mRNA (Genbank accession number XM_015093243.4). The results indicated that pCHECK-W and pCHECK-M vectors were successfully constructed.

Dual Luciferase Activity Assay of SLC24A2 Targeted by oar-miR-377

The results showed that the relative luciferase activity in pCHECK-W and mimic was significantly lower than the pCHECK-W and mimic NC (negative control), psiCHECK-2 and mimic NC (negative control) by using dual luciferase activity assay ($P<0.05$) *(Fig. 1)*. The

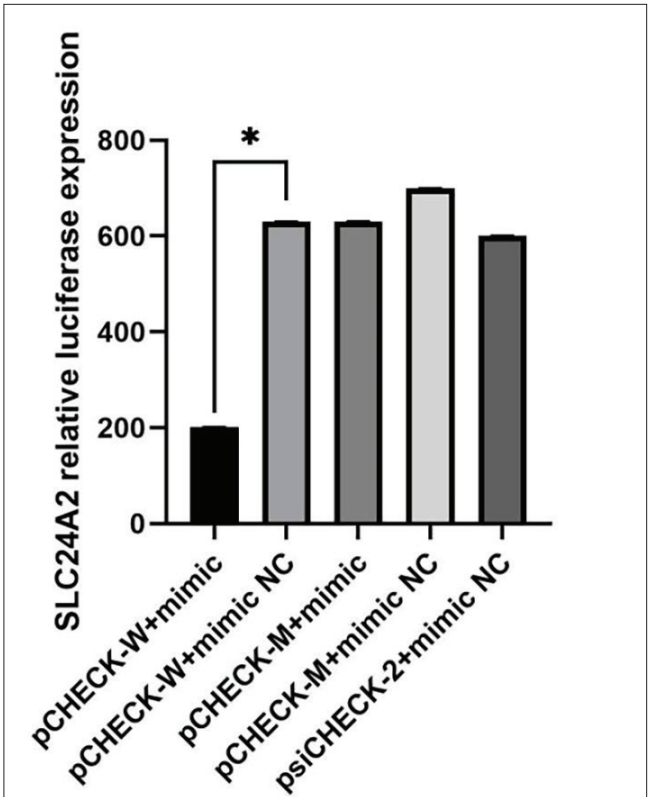

Fig 1. Analysis of dual-luciferase reporter assay

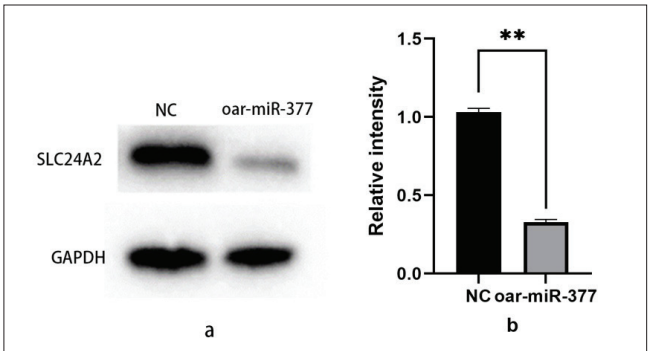

Fig 2. Protein levels of SLC24A2 regulated by oar-miR-377. **A:** Western blot images, **B:** Relative intensity of Western blot

relative luciferase activities were not significantly different among pCHECK-M and mimic, pCHECK-M and mimic NC, psiCHECK-2 and mimic NC (P>0.05). These results indicated that oar-miR-377 was binding to the 3'-UTR in the regulation of SLC24A2 mRNA.

The Effect of SLC24A2 Protein Level Regulated by oar-miR-377

The regulation of SLC24A2 protein level by oar-miR-377 in sheep skin fibroblasts was detected by Western blot *(Fig. 2-a)*. The results show that the protein relative expression of SLC24A2 regulated by oar-miR-377 was extremely significantly lower than NC (P<0.01), the expression level

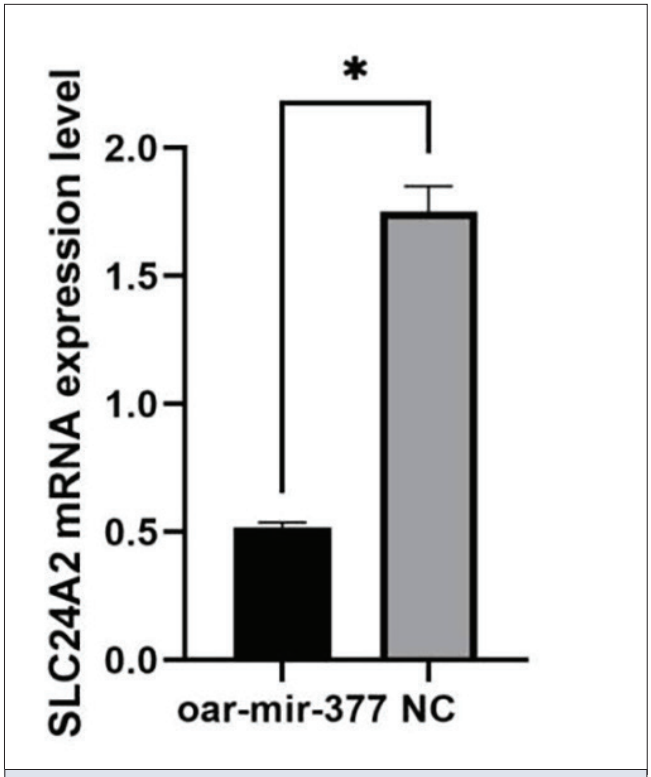

Fig 3. mRNA expression levels of SLC24A2 gene regulated by oar-miR-377

of oar-miR-377 was 3.28 times lower than the negative control *(Fig. 2-b)*.

The Effect of SLC24A2 Gene Expression Level Regulated by oar-miR-377

The mRNA expression level of SLC24A2 was regulated by oar-miR-377 and negative control was detected using RT-qPCR. The results showed that SLC24A2 relative expression level was significantly lower than NC (P<0.05), the expression level of SLC24A2 was 3.37 times lower than the NC *(Fig. 3)*.

PCR Amplification and SNP Identification of oar-miR-377 Precursors

PCR productions of oar-miR-377 were detected by 1.5% agarose gel electrophoresis, and the 460bp bands were observed. The sequence of PCR productions was aligned to the known oar-miR-377 precursors sequence after DNA sequencing. T > C mutation site was detected at 276bp upstream flanking region of oar-miR-377 by miR-377-1 primer in Chinese Merino sheep, and located in chr18: 64559086 against the miRBase miRNA database *(Fig. 4)*.

Association of oar-miR-377 Polymorphisms with Wool Traits

Three genotypes of TT, TC, and CC were identified in Chinese Merino sheep (Xinjiang Junken type), by using genetic polymorphism analysis of -276T>C locus of oar-

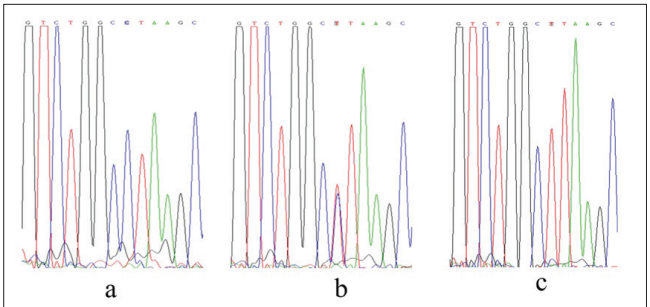

Fig 4. Three genotypes of oar-miR-377 gene by sequencing. **A:** CC genotype, **B:** CT genotype, **C:** TT genotype

Table 4. *The distribution of genotypic frequency and gene frequency of -276T>C in oar-miR-377 in Chinese Merino sheep*

Genotype Frequency			Allele Frequency		χ^2	P-value
CC	TC	TT	C	T		
0.39 (103)	0.55 (146)	0.06 (16)	0.67	0.33	1.7	0.19

Table 5. *Effects of oar-miR-377 SNP on wool traits (LSM)*

Genotype	Numbers	Wool Fiber Diameter (μm)	Wool Fiber Diameter Standard Deviation	Coefficient of Variation of Wool Fiber Diameter
CC	103	19.64±0.19	3.94±0.06	19.96±0.21 ^{A}
TC	146	19.48±0.16	3.86±0.05	18.02±0.18 ^{B}
TT	16	19.72±0.56	3.89±0.19	17.81±0.62 ^{B}

Different uppercase letters indicate the difference is extremely significant (P<0.01).

miR-377. The genotype frequencies of CC, TC, and TT in the Chinese Merino sheep were 0.39, 0.55, and 0.06 *(Table 4)*. The SNP site was under the Hardy-Weinberg equilibrium in Chinese Merino sheep.

The results of the LSM analysis showed that the coefficient of variation of wool fiber diameter of the CC genotype was extremely significantly larger than that of the TC and TT genotypes (P<0.01). The wool fiber diameter was no significant difference among the CC, TT, and TC genotypes. The wool fiber diameter standard deviation was no significant difference among the CC, TT, and TC genotypes *(Table 5)*.

Discussion

MicroRNA is a small non-coding RNA [28], several research had proved that miRNAs were related to the development of hair follicles, 22 new miRNAs and 316 conserved miRNAs were identified in the growth of skin and hair follicles in adult inner Mongolia cashmere goats [29]. MiR-203 was abundantly expressed in the epidermis and hair follicles, and closely related to the development of skin and hair follicles [18]. MiR-206 regulated the periodic changes of hair follicles by affecting the expression of genes related to hair follicle initiation and development in Shanbei white cashmere goats [19]. MiR-125b may act as a repressor to suppress hair follicle stem cell differentiation [20]. MiR-205 has a positive effect on hair follicle stem cells and the proliferation of their progenies [21].

oar-miR-377 has been reported to enhance fibronectin protein production, regulate angiogenesis, suppress cell proliferation, predict clinical outcomes in patients with gastric cancer, induce tumorigenesis, and promote oxidative stress [30]. Owing to the pleiotropic functions and DNMT1 targeting potential of oar-miR-377 may regulate human skin fibroblast (HSF) senescence by targeting DNMT1 [31]. Studies have shown that oar-miR-377 controls the occurrence and development of esophageal cancer by inhibiting the expression of CD133 and VEGF [32], and can regulate the NF-κB signaling pathway in melanoma cells by targeting the E2F3 gene [33]. The SLC24A2 gene is a cation exchanger and serves as the second member of the solute carrier 24 family. Although there is no direct evidence that SLC24A2 is related to skin hair follicles, SLC24A5 is related to animal hair color. Due to the mutation of the SLC24A5 gene, the golden mutation is accompanied by the decreased pigmentation of the skin melanophore and the retinal epithelium, and the number and density of melanosomes are reduced, resulting in the delay and reduction of melanin deposition. In this study, the Dual-Luciferase reporter assay was used to study the SLC24A2 targeting relationship with oar-miR-377,

and results showed that oar-miR-377 could significantly reduce the gene expression and protein expression of the SLC24A2 gene compared with NC, it was indicated that SLC24A2 is also regulated by oar-miR-377 to affect the quality of wool in sheep. Previous studies found that oar-miR-377 can regulate CD133, VEGF, and E2F3 genes in humans, and this study found that oar-miR-377 can regulate the SLC24A2 gene in sheep. This result indicates that oar-miR-377 is species-specific gene regulation.

The SNPs affect gene expression and complex diseases. However, because the thermodynamics of RNA-RNA binding plays a crucial role in the interaction of miRNA with target mRNA, it can be expected that sequence variants such as SNP in miRNA binding sites may affect the expression of miRNA targets. There was also evidence that SNPs in miRNA binding sites in oncogenes are associated with increased gene expression in papillary thyroid cancer [34]. Due to the mutation of the SLC24A5 gene, the golden mutation is accompanied by the decreased pigmentation of the skin melanophore and the retinal epithelium, and the number and density of melanosomes are reduced, resulting in the delay and reduction of melanin deposition [35]. In this study, T > C mutation was found at 276bp upstream flanking region of the oar-miR-377 by sequencing and sequence alignment. TT, TC, and CC genotypes were identified in Chinese Merino sheep (Xinjiang Junken type), and coefficient of variation of wool fiber diameter of the TC and TT genotypes was extremely significantly less than that of the CC genotype, it was indicated that the T allele is closely related to wool quality. These results suggest that SNP at 276bp upstream flanking region of the oar-miR-377 affected the binding with SLC24A2 and regulation of SLC24A2 expression. Currently, there were lack of clear research on the mutation of miRNA relationship to phenotypic traits, we speculated that this SNP of oar-miR-377 flanking region affected the expression level of mature miR-377 and SLC24A2 target gene, and indirectly regulated secondary hair follicle development and wool quality. As a result, the mutation of oar-miR-377 influences the coefficient of variation of wool fiber diameter. It was indicated that oar-miR-377 regulated hair follicle development, the mutation of oar-miR-377 affected wool quality. The T allele of 276T>C in oar-miR-377 could promote the secondary hair follicle development, the individuals with T allele could be selected in fine wool sheep breeding.

oar-miR-377 could significantly reduce the gene expression of the SLC24A2 at the level of transcription and translation, SLC24A2 was regulated by oar-miR-377 to affect the wool quality. T > C mutation found at 276bp upstream flanking region of the oar-miR-377 extremely affected coefficient of variation of wool fiber diameter in Chinese Merino sheep (Xinjiang Junken type), TC and TT genotypes could be favorable genotypes for improving wool quality in fine wool sheep breeding.

Declarations

Availability of Data and Materials: The data presented in this study are available on request from the corresponding author (H. Yang).

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Ethical Approval: This study was carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals and approved by the Experimental Animal Care and Use Committee of Xinjiang Academy of Agricultural and Reclamation Sciences (Shihezi, China, ethic committee approval number: XJNKKXY-2020-34, 30 December 2020).

Competing Interests: The authors declared that there is no competing interest.

Declaration of Generative Artificial Intelligence: The article, tables and figures were not written by AI and AI-assisted technologies.

Authors Contributions: Conceptualization and writing-original draft preparation: H. Zhou and H. Yang; Methodology and data curation: H. Yang; Validation: W. Fan; Investigation and samples: Qian Yu and Wenzhe Zhang; Writing-review and editing: Z. Zhao and Y. Yang; Funding acquisition: H. Yang. All authors have read and agreed to the published version of the manuscript.

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RESEARCH ARTICLE

Determination of Antioxidant and Immune Responses with Bile Acids Supplementation in Geese

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Abstract

This study investigated the impact of varying levels of bile acids (BAs) on the immune and antioxidant functions in the geese. A total of 168 male Hortobágyi geese, aged 28 days, were randomly assigned to four groups: the control group received a basic diet, while the other groups received diets supplemented with 75 mg/kg, 150 mg/kg, and 300 mg/kg of BAs. The trial lasted for 35 days, after which samples were collected for the analysis of antioxidant and immune indicators. Results showed that, compared to the control group, the supplementation of BAs in the feed did not significantly affect the indices of immune organs or serum immune levels ($P>0.05$). However, a supplementation with 300 mg/kg BAs significantly increased the expression of *IL-10* mRNA in the geese's spleens. Regarding antioxidant indicators, the addition of 150 mg/kg and 300 mg/kg BAs significantly enhanced the serum levels of Superoxide Dismutase (SOD), Glutathione Peroxidase (GSH-Px), and Total Antioxidant Capacity (T-AOC) ($P<0.05$), as well as upregulated the expression of liver *Nrf2*, *GPX-1* and *SOD1* mRNA. In conclusion, within the range of BAs supplementation used in this experiment, the improvement in geese's immune status was limited. Although higher levels of BAs might influence the immune status, the supplementation significantly enhanced the antioxidant capacity in serum and liver, with an optimal addition level of 150 mg/kg.

Keywords: Antioxidant, Bile acids, Geese, Immune response

INTRODUCTION

The Hortobágyi goose, developed by the Hungarian Hortobágyi Goose Joint Stock Company, stands out as a kombine breed for meat, down, and egg production. It stands out, experiencing a growing market demand in Central Asia and Europe, with a notable surge in popularity, especially in China [1]. The Hortobágyi goose is primarily raised in Heilongjiang and Yunnan provinces in China. It typically produces 40-50 eggs annually, with each egg weighing around 170-190 g. The breed also has a strong growth rate, with adult geese reaching 6-8 kg, and males weighing up to 12 kg by the end of the breeding cycle. In recent years, goose farming systems, driven by economic benefits and environmental protection pressures, have been shifting from traditional mixed water-land farming to modern, intensive, land-based farming [2]. In modern high-density, enclosed rearing conditions, animals face increased stress, leading to reduced immune strength, weakened antioxidant capacity, impaired digestive function, decreased feed conversion rates, and higher morbidity and mortality rates [3,4]. The growth stage from gosling to market readiness is particularly crucial for geese, as their antioxidant and immune responses during this period significantly influence their overall health and development. The antioxidant and immune systems serve as crucial pillars in maintaining animal health, with the antioxidant system combating cellular damage caused by free radicals, and a robust immune system effectively warding off diseases [5,6]. Both systems are indispensable for coping with environmental stress and promoting growth. One potential strategy to reduce stress in geese is the supplementation of BAs, which have been shown to alleviate stress responses and improve overall resilience. Therefore, implementing appropriate strategies to reduce the stress response in geese has become a pressing issue to address.

Bile acids (BAs), as amphipathic steroidal compounds, feature a unique molecular structure where hydroxyl and carboxyl groups on the side chain create a hydrophilic surface, while alkyl groups and hydrocarbon nuclei form a hydrophobic surface [7]. This special molecular configuration endows BAs with both hydrophilic and lipophilic properties, enabling them to effectively

emulsify lipids, forming oil-water mixtures, and increase the contact area with lipases, thereby accelerating fat digestion. The digestion products, encapsulated in BAs micelles, are absorbed by the villi in the small intestine [8,9]. Extensive research indicates that regulating BAs plays a significant role in improving lipid metabolism [10,11]. BAs function as potent antimicrobial agents. Their binding with *farnesol X receptor (FXR)* regulates the expression of antimicrobial substances in the gut, preventing bacterial overgrowth by activating the defense system of the small intestine. BAs receptors present in intestinal endothelial cells, immune cells, and epithelial cells contribute to the immunomodulation of the gut mucosa, playing a crucial role in maintaining intestinal immunity [12,13]. Moreover, BAs can suppress the expression of nuclear factor κB (nf-κb) by upregulating the expression of *FXR*, thus reducing inflammatory responses and enhancing immunity [14]. While current applications of BAs are predominantly in aquatic animals, for instance, adding 130 mg/kg of BAs to improve serum immunity in Thinlip mullet or *Litopenaeus vannamei* [15,16]. *NF-E2 related factor 2 (Nrf2)* is a critical transcription factor that regulates the expression of various antioxidant genes. BAs indirectly enhance cellular antioxidant capacity by affecting the *Nrf2* pathway [17]. In a previous study on broilers, dietary supplementation with BAs increased antioxidant enzyme activities and reduced oxidative stress by regulating *Nrf2* expression through binding with Kelch-like ECH-associated protein 1 (keap1) [18]. Multiple studies also show that appropriate addition of BAs to the diet can enhance serum levels SOD and GSH-Px [15,19]. However, the impact of BAs on the immune and antioxidant status of geese remains unexplored. This research aims to determine the effect of dietary BAs supplementation on the antioxidant capacity and immunity of geese, thereby providing foundational data for the application of BAs in goose diets.

Material and Methods

Ethical Statement

All animal procedures were performed according to guidelines provided by the China Council on Animal Care. All animal experiments were approved by the Animal Care and Use Committee of West Anhui University (Approval no: SYDW-P20210823021).

Experimental Design and Diet

The BAs used in this experiment were purchased from Zhengzhou Shangshui Biotechnology Co., Ltd, Henan Province, China. These BAs comprised 15% cholic acid, 44% hyodeoxycholic acid, and 40.50% chenodeoxycholic acid. A total of 168 male Hortobágyi geese, aged 28 days, were purchased from the Xiangtiange Farm in Ma'anshan City, Anhui Province, China. The geese were randomly allocated to four groups, each receiving a diet with different levels of BAs (0, 75, 150, and 300 mg/kg, respectively). The addition level of BAs in each group in the experiment was determined according to previous studies and through pre-experiments [20,21]. Each group consisted of six replicates, with each replicate comprising seven geese. The trial lasted 35 days. The selected intervention period (28-63 d) spans from the end of brooding to the market weight stage, which is the critical period for growth in geese. At 28 days, geese have developed some immunity from early vaccinations and maternal antibodies. However, other potential variables, such as diet, environmental conditions, and health status, were carefully controlled to minimize any confounding effects on the experimental results. The feed formulation strictly adhered to the recommendations of NRC 1994 [22], with appropriate modifications made according to the nutritional needs of goose rearing in China *(Table 1)*. All geese in the experiment were raised in an enclosed environment with a stocking density of 0.5 m^2 per goose. The ambient temperature was maintained at around 15°C. The geese were exposed to natural daylight during the day [23]. Throughout the experiment, the geese had unrestricted access to feed and water, and their health status and vaccination records were regularly monitored. The following vaccinations were administered during the study: At 28 days of age: H5+H7 inactivated vaccine (Qingdao Yibang Biotechnology Co., Ltd, Qingdao, China). At 40 days of age: Goose peritonitis + *E. coli* inactivated vaccine (Shandong Binzhou Wohua Biotechnology Co., Ltd, Binzhou, China).

Sample Collection

At the end of the experiment (63 d), the geese from each replicate underwent an 8-h fast, and their weight was measured. One goose close to the average weight was selected from each replicate for further analysis. A 5 mL blood sample was collected from the wing vein, and serum was prepared and stored in a -20°C freezer for subsequent analysis. Subsequently, the geese were euthanized using cervical dislocation, and the thymus, bursa of Fabricius, and spleen immune organs were excised and weighed. The immune organ index was calculated (immune organ index = weight of immune organ, g/live weight of the animal, kg) [24,25].

Serum Immune and Antioxidant Indexes

The antioxidant indexes of the serum were determined following the instructions of the test kits provided by Shanghai Renjie Biotechnology Co., Ltd., Shanghai, China. The total antioxidant capacity (TAC) of the serum was measured using the Fe^{2+} reduction method. Glutathione Peroxidase (GSH-Px) activity was assessed using the enzymatic colorimetric method. Catalase (CAT) activity was determined by the ammonium molybdate

Table 1. *Composition and nutrient level of experiment diets (air-dry basis)*

Ingredients	Content (%)	Nutrient Level	Content (%)
Corn	67.92	CP	16.00
Soybean meal	24.90	ME (MJ/kg)[b]	12.40
Soybean oil	2.00	CF	2.56
Lys	0.09	Ca	0.79
Met	0.09	P	0.51
Premix[a]	5.00	Lys	0.90
Total	100.00	Met+Cys	0.66
		Thr	0.63

[a] *One kilogram of the premix contained the following: Fe: 100 mg, Cu: 8 mg, Mn: 120 mg, Zn: 100 mg, Se: 0.4 mg, Co: 1.0 mg, I: 0.4 mg, Vit. A: 8330 IU, Vit. B_1: 2.0 mg, Vit. B_2: 0.8 mg, Vit. B_6: 1.2 mg, Vit. B_{12}: 0.03 mg, Vit. D_3: 1440 IU, Vit. E: 30 IU, Biotin: 0.2 mg, Folic acid: 2.0 mg, Pantothenic acid: 20 mg, Niacin acid: 40 mg*
[b] *Nutrient levels were all calculated values*

Table 2. *Primer sequences for genes used in RT-qPCR*

Genes	Primer Sequence 5′-3′	Genbank	Amplification Length
IL-6	F:AAGCATCTGGCAACGACGATAAGG	XM_048070285.1	90
	R:TGTGAGGAGGGATTTCTGGGTAGC		
IL-10	F:TGCCAGTCGGTGTCGGAGATG	XM_048071022.1	81
	R:CTGGTGGTGCTCGCTGTTCTTG		
SOD-1	F:ATCCTGAGGGCAAGAAGCA	XM_013192917.1	188
	R:TTTACCCAGGTCATCGCTTT		
GPX-1	F:GCAAGGGGTACAAGCCCAACT	XM_013201826.1	178
	R:GATGATGTACTGCGGGTTGGTC		
CAT	F:TGTAGAGGAAGCAGGAAGGC	XM_013194546.1	98
	R:AAGACCAGGATGGGTAGTTGC		
Nrf2	F:CGCCTTGAAGCTCATCTCAC	D_49365.1	176
	R:TTCTTGCCTCTCCTGCGTAT		
β-actin	F:TCCGTGACATCAAGGAGAAG	XM_013174886.1	144
	F:TCCGTGACATCAAGGAGAAG		

method. Total Superoxide Dismutase (T-SOD) activity was evaluated using the xanthine oxidase method, and the content of Malondialdehyde (MDA) was measured by the thiobarbituric acid method.

Immune indexes were analyzed using Enzyme-Linked Immunosorbent Assay (ELISA) kits (Shanghai Renjie Biotechnology Co., Ltd., Shanghai, China). These tests measured the concentrations of Immunoglobulin A (IgA), Immunoglobulin Y (IgY), Immunoglobulin M (IgM), and inflammatory markers including Interleukin-6 (IL-6) and Interleukin-10 (IL-10), in the serum.

Gene Expression

RNA was extracted from the liver and spleen using Trizol (Invitrogen, Carlsbad, CA, USA). Subsequently, total RNA was transcribed into cDNA using a reverse transcription kit (TransGen, Beijing, China). The concentration and purity of the cDNA were measured using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR to evaluate the expression levels of target genes was performed using the TransGen TB Green kit (TransGen Bio Inc., Beijing, China) and a QuantStudio 5 fluorescence quantitation system (Thermo Fisher Scientific, MA, USA). The target genes included IL6, IL10, SOD1, GPX1, CAT, with β-actin serving as the internal reference gene. The primer sequences for these genes are listed in *Table 2*. Data analysis was performed using the relative quantification method ($2^{-\Delta\Delta CT}$).

Data Analysis

Following initial processing using Excel 2019, the experimental data were analyzed using SPSS statistical software (version 26.0, SPSS Inc., Chicago, USA) for One-Way ANOVA. Group differences were evaluated through

Tukey's multiple comparison analysis to determine the significance of differences, with statistical significance denoted by P<0.05. The results are reported as mean values ± SEM.

Results

Immune Organ Indexes

During the entire study period, no deaths occurred in any of the experimental groups. The effects of different levels of BAs on the immune organ indexes of geese are displayed in *Table 3*. It was observed that, within the specified range of BAs concentrations for this experiment, there was no significant effect on the spleen index, bursa of Fabricius index, or thymus index in geese (P>0.05).

Serum Immune Indexes

To further assess the impact of BAs on the immune status of geese, we measured the levels of immune factors in the serum *(Fig. 1)*. The analysis revealed a decreasing trend in the level of IL-6 with increasing BAs concentrations. For IL-10, the group receiving 300 mg/kg BAs showed the highest level. The 150 mg/kg BAs group had the highest levels of serum IgA and IgM, and there was a trend of increasing IgG levels with higher BAs supplementation. However, no significant differences were observed between the groups (P>0.05).

Spleen Immune Gene Expression

The effects of different levels of BAs on the expression of *IL-6* and *IL-10* mRNA in the spleen are shown in *Fig. 2*. It was observed that 300 mg/kg of BAs significantly increased the expression of *IL-10* mRNA in the liver (P<0.05). Additionally, a significant difference was noted between the 75 mg/kg and 300 mg/kg BAs groups (P<0.05).

Serum Antioxidant Indexes

As illustrated in *Fig. 3*, it is apparent that serum levels of CAT and MDA did not exhibit significant differences across all groups. The supplementation of BAs at levels ranging from 75 mg/kg to 300 mg/kg significantly increased serum levels of SOD and GSH-Px (P<0.05), with no significant differences observed among the various BAs supplementation groups. Notably, serum SOD levels continuously increased, peaking at 150 mg/kg for GSH-Px. Additionally, the supplementation with 150 mg/kg or 300 mg/kg BAs significantly enhanced serum TAC levels (P<0.05).

Table 3. *The effects of supplement BAs on the immune organ indexes of geese*

Items	Treatment				P-value
	0 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	
Spleen index, g/kg	0.73±0.08	0.64±0.07	0.70±0.07	0.67±0.09	0.858
Bursa of Fabricius index, g/kg	2.49±0.26	2.73±0.84	2.67±1.15	1.71±0.26	0.102
Thymus index, g/kg	0.56±0.03	0.64±0.09	0.51±0.05	0.58±0.04	0.425

[a,b,c] *Values within a row with different superscripts differ significantly at P<0.05*

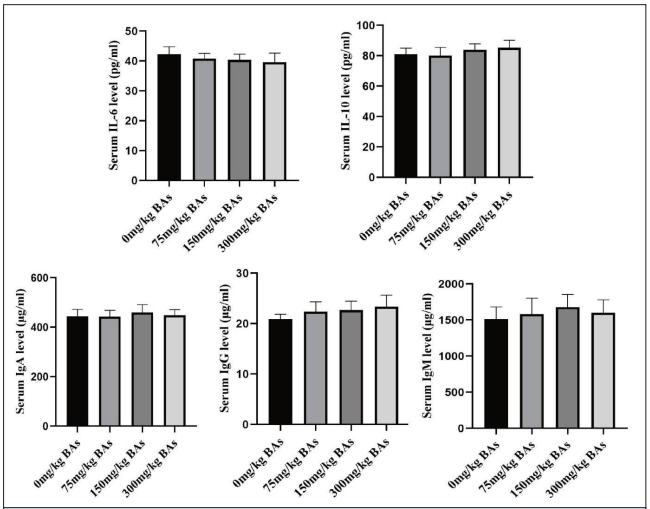

Fig 1. Effects of different BAs levels on serum immune indexes. The analysis of the impact was conducted using one-way ANOVA, with group differences deemed significant at P<0.05. Distinct means, labeled as a, b, and c, are significantly different when the P<0.05

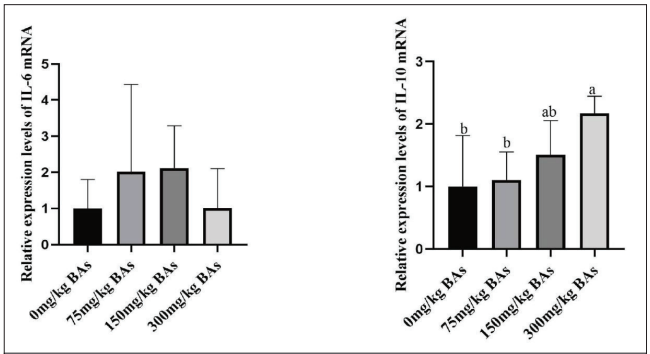

Fig 2. Effects of different BAs levels on spleen immune gene expression. The analysis of the impact was conducted using one-way ANOVA, with group differences deemed significant at P<0.05. Distinct means, labeled as a, b, and c, are significantly different when the P<0.05

Liver Antioxidant Gene Expression

The *Fig. 4* presents the expression of antioxidant genes in the liver across different groups. Similar to the serum

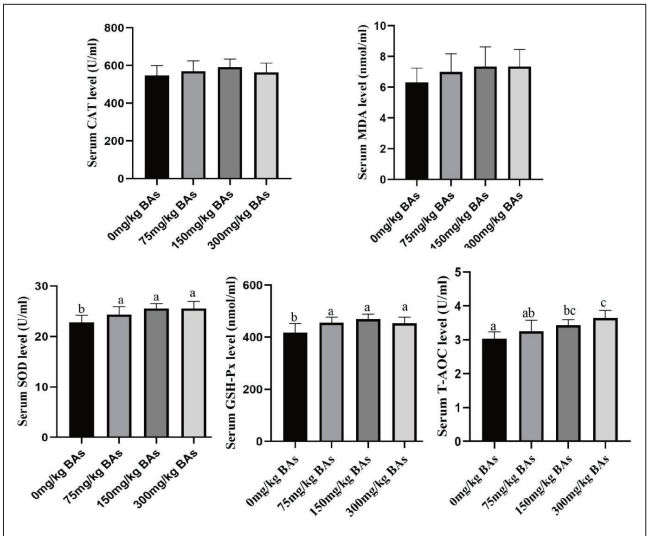

Fig 3. Effects of different BAs levels on serum antioxidant indexes. The analysis of the impact was conducted using one-way ANOVA, with group differences deemed significant at P<0.05. Distinct means, labeled as a, b, and c, are significantly different when the P<0.05

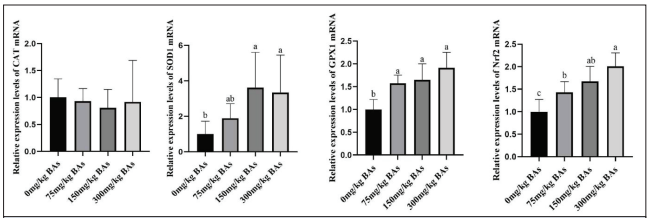

Fig 4. Effects of different BAs levels on Liver antioxidant gene expression. The analysis of the impact was conducted using one-way ANOVA, with group differences deemed significant at P<0.05. Distinct means, labeled as a, b, and c, are significantly different when the P<0.05

antioxidant levels, there were no significant differences in the expression of *CAT* mRNA in the liver among the groups (P>0.05). However, supplementation with 150 mg/kg or 300 mg/kg of BAs significantly increased the expression of *SOD1*, *GPX-1*, and *Nrf2* mRNA in the liver (P<0.05).

Discussion

Maintaining an optimal level of immune activity is crucial for the healthy growth of animals in poultry farms [26]. Immune organs, including the thymus, spleen, and bursa of Fabricius, play crucial roles in lymphocyte production, immune response [27,28]. In this study, we investigated the effects of BAs supplementation on the immune status of geese by measuring immune organ indices and serum immunoglobulins. We found no significant differences in the immune organ indices across different levels of BAs. This suggests that at the concentrations used, BAs may not have a direct effect on the development or function of these primary immune organs. These results align with some previous studies, where BAs did not significantly affect immune organ indices, which might indicate that their immune-modulating effects are not primarily exerted on immune organs themselves [29]. In contrast, studies have shown that BAs can influence immune responses, particularly within the gut, rather than the immune organs per se. For instance, BAs have been found to modulate gut-associated lymphoid tissue and influence immune cell responses in the gut. This suggests that the immune effects of BAs may be more localized, acting on mucosal immunity rather than systemic immune organs [30]. Furthermore, regarding humoral immunity, our study did not observe any significant changes in serum IgA, IgM, or IgG levels. This is consistent with other studies in which BAs did not significantly affect immunoglobulin levels in serum [31]. However, some research has shown that BAs can elevate intestinal IgA levels, particularly in animals with altered gut microbiota or those exposed to high-fat diets [28]. These studies suggest that BAs may play a more significant role in regulating gut immunity rather than circulating immunoglobulin levels. One notable finding in our study was the increased expression of IL-10 in the spleen at 300 mg/kg of BAs supplementation. This is in line with previous studies showing that BAs can reduce pro-inflammatory cytokines such as IL-6, while enhancing anti-inflammatory cytokines like IL-10 [32]. The increase in IL-10 expression in the spleen may indicate a local immune modulation effect by BAs, potentially through interactions with immune cells in the spleen. This is an area that requires further investigation, as the precise mechanisms of BAs in regulating immune responses at the molecular level are not fully understood.

Under normal circumstances, the body can neutralize surplus free radicals through its enzymatic antioxidant defense system, providing protection against oxidative damage. The assessment of oxidative damage involves measuring the activities of antioxidant enzymes and the content of MDA [33]. TAC, serving as a comprehensive indicator of the antioxidant system, reflects the cumulative effect of antioxidants in the body. Generally, higher TAC values within a certain range indicate better antioxidant capabilities of the organism [34]. One of the primary physiological functions of BAs is their antioxidant activity. Numerous studies have shown that BAs have beneficial effects on the body's redox balance. For instance, BAs can influence the PI3K/Akt signaling pathway, closely related to cellular survival, proliferation, and antioxidant defense mechanisms [35]. Serum antioxidant indicators most directly reflect the oxidative stress state of animals, and a certain amount of BAs can significantly increase serum levels of SOD and GSH-Px in pigs [36]. Further studies indicate that adding BAs to the diet of aquatic animals can effectively prevent oxidative damage and improve the health status of farmed fish [37]. This experiment demonstrates that adding BAs to the diet of geese

significantly enhances the antioxidant capacities of SOD, GSH-Px, and TAC in serum. These results underscore that BAs have the potential to improve the serum antioxidant capacity in geese.

Playing a crucial role in digestion, absorption, substance metabolism, and immune response within the animal body, the liver stands as a vital organ in livestock and poultry. Exposed to endogenous toxins delivered via the portal vein, the liver is a primary site of oxidative stress [38]. The *Nrf2* pathway plays a crucial role in combating oxidative stress and inflammation. *Nrf2*, identified over a decade ago as an essential transcription activator of antioxidative genes, exerts significant antioxidative protective effects. It is activated by many phenolic antioxidants, whose antioxidative activities are largely regulated through the *Nrf2* mechanism [39]. Upon oxidative stress stimulation, *Nrf2* binds to the antioxidant response element, regulating Phase I detoxifying enzymes and heme oxygenase-1, among other antioxidative enzymes to combat the cytotoxicity caused by oxidative stress, ultimately restoring cellular redox homeostasis [40]. In our experiment, a significant upregulation trend in liver *Nrf2* mRNA expression was observed with the addition of BAs to geese diets. Similarly, in chicken diets, the addition of BAs can regulate *Nrf2* expression by binding to *Keap1*, thereby reducing oxidative stress. It is noteworthy that some studies suggest that oxidative interference can hinder *Keap1*-mediated *Nrf2* ubiquitination but does not disrupt the *Nrf2*/*Keap1* binding [41]. Therefore, it can be inferred that under severe oxidative stress, the probability of *Nrf2* dissociating from *Keap1* and entering the nucleus to activate antioxidative genes increases. Furthermore, *Nrf2* directly controls glutamate-cysteine ligase and glutathione synthetase to regulate GSH levels. Beyond GSH synthesis, *Nrf2* also plays a role in maintaining GSH [42]. By regulating the transcription of many ROS-detoxifying enzymes, *Nrf2* reduces ROS production by improving mitochondrial function and reducing oxidative stress. Mitochondria, critical sites of ROS production within cells, can have reduced ROS production with BAs maintaining mitochondrial integrity and function [43]. This study also found that 150 mg/kg BAs or 300 mg/kg BAs increased liver *Nrf2* expression, subsequently enhancing the expression of *SOD1* and *GPX-1*. This indicates that BAs supplementation effectively improved the antioxidative state of the liver.

In summary, we conclude that the expression levels of *IL-10* in the spleen increased with the supplementation of 300mg/kg BAs in geese diets, while the expression levels of *SOD1*, *GPX-1*, and *Nrf2* genes in the liver were significantly enhanced with 150 mg/kg or 300 mg/kg BAs. Consequently, this led to an increase in the levels of serum SOD, GSH-Px, and TAC. Therefore, the antioxidative status of geese can be effectively improved with BAs. The inclusion of exogenous BAs in the diet of geese can ameliorate their stress status, with the optimal supplementation level being 150 mg/kg.

Declarations

Availability of Data and Materials: The original data of the paper are available upon request from the corresponding author (G. Xu).

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Ethical Statement: All animal procedures were performed according to guidelines provided by the China Council on Animal Care. All animal experiments were approved by the Animal Care and Use Committee of West Anhui University (Approval no: SYDW-P20210823021).

Competing Interests: The authors declared that there is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The author declare that the article tables and figures were not written or created by AI and AI-assisted technologies.

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Research Article

The Effect of Systemic Hypertension on Prostatic Arterial Hemodynamics in Dogs with Benign Prostate Hyperplasia

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Abstract

This study aimed to examine how systemic hypertension impacts resistance indices in the prostate artery by comparing ultrasonographic assessments of the prostate gland between normotensive and hypertensive dogs with benign prostatic hyperplasia (BPH). Dogs presenting with symptoms such as frequent urination, painful urination, and blood in the urine, and diagnosed with BPH, were divided into two groups: normotensive and hypertensive, with each group consisting of ten dogs. Blood pressure measurements, as well as B-mode and Doppler ultrasonographic evaluations, were conducted. The mean age for the normotensive and hypertensive groups were 9.80±1.81 and 11.10±1.79 years, respectively. The mean weight for the normotensive and hypertensive groups were 27.60±6.02 and 29.00±4.00 kg, respectively. The mean prostate volume for the normotensive and hypertensive groups were 22.35±2.76 and 22.78±2.35 years, respectively (P=0.708). Dogs with BPH who also had hypertension exhibited significantly higher resistive indices in the prostate arteries compared to those with BPH who were normotensive (P<0.001). While the RI value was 0.76±0.03 in BPH dogs with hypertension, the RI value was 0.62±0.05 in normotensive BPH dogs (P<0.001). Finally, in veterinary practice, when dealing with a hypertensive patient, it is important to consider potential risk factors for prostatic vascular dysfunction. Additionally, the possibility of clinically significant BPH in hypertensive patients should be considered, and appropriate diagnostic tests should be conducted.

Keywords: Benign prostatic hyperplasia, Doppler ultrasonography, Hypertension, Resistive index

Introduction

Systemic hypertension (SH) refers to the abnormal and persistent increase in the pressure exerted by arterial blood on the vascular walls and the organs supplied by these vessels [1]. In veterinary medicine, SH is classified into 3 categories. These are situational, secondary and idiopathic systemic hypertension [2]. In current guidelines, normotensive systolic and diastolic pressure in cats and dogs are accepted as 120-130 and 60-90 mmHg, respectively [3,4]. However, it has been reported that systolic pressure higher than 150 mmHg increases the risk of target organ damage. For this reason, senior life recommends measuring blood pressure in dogs and cats at some stage and performing detailed examination in all animals with blood pressure more than 150 mmHg [5].

Benign prostatic hyperplasia (BPH) is the most common prostate disease affecting male dogs and develops spontaneously as glandular hyperplasia [6,7]. It is most often the result of aging and increased levels of dihydrotestosterone (DHT) in unspayed male dogs, which causes an increase in the size (hypertrophy) and number (hyperplasia) of prostate epithelial cells (hyperplasia: main mechanism in dogs), forming BPH [8,9]. Methods used to diagnose BPH include anamnesis, clinical findings, physical examination, rectal palpation of the prostate contour, radiographic measurement of prostate size, ultrasonographic measurement of prostate volume and parenchyma, ultrasound-guided fine needle aspiration, and excisional biopsy [10]. Since BPH and many other prostate diseases are associated with enlargement of the prostate, measuring prostate size is very important for diagnosis [11]. Ultrasonography is the most important diagnostic method of choice to examine the prostate and allows assessing both the size of the gland and the homogeneity of its parenchyma [12]. In addition, ultrasonographic examination provides information about the shape, contour, echogenicity and symmetry of the prostate and also provides information about

the adjacent soft tissue [13]. In recent years, the resistive index (RI) of the prostatic artery measured by Doppler ultrasonography has been used to evaluate patients with BPH, and RI has been reported to be increased in dogs with BPH [14-16].

Research in human medicine shows that systemic hypertension (SH) is involved in the etiology of BPH [17,18]. Additionally, BPH symptom scores have been reported to be higher in BPH patients with SH compared to healthy men [19]. The prevalence of SH and BPH are both emerging as a function of increasing age [18]. However, some studies have shown that, regardless of age, patients with symptoms of cardiovascular disease/atherosclerosis/hypertension are at much higher risk for BPH than those without the disease [20]. Systemic vascular dysfunctions may specifically affect the prostate gland [21]. The vascular system of the prostate is an important component of prostate growth and regulation, and therefore the idea was developed that dysfunction of blood flow in the prostate gland is involved in causing and controlling BPH [22].

The aim of this study was to find out whether there is a significant difference between the prostatic arterial resistive indexes of systemic hypertension dogs with BPH and the prostatic arterial resistive indexes of normotensive dogs with BPH. We hypothesized that the prostatic arterial resistive index would be significantly higher in systemic hypertension dogs with BPH than normotensive dogs with BPH.

Materials and Methods

Ethical Statement

This study was approved by the Ondokuz Mayıs University, Animal Experiments Local Ethics Committee (Approval no: E-68489742-604.01-2400102486). In addition, an Informed Consent Form (for each patient) was obtained from dog owners.

Animal and Groups

Twenty dogs belonging to clients at the Ondokuz Mayıs University Veterinary Hospital were included in this study. In the control group, 10 patients with different breeds; the breeds were mixed-breed dogs (n=4), Golden retrievers (n=2), English setter (n=1), German pointer (n=1), Siberian husky (n=1), and Belgian shepherd (n=1). In the study group, 10 patients had different breeds; the breeds were mixed-breed dogs (n=3), Golden retrievers (n=2), German shepherds (n=3), and Labrador retrievers (n=2). Dogs presenting with symptoms such as frequent urination, painful urination, constipation, and blood in the urine, and diagnosed with BPH, were divided into two groups: normotensive BPH and hypertensive BPH group, with each group consisting of ten dogs. Also, the study group (hypertensive BPH) consisted of 10 dogs with BPH diagnosed with hypertension that presented to our hospital with blood pressure problems (nervous system symptoms such as dilated eyes, depression, head tilt, weakness, heart murmurs, or abnormal heart rhythm symptoms). The control group (normotensive BPH) consisted of 10 dogs with BPH without any disease other than the diagnosis of BPH.

Hematological and serum biochemical analyses were performed on dogs to exclude infection, metabolic or other diseases. Four dogs with infection, metabolic or other diseases were excluded from the study. Limitations of this study are that the marker of BPH was not supported by biopsy and hormonal imbalances involving dihydrotestosterone (DHT), estrogen, and testosterone were not determined in the formation and progression of BPH.

Study Design

Inclusion in the hypertensive group (systemic hypertension) was determined based on documentation in the medical record of an elevated blood pressure in the range for systemic hypertension. Systolic blood pressure was measured in all dogs using the Doppler Blood Pressure System method (Vet-Dop2, USA, Vmed Technology, Washington) placed between the tarsal and metatarsal pad of the hind limb *(Fig. 1)*. The final value of systemic blood pressure (SBP) was the mean value of 5 consecutive consistent measurements after, at least, 5 min acclimation period and before performing any other procedure. Systemic hypertension was classified as mild (150-159 mmHg), moderate (160-179 mmHg), or severe >180 mmHg [2]. Animals were included in the hypertensive group when SBP was ≥150 mmHg. The same investigator included dogs in the control group if they had a recorded normal blood pressure (less than 150 mmHg).

Prostatic ultrasonographic evaluation was performed from the abdominal region with a Mindray Vetus 9 color Doppler ultrasound device and a microconvex probe 6.5-7.5 MHz transducer. Dogs were placed in dorsal recumbency and caudal abdominal region was sheaved before ultrasonographic scanning [23]. Prostatic volume (PV) was evaluated by B-mode ultrasonography using the bladder as a window, measuring height and length in the sagittal plane and width in the axial plane *(Fig. 2)*. PV was calculated using the formula: PV (cm^3)=0.487×L×W×(DL+DT):2+6.38 (L=length; DL=depth on longitudinal section; DT=depth on transverse section; W=width) [24]. The expected prostate volume (EPV) was used to predicted the prostatic volume according to the dog weight, i.e., a prostatic volume control for each dog body weight, considering the following formula: EPV=8.48+(0.238×kg body

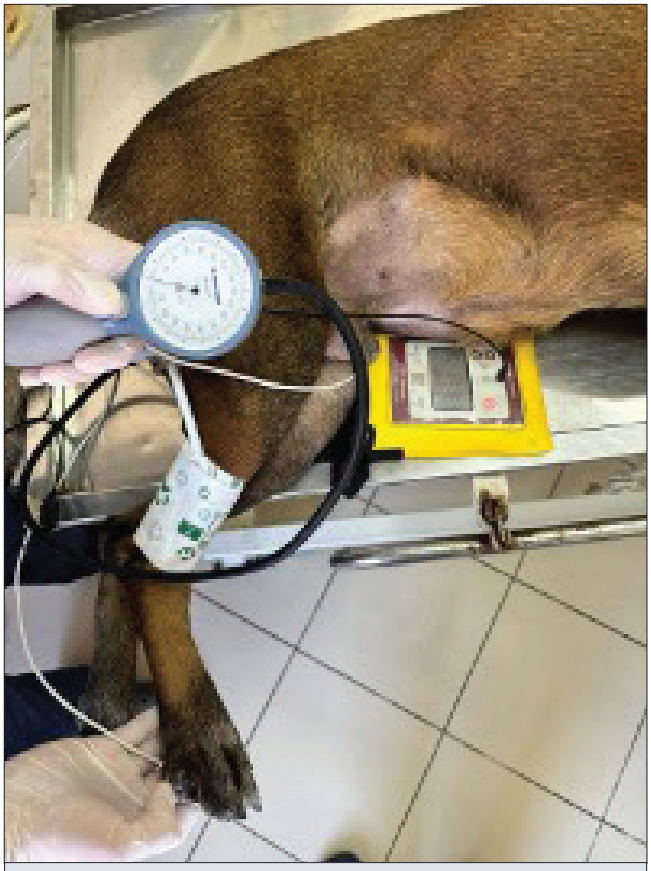

Fig 1. Systolic blood pressure obtained while lying in a lateral position using Vet-Dop2. This dog's systolic blood pressure was 175

weight) [24,25]. Prostatic tissue perfusion and blood flow velocity of the prostatic artery were evaluated by Doppler ultrasonography *(Fig. 3)*. Prostatic artery was scanned at the hypogastric abdominal region and located cranio-dorsal to the prostate gland [22,26,27]. The size of the sample volume, which determines Doppler information, was kept constant at 1 mm. Color flow Doppler was used to map the vessel and subsequently pulsed-wave Doppler was used to characterize the waveform. Blood sample volume was positioned at the artery center and all measurements were obtained with an angle of ≤60°, making proper angle correction whenever necessary. A total of 6 stable waves of the prostatic artery were obtained to calculate the average of each variable. Spectral waveform analysis was performed after the blood flow samplings were performed. Thereby, the mean values of RI, and PI ratio were calculated after 3 consecutive measurements were performed. The following blood flow velocity parameters were automatically calculated by the Doppler machine software, using mathematical formulas or Pourcelot index: peak systolic velocity (PSV), end diastolic velocity (EDV), resistance index [RI=(PSV-EDV) /PSV], pulsatility index [PI=(PSV-EDV)/mean velocity], time average maximum velocity (TAMAX) and peak systolic: diastolic velocity [S/D=(PSV/EDV)]. Analysis was performed always by only one analyzer.

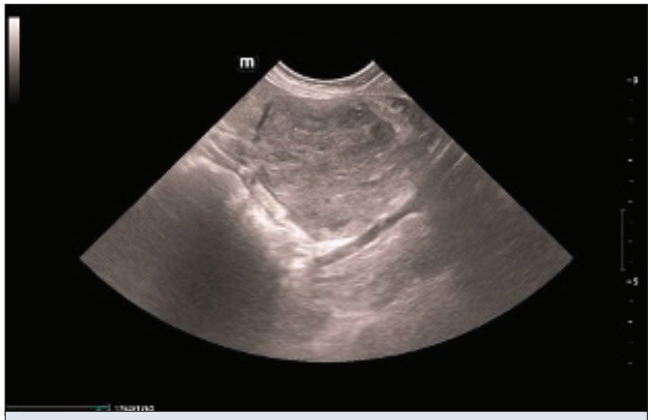

Fig 2. B-mode imaging of a benign prostatic hyperplasia. Hyperechoic parenchyma and enlarged prostate

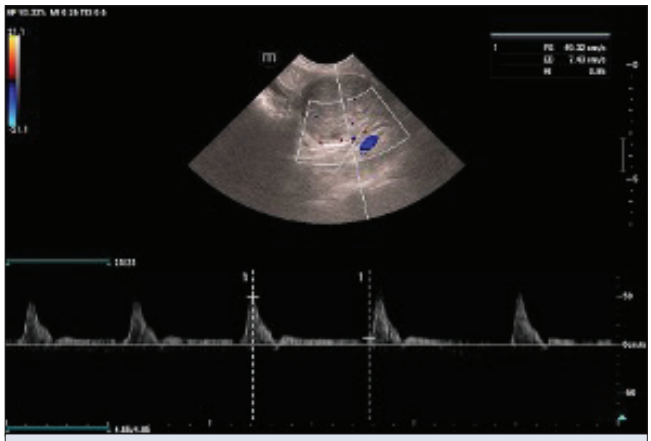

Fig 3. Longitudinal ultrasound image obtained in right lateral recumbency using Mindray Vetus 9. This dog presented with systolic blood pressure of 180, and a prostatic arterial RI of 0.85

Statistical Analysis

The data were analyzed using the IBM SPSS Statistics for Windows (IBM Corp., Armonk, N.Y., USA). Means and standard deviation were obtained for the continuous variables. Quantitative data were assessed for normality using the Kolmogorove-Smirnov test, Shapiroe-Wilk test, and direct data visualization methods. Quantitative data were compared between the study groups using independent t-test for normally distributed quantitative variables. P values less than 0.05 were considered significant. Correlations between measured variables were evaluated with Pearson correlation coefficient.

Results

No significant difference was found between the groups in terms of age, weight and prostate volumes. However, there was a significant difference (P<0.001) in systolic and diastolic blood pressure between the hypertensive and normotensive BPH groups *(Table 1)*.

When hemodynamic changes between the two groups were examined; PSV in the hypertensive BPH group was

Table 1. *Age, weight, prostate volume and blood pressure findings of the control and study groups*

Parameters	Normotensive BPH Group Mean±S.D.	Hypertensive BPH Group Mean±S.D.	P Value
Age (years)	9.80±1.81	11.10±1.79	0.548
Weight (kg)	27.60±6.02	29.00±4.00	0.124
PV (cm^3)	22.35±2.76	22.78±2.35	0.708
EPV (cm^3)	15.04±1.43	15.37±0.95	0.548
SBP (mmHg)	124.00±6.14	179.50±4.97	**<0.001**
DBP (mmHg)	84.00±3.94	103.00±6.32	**<0.001**

PV: prostatic volume; EPV: expected prostatic volume; DBP: diastolic blood pressure; SBP: systolic blood pressure

Table 2. *Prostatic arterial Doppler hemodynamic findings of the control and study groups*

Parameters	Normotensive BPH Group Mean±S.D.	Hypertensive BPH Group Mean±S.D.	P Value
PSV (cm/s)	16.21±2.15	23.03±3.42	**<0.001**
EDV (cm/s)	5.99±1.06	5.35±1.18	0.215
RI	0.62±0.05	0.76±0.03	**<0.001**
PI	1.06±0.11	1.34±0.07	**<0.001**

PSV: peak systolic velocity; EDV: end diastolic velocity; RI: Resistive Index; PI: Pulsatility Index

Table 3. *Correlation of PV, PSV, EDV, RI and PI of benign prostatic hyperplasia dogs (n=20) with other parameters*

Parameters	PV (cm^3)		PSV (cm/s)		EDV (cm/s)		RI		PI	
	r	P Value	r	P Value	r	P Value	r	P Value	r	P Value
Age (years)	-0.005	0.983	0.302	0.196	0.255	0.278	0.050	0.835	0.271	0.247
Weight (kg)	**0.881***	**<0.001**	0.235	0.318	0.245	0.298	-0.027	0.910	0.032	0.893
SBP (mmHg)	0.109	0.647	**0.833***	**<0.001**	-0.265	0.259	**0.866***	**<0.001**	**0.878***	**<0.001**
DBP (mmHg)	0.203	0.391	**0.773***	**<0.001**	-0.186	0.434	**0.740***	**<0.001**	**0.748***	**<0.001**
PV (cm^3)	1	NA	0.212	0.369	0.180	0.448	0.014	0.952	0.162	0.496
PSV (cm/s)	0.212	0.369	1	NA	0.176	0.458	**0.670***	**0.001**	**0.752***	**<0.001**
EDV (cm/s)	0.180	0.448	0.176	0.458	1	NA	**-0.603***	**0.005**	-0.275	0.240
RI	0.014	0.952	**0.670***	**0.001**	**-0.603***	**0.005**	1	NA	**0.826***	**<0.001**
PI	0.162	0.496	**0.752***	**<0.001**	-0.275	0.240	**0.826***	**<0.001**	1	NA

** statistically significant; -: negative correlation; PV: prostatic volume; PSV: peak systolic velocity; EDV: end diastolic velocity; RI: Resistive Index; PI: Pulsatility Index; SBP: systolic blood pressure; DBP: diastolic blood pressure; NA: not applicable*

higher than the normotensive BPH group, and this was found to be a significant difference (P<0.001). RI and PI were significantly higher (P<0.001) in the hypertensive BPH group than in the normotensive BPH group. No significant difference was found between the groups in terms of EDV (*Table 2*).

A positive correlation was found between weight and prostatic volume, systolic blood pressure and peak systolic velocity, diastolic blood pressure and peak systolic velocity, systolic blood pressure and resistive index. Also positive correlation was found diastolic blood pressure and resistive index, peak systolic velocity and resistive index, systolic blood pressure and pulsatility index, diastolic blood pressure and pulsatility index, peak systolic velocity and pulsatility index, resistive index and pulsatility index. Resistive index and Pulsatility index were also positively

correlated with peak systolic velocity. It was noted that there was a negative correlation between the resistive index and end diastolic velocity *(Table 3)*.

Discussion

In this study, prostatic hyperplasia (PH) and prostatic arterial hemodynamics were investigated in hypertensive and normotensive dogs.

The relationship between SH and BPH first emerged in the field of human medicine in 1966 when the etiologic similarities of these two diseases were reported and has been studied for more than 50 years [17]. Furthermore, the pathogenesis of BPH is still unclear, possibly due to a number of complex mechanisms. This aspect is a current issue that is attracting the attention of more researchers.

Systemic hypertension appears to play a specific role in the pathogenesis of BPH through both static and dynamic components. Systemic vascular dysfunction can affect the prostate gland and cause dysfunction of blood flow in the prostate gland. Systemic hypertension has been proven to have a higher prevalence of BPH and lower urinary tract symptoms (LUTS) in humans, in various animal models and in epidemiologic studies [28].

Studies in human medicine have reported that prostatic arterial RI is higher in patients with hypertension than in normotensive men [29]. A previous study has shown that hypertensive men have more severe urinary tract symptoms and are more prone to prostatomegaly than normotensive men [30]. Hammarsten et al.[28] reported that individuals with treated hypertension had a larger prostate volume and higher BPH growth rate than normotensive individuals.

In addition, the relationship between SH and PH has also been supported by studies in veterinary medicine using animal models. Spontaneously hypertensive rats (SHR), an animal model of systemic hypertension, have been found to exhibit abnormal lower urinary tract function similar to patients with BPH [31]. In a different study, it was reported that SHR rats with prostatic hyperplasia had increased development of glandular epithelium of the prostate. SHR rats were found to develop BPH-like features in the absence of any inductive exogenous agent. Conversely, normotensive rats were reported not to develop such features [32].

Statistical differences were found between the targeted parameters in SH dogs in the study. There are many proposed mechanisms to link the development of hypertension and BPH, such as age and weight [18,33]. Weight and age were kept similar in the groups of dogs presented in the study. Age is associated with hypertension and BPH, as found in a previous study [18]. An age-related increase in BPH in dogs is also well known, so age and weight differences were eliminated between the groups of dogs in the study to ensure that age and weight were not a factor in the development of BPH in hypertensive dogs [34]. In addition, a study has determined that there is a significant, age-independent relationship between BPH symptoms and hypertension. This finding points to a common pathophysiological factor for both disease states, such as increased sympathetic activity [19]. Consistent with previous findings, our current study showed that hypertension can often be accompanied by BPH or changes in prostatic arterial hemodynamics. In a 2023 study [29], patients with BPH and hypertension had significantly higher prostate artery resistance indices than normotensives with BPH. Even in patients with BPH and controlled hypertension, prostate artery resistance indices were still higher than normotensive men with BPH. The incidence of BPH may be significantly increased in dogs with hypertension. Prostatic arterial hemodynamics may be impaired in hypertensive dogs and it is suggested that hypertension may be an important factor influencing the incidence of BPH. We suggest future large-scale studies to further confirm our results.

In conclusion the studies presented, it was concluded that diabetes mellitus, hypertension, obesity, ischemic heart disease, excessive carbohydrate and fat intake, hyperinsulinemia, insulin resistance and dyslipidemia are risk factors in the development of BPH [29,33,35]. In addition, vascular dysfunctions are known to specifically affect the prostate gland [21]. In veterinary medicine, when confronted with a hypertensive patient, the possible presence of risk factors for dysfunction of the prostatic vascular system should be considered. The possibility of a clinically significant BPH in hypertensive patients should also be kept in mind and necessary examinations should be performed.

Declarations

Availability of Data and Materials: The datasets during and analysed during the current study available from the corresponding author (Ç. Esin) on reasonable request.

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Competing Interest: The author declared that there is no conflict of interest.

Ethical Approval: This study was approved by the Ondokuz Mayıs University, Animal Experiments Local Ethics Committee (Approval no: E-68489742-604.01-2400102486).

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Research Article

Epidemiological Survey on Tick Borne Diseases of Pet Dogs in Korla, Northwestern China

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Abstract

Pet dogs pose a potential risk to transmitting zoonotic pathogens by ticks. However information about the prevalence status in pet dogs of tick-borne diseases is currently limited. In this study, 196 blood samples and 223 ticks were collected from pet dogs in Korla, northwestern China. Based on morphological and molecular characteristics, all ticks were identified as *Rhipicephalus turanicus* sensu stricto. We used primers targeting the 16S ribosomal *(16S rRNA)* gene for detection of *Anaplasma bovis* species, targeting the small subunit 18S ribosomal RNA(*18S rRNA*) gene for detection of *Hepatozoon canis* species and targeting htpAB-associated repetitive element gene (IS111) for detection of *Coxiella burnetii* species The nested PCR(nPCR)-positive products were sequenced, aligned, and phylogenetically analyzed. three tick-borne pathogens were detected in the samples. *Coxiella burnetii* were detected both in parasitic ticks and in blood samples with a detection rate of 17.93% (40/233) in tick and 79.1% (155/196) in blood samples, followed by 21.52% *H. canis* (48/233) in tick, 2.5% A. bovis (5/196) in blood samples. This study provided molecular evidence for the occurrence of *A. bovis, H. canis* and *C. burnetii* circulating in pet dogs and their ticks in northwestern China. Understanding the prevalence of Tick-borne diseases in pet dog is essential for developing effective strategies for disease control and management.

Keywords: Pet dogs, Ticks, *Anaplasma bovis*, *Coxiella burnetii*, *Hepatozoon canis*, Northwestern China

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Introduction

Tick-borne diseases (TBDs) are relatively common in pet dogs, and dog-human relationships may facilitate the spread of tick-borne pathogens among people [1]. Many factors can affect the spread of tick-borne diseases in pet dogs, such as the pet dog's living environment, parasite control, and the health awareness of pet dog owners [2]. However, parasite control in pet dogs is often neglected due to the lack of knowledge about parasite hazards by dog owners [3]. Due to the increasing number of pet dogs in China and their close relationship with humans, there is a need to study the epidemiological status of tick-borne zoonotic pathogens [4].

Korla City is listed as an important transportation hub and material distribution center of Xinjiang Uygur Autonomous Region (XUAR, northwestern China) with more than 477.000 residents. It is located in the northeast edge of the Taklamakan Desert, which is the second largest desert in the world. Extreme dryness with an average annual precipitation of 58.6 mm and an annual maximum evaporation of 2788.2 mm is its climatic characteristics [5]. *Rhipicephalus turanicus*, *Dermacentor marginatus* and *Hyalomma asiaticum* were previously reported as dominant tick species in the oasis of Taklamakan desert [6]. TBPs in pet dogs and their ticks, such as *Candidatus Rickettsia barbariae*, *Rickettsia massiliae*, *Rickettsia conorii*,

Rickettsia sibirica, *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, *Anaplasma ovis* and *Brucella* spp., were previously reported in north region of XUAR [7,8]. In present study, we further investigated the blood samples of pet dogs and tick,which will help disease prevention and control in Korla City, .

Material and Methods

Ethical Statement

This study was reviewed and approved by the ethics committee of School of Medicine, Shihezi University in accordance with the medical regulations of China (Approval numbers A2020-113-01).

Sample Collection

Two shelters for stray dogs and five pet hospitals close to pastures in Korla City (934 m above sea level; 41°14′N 85°11′E) in Tarim Basin, XUAR were selected between late April to mid-May 2021(coinciding with the peak activities of adult ticks), blood and tick samples were collected from pet dogs based on clinical symptoms that include depression, weight loss, and anorexia. All samples were collected with the permission of the pet owner and sample collection was carried out by a local veterinarian. The blood samples are collected into a vacuum tube containing ethylene diamine tetraacetic acid (EDTA) anticoagulant while ticks were picked from dogs and placed in tubes containing 75% ethanol and 5% glycerol.

Identification of Ticks

Extracted total DNA from 200 µL whole blood samples using a blood DNA extraction kit (Omega Bio-tek, Norcross, USA) and genomic DNA from whole ticks using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China) following the manufacturer's instructions. All ticks were identified based on morphology before DNA is extracted, as previously described [9,10]. Subsequently, 30 representative ticks, with 4-6 ticks at each veterinary clinic, were subjected to molecular classification analysis based on partial mitochondrial 16S ribosomal RNA *[16S* rRNA (460bp)] gene sequences to confirm tick species [11].

Detection of Tick-Borne Pathogens

We used a partial *16S rRNA* (450bp) gene to detect *A. bovis* [12]. The molecular detection of *C. burnetii* was per6formed using the IS111 (260bp) [13]. *Hepatozoon canis* were detected targeting 18S ribosomal RNA *(18S rRNA)* [14]. DNA from our laboratory was used as positive controls for *A. bovis*, *C. burnetii* and *H. canis*. Double distilled water was used as a negative control (Dongsheng, Guangzhou, China). Characteristics of the amplified fragments and corresponding primer sequences are provided in *Table 1*.

Sequencing and Data Analyses

Sequencing data were subjected to Basic Local Alignment Search Tool (BLAST) searches *(http://www.ncbi.nlm.nih.gov/blast/)* and then aligned and analyzed with reference sequences downloaded from GenBank. Phylogenetic trees

Table 1. *Characteristics of amplified fragments and corresponding primer sequences*

Targeted DNA	Gene	Primer sequence (5'-3)	Fragment	Cycling Conditions of PCR Assays	Reference
Tick	16S rRNA	Forward 1 (CTGCTCAATGATTTTTTAAATTGCTGTGG)	460bp	94°C for 5 min, followed by 37 cycles at 92°C for 30 s, 54°C for 30s, and 72°C for 30s, with a final extension at 72°C for 8 min	[11]
		Reverse 1 (CCGGTCTGAACTCAGATCAAGT)			
Hepatozoon canis	*18S rRNA*	Forward 2 (ATACATGAGCAAAATCTCAAC)	666bp	95°C for 5 min, followed by 35 cycles at 95°C for 60s, 58°C for 60 s, and 72°C for 60 sec, with a final extension at 72°C for 5 min	[14]
		Reverse 2 (CTTATTATTCCATGCTGCAG)			
Anaplasma bovis	16S rRNA	Forward 1 (TTGAGAGTTTGATCCTGGCTCAGAACG)	450bp	94°C for 5 min, followed by 40 cycles at 94°C for 45s, 55°C for 50 s, and 72°C for 1 min, with a final extension at 72°C for 5 min	[12]
		Reverse 1 (CACCTCTACACTAGGAATTCCGCTATC)			
		Forward 2 (TTGAGAGTTTGATCCTGGCTCAGAACG)			
		Reverse 2 (GTACCGTCATTATCTTCCCTA)			
Coxiella burnetii	IS111	Forward 1 (TACTGGGTGTTGATATTGC)	260bp	95°C for 8 min; followed by 35 cycles at 95°C for 15 s, 52°C for 5s, and 72°C for 1 min; and extension at 68°C for 10 min.	[13]
		Reverse 1 (CCGTT TCATCCGCGGTG)			
		Forward 2 (GTAAAGTGATCTACACGA)			
		Reverse 2 (TTAACAGCGCTTGAACGT)			

were constructed based on the sequence distance method using the neighbor-joining algorithms implemented in the Molecular Evolutionary Genetics Analysis MEGA 7.0 *(http://www.megasoftware.net)* software.

Results

All ticks (72 male and 151 female) were collected and morphologically identified as *Rhipicephalus turanicus* sensu stricto (s.s.) *(Fig. 1)*. The obtained sequences of *Rh. turanicus* s.s. have been deposited in the GenBank database. Phylogenetic trees analysis further confirmed these results *(Fig. 2)*.

Three tick-borne pathogens were detected in this test, among which the highest detection rate was *C. burnetii,* which was detected in both ticks and blood samples, with a detection rate of 17.94% (40/223) in ticks and 79.1% (155/196) in blood samples, followed by *H. canis* 21.52% (48/223) in ticks and *A. bovis* 2.5% (5/196) in blood samples. Among them, co-detection with *H. canis* and *C. burnetii* was detected in 12 blood samples. All ticks and blood samples were also tested for *Borrelia burgdorferi* and *Leptospira sp.*, However, DNA from these pathogens was not detected in any of the samples.

Among all the positive ticks and blood samples, *A. bovis* and *C. burnetii* showed 99.78% and 99.63 identity to the corresponding sequences of *A. bovis* (MH255939) from Shaanxi Province and *C. burnetii* (KX852471) from

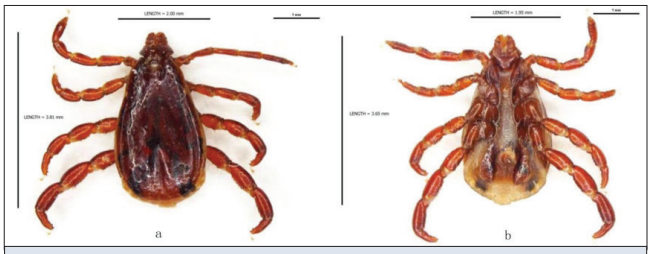

Fig 1. Morphological analysis of *Rhipicephalus turanicus* sensu stricto collected from pet dogs. a Male, dorsal; b male, ventral

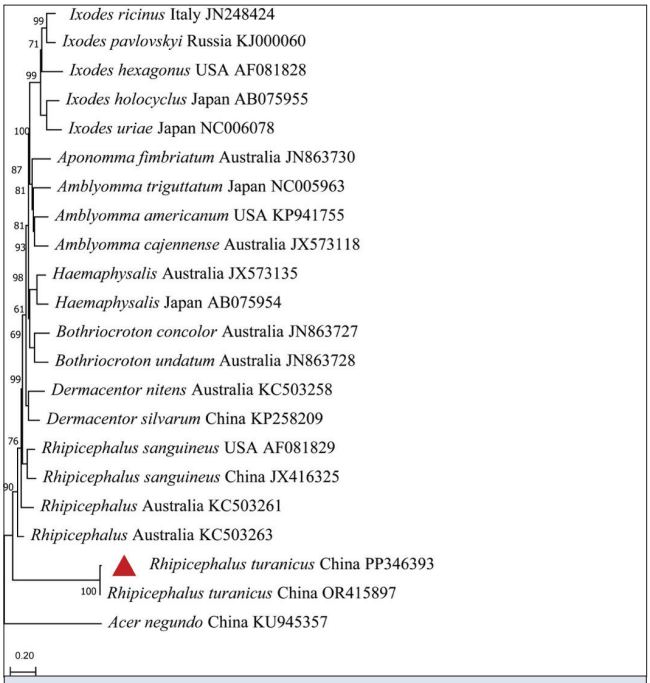

Fig 2. Phylogenetic tree based on partial gene of the 16S rRNA of *Rhipicephalus turanicus* sensu stricto (▲) collected from pet dogs obtained in this study in northwestern China. The evolutionary history was inferred using the neighbor-joining method (bootstrap replicates: 1000) with MEGA 7.0

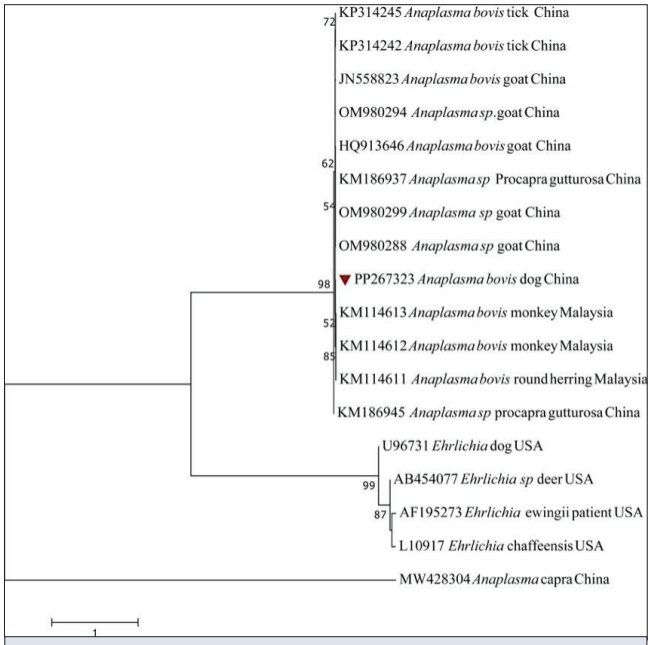

Fig 3. Phylogenetic tree based on partial gene of the 16S rRNA of *Anaplasma bovis* (▼) from blood samples obtained in this study in northwestern China. The evolutionary history was inferred using the neighbor-joining method (bootstrap replicates: 1000) with MEGA 7.0

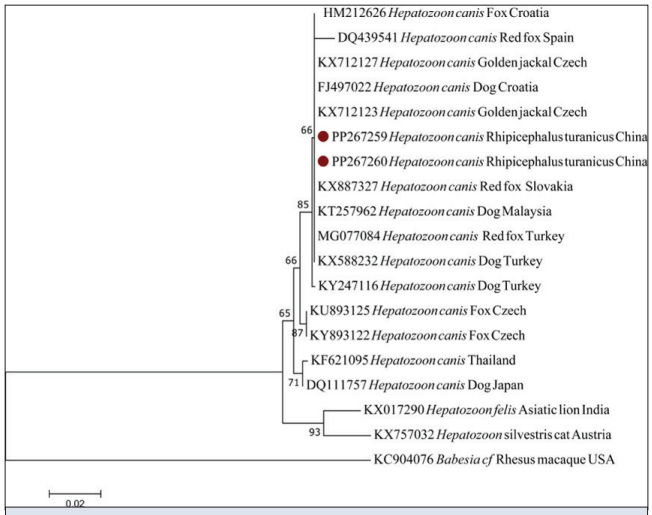

Fig 4. Phylogenetic tree based on partial gene of the 18S rRNA sequence of *Hepatozoon canis* (●) from *Rhipicephalus turanicus* sensu stricto obtained in this study in northwestern China. The evolutionary history was inferred using the neighbor-joining method (bootstrap replicates: 1000) with MEGA 7.0

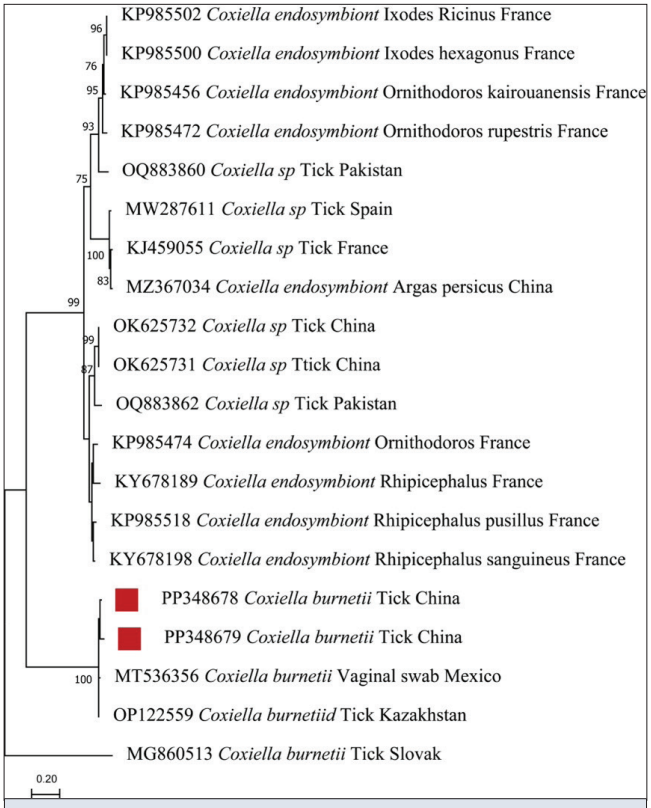

Fig 5. Phylogenetic tree based on partial gene of the htpABgene sequence of *Coxiella burnetii* ( ) from *Rhipicephalus turanicus* sensu stricto and blood samples obtained in this study in northwestern China. The evolutionary history was inferred using the neighbor-joining method (bootstrap replicates: 1000) with MEGA 7.0.

XUAR, respectively. *Hepatozoon canis* showed 99.84% identity to the corresponding sequences from Czech Republic (KX712129). Phylogenetic trees analysis further confirmed these results *(A. bovis - Fig. 3; H. canis - Fig. 4; C. burnetii - Fig. 5).*

All sequences from this study were deposited in the GenBank *(http://www. ncbi.nlm.nih.gov)* database *(Rh. turanicus* s.s PP346393; *A. bovis* PP267323; *C. burnetii* PP348678-PP472476; *H. canis* PP267259-PP267260).

Discussion

There have been increasing numbers of cases of zoonotic tick borne diseases in humans and pet dogs [15]. In this study, *C. burnetii, H. canis* and *A. bovis* were screened out in pet dogs and their parasitic ticks. To the best of our knowledge, this is the first report of *C. burnetii* and *H. canis* in *Rh. turanicus s.s.* in China.

Anaplasma spp. is transmitted by ticks and contains seven proven species. Two of these species, *A. phagocytophilum* and *A. capra*, commonly cause disease in humans [16]. *Anaplasma bovis* was initially thought to be just an animal pathogen until the first patient case was reported in 2019 [17,18]. In this study, *A. bovis* was detected both in dog ticks and blood samples. This finding suggests that it is vital to further survey *A. bovis* among pet dogs, ticks and dog owners especially in oasis of Taklamakan Desert in the future.

Coxiella burnetii can infect a variety of domestic and wild animals, including mammals, birds, and reptiles. Previously, cattle, sheep, and goats were considered the primary hosts [19]. At the same time, dogs and cats are classified as mammals susceptible to *C. burnetii* [20]. Pet animals, especially those in close contact with their owners, play an important role in reservoirs of *C. burnetii*, which causes urban Q fever and sporadic Q fever [21]. In this study, it was not only found in the samples of dog ticks, but also in dog blood samples with 79.1% (155/196) positive rate. This result gives a strong warning to public health security against Q fever.

To date, *Hepatozoon* spp. includes at least 340 species and can infect a wide range of vertebrate hosts, such as mammals, reptiles, birds, fish, and invertebrates. In terms of its primary vectors, *Amblyomma ovale*, *Rhipicephalus microplus*, *Haemaphysalis longicornis* and *Haemaphysalis flava* have been identified as definite hosts for *Hepatozoon* [22-24]. In this study, we found *H. canis* with 21.52% (48/233) positivity in *Rh. turanicus s.s.* Although this study does not confirm *Rh. turanicus s.s* being vectored as *H. canis*, we still believe *H. canis* pose a potential risk to dogs and dog owners in local people.

Co-infection is common in tick-bitten mammals. Previously, some scholars have discovered that co-infections were identified in 16.7% of *Ixodes ricinus* (89/534), which accounted for 64.5% (89/138) of all infected ticks. Co-infection prevalence was 14.3% (11/77) in adults and 17.1% (78/457) in nymphs [25]. Meanwhile, ticks can acquire a variety of pathogenic species (such as parasites, bacteria or viruses) through blood-sucking to different vertebrate hosts or through systemic transmission of common feeding or co-feeding [26]. In this study, *H. canis* and *C. burnetii* were simultaneously detected in 12 dog blood samples. This study extends tick-borne pathogen co-detection in pet dogs.

With the number of pet dogs increasing in China, it is necessary to strengthen the supervision of pet dogs and stray dogs in order to control tick-borne zoonotic diseases in the horizon of "One World One Health".

Declarations

Availability of Data and Materials: The datasets generated during and/or analysed during the current study are available from the corresponding author (XW) on reasonable request.

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Research Article

Morphological and Molecular Changes in Renal Tissue Following Experimental Unilateral Ureteral Obstruction in Rat Kidneys

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Abstract

This study was designed to investigate the pathological, molecular, and biochemical changes subjected to experimental unilateral ureteral obstruction (UUO) during the acute phase, specifically within a seven-day period. The kidney's reaction to obstructions, such as unilateral ureteral obstruction (UUO), is highly complex and involves a dynamic interplay of various molecular processes. A total of 72 Wistar Albino rats were divided into nine groups, each consisting of eight rats. The left kidneys of the first seven groups underwent UUO surgery, with one group being necropsied daily. All left kidneys were examined using histopathological, immunohistochemical, biochemical, and molecular methods. On the first day of unilateral ureteral obstruction (UUO), significant pathological and biochemical changes were observed in the kidneys. These included the formation of cystic dilated tubules, a decrease in cyclooxygenase-2 (COX-2) gene expression, and a reduction in glutathione peroxidase (GSH-Px) levels. By the second day, hyperemia, increased tumor necrosis factor-alpha (*TNFα*) gene expression, and elevated malondialdehyde (MDA) levels were evident. In the third day; there was mild interstitial mononuclear cell infiltration roteinaceous filtrate in tubules, heightened transforming growth factor beta 1 (TGF-β1) gene and protein expression, and increased angiotensin II (ANG-II) and interleukin-10 (IL-10) protein expression. By day five, kidneys subjected to UUO exhibited hydropic degeneration, TNF-α protein expression, and anti-RELA antibody expression. On the sixth day, significant increase in *IL-10* gene expression was noted. In conclusion; these results provide valuable insights for future studies on UUO pathogenesis and research into potential treatment modalities.

Keywords: Angiotensin II, Interleukin-10, Oxidative stress, Transforming growth factor beta 1, Unilateral ureteral obstruction

Introduction

Ureteral obstructions results in kidney enlargement due to urine accumulation in the renal pelvis or calyces [1-3]. When left untreated, these highly complicated obstructions can lead to hydronephrosis and gradual deterioration of kidney function [1,3,4].

The UUO model serves as a standard for comprehending the origins and mechanisms of renal interstitial fibrosis in mice and rats, with manifestation in mice within a week and rats within 2-3 weeks [1,5,6]. In response to mechanical stress, ligated kidney tissses in the UUO model synthesize chemoattractants within the first 4 h, triggering interstitial monocyte and T lymphocyte infiltration. These infiltrating cells further exacerbate the disease by synthesizing transforming growth factor beta 1 (TGF-β1), diminishing renal blood flow, and reducing glomerular filtration rates [7].

Proinflammatory cytokines, Nuclear factor kappa B (NF-κB) activation, and oxidative stress are key drivers in progressive renal damage caused by obstructive nephropathy. They induce tubular cell apoptosis and interstitial fibrosis. Increased angiotensin II (ANG-II) production, heightened oxidative stress, and elevated pro-inflammatory cytokines contribute to NF-κB activation. This, in turn, prompts the expression of adhesion molecules and chemokines responsible for leukocyte

recruitment, along with an overexpression of cytokines that intensify the inflammatory response in the damaged kidney [6,8,9].

The UUO model has been studied in the context of chronic kidney diseases, typically spanning around 7 days or extending to 14 days or more [1,3]. The present study investigated the daily changes occurring in rat kidneys subjected to experimental UUO, employing histopathological, immunohistochemical (Anti-RELA), biochemical [oxidative stress, angiotensin II (ANG II), NF-κB, interleukin-10 (IL-10), TGF-β1, tumor necrosis factor-alpha (TNF-α)], and molecular [expression levels of cyclooxygenase-2 (*COX-2*), *TGF-β1*, *TNFα*, *IL-10* genes] methods over a week.

Materials and Methods

Ethical Statement

This study was approved by the Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee (Approval no: 2022/03-02).

Animals and Experimental Protocol

In this study, 72 male Wistar Albino rats, aged 3 months and weighing between 300-350 g, were utilized. Rats were kept under constant environmental conditions (12 h day/night cycles, 24±3°C), received the standard commercial rat food (pellet feed) and had free access to tap water. The rats were divided into 9 groups, each comprising 8 rats. To induce hydronephrosis, the first seven groups underwent UUO surgery, with necropsies conducted on one group daily (day 1: Group 1; day 2: Group 2; day 3: Group 3; day 4: Group 4; day 5: Group 5; day 6: Group 6; day 7: Group 7). The sham group (Group 8) had their abdominal cavity opened and closed without creating UUO and was euthanized on the 1st day. The control group (Group 9) was euthanized on the 1st day without any intervention.

During the UUO procedure for hydronephrosis induction, all rats were anesthetized using xylazine HCL [10 mg/kg, intraperitoneal (IP)] and ketamine HCL (50 mg/kg, IP). The abdominal areas of the anesthetized rats were shaved, and the incision areas were made aseptic while the rats were positioned supinely. Following a midline incision, access to the abdominal cavity was achieved. After exposing the left kidney, the left ureter was identified. Two ligatures with 5/0 non-absorbable thread were placed on the midline of the left ureter during the procedure, and the ureter was cut between the two ligatures. Subsequently, the peritoneum, muscles, and skin were closed routinely using 3/0 absorbable sutures [5].

Finally, the rats were euthanized under xylazine HCl (10 mg/kg, IP) and ketamine HCl (50 mg/kg, IP) anesthesia, and kidney tissues were collected after necropsy.

Histopathological Analysis

Following the experiment, a portion of the left kidney was preserved in 10% buffered formalin for subsequent pathological examination. The samples were washed under tap water, dehydrated in ascending grades of ethanol (70, 80, 90, 96 and 100%), cleared in xylene, and embedded in paraffin. Kidney sections of 4 µm thickness were prepared and stained with haematoxylin and eosin (H&E) for morphological changes. Histopathological alterations, such as mononuclear cell infiltration and cystic dilated tubules in the kidneys, were assessed in five randomly selected areas (at 100x magnification), adhering to the criteria described by Otunctemur et al.[10] and Hassan et al.[9]: 0 denoting no change, 1 indicating changes affecting <25% of the area, 2 signifying changes affecting 25-50% of the area, and 3 representing changes affecting >50% of the area.

Immunohistochemical Analysis

For immunohistochemical analysis, tissue sections were derived from the same blocks utilized in the histopathological examination and placed on adhesive slides. Avidin-Biotin Peroxidase Complex (ABC) technique was performed according to the manufacturer guidelines (SensiTek HRP, ScyTek Laboratories, Logan, UT) to show the expression and localization of anti-RELA in tissues. Paraffin-embedded kidney sections were deparaffinised, hydrated and incubated with 3% H_2O_2 to block peroxidase activity. Proteinase K (Abcam, ab64220) was applied as antigen retrieval to the paraffin sections. Afterward, the sections incubated with anti-RELA antibody [ST.Johns Laboratorıes, STJ94473-100, Anti-RELA antibody (220-300), 1/100 dilution, 45min/45°C]. The binding sites of antibody were visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB, ScyTek Laboratories, Logan, UT). The samples were counterstained with H&E. Immunohistochemical grading of kidney tissues followed the methodology established by Hassan et al.[9], utilizing a scoring system of 0, 1, 2, and 3.

Molecular Analysis

Post-sacrifice, kidney tissues were promptly washed with PBS solution (pH: 7.4), then placed in nuclease free tubes and frozen in liquid nitrogen. Upon arrival at the laboratory, the frozen samples were stored at -80°C until molecular analysis. For gene expression analyses, total RNA was isolated from kidney tissues using the modified Trizol method through the Hibrizol kit (Hibrigen, TÜRKİYE) [11]. For this purpose, approximately 50 mg of tissue was homogenized in 1 mL Hibrizol in 2 mL nuclease-free tubes using the Bioprep-6 homogenizer (AllSheng, CHINA). The RNA pellet was obtained through successive steps involving chloroform, isopropyl alcohol, and ethyl alcohol, following the kit protocol. The resulting

RNA pellets were diluted with 20-50 µL of nuclease-free water after approximately 10 min of incubation at room temperature. Purity (A260/A280) and concentration values of the isolated RNAs were assessed via SMA-1000 Spectrophotometer (Merinton, CHINA), and their quality was electrophoretically validated through 1% agarose gel electrophoresis.

The isolated RNA samples were adjusted to a concentration of 1000 ng/µL with nuclease-free water and subsequently treated with DNase I (EN0521, Thermo Scientific, USA) to eliminate potential DNA contamination. cDNA synthesis was carried out using the Onescript Plus cDNA Synthesis Kit (G236, ABM, CANADA) following the kit protocols. For this process, samples were incubated in a thermal cycler (BioRad T100, USA) at 55ºC for 15 min and at 85ºC for 5 min. After the reaction, the samples were completed to 200 µL with nuclease-free water and stored at -80°C until qPCR analysis.

Expression levels of *TNFα, COX-2, TGFβ-1,* and *IL-10* genes were determined by qPCR (Rotorgene Q MDx 5plex HRM, Qiagen USA) with the EnTurbo™ SYBR Green PCR SuperMix kit (EQ014, ELK Biotechnology, China). The *ACTB* were used as the housekeeping gene to determine the expression levels of the target genes, and all samples were analyzed in duplicate. qPCR reaction conditions were set at 15 sec at 95ºC, 60 sec at 60ºC, and 30 sec at 72ºC for 40 cycles, following a 10 min incubation at 95ºC. The forward and reverse sequences of the primers used for gene amplification are detailed in *Table 1*.

Biochemical Analysis

Protein analyses for ANG-II, NF-κB, IL-10, TGF-β1, and TNF-α in kidney tissue were conducted using an ELISA plate reader (Bio-Tek, Winooski, VT) and commercial kits (ELK BIOTEK), as per the manufacturer's specified protocol.

For analyses related to oxidative stress and antioxidant activity, tissue samples underwent a 1/10 dilution with Tris buffer (pH 7.4) and were subsequently homogenized using a homogenizer. The determination of total protein in the tissue followed the Lowry method [17].

The assessment of lipid peroxidation (LPO) levels relied on measuring the amount of malondialdehyde (MDA) produced in the tissue. MDA levels were determined according to the spectrophotometric method described by Placer et al.[18]. The pink complex formed by MDA with thiobarbituric acid was measured spectrophotometrically at 532 nm, and the MDA level was expressed as nmol/g protein.

The of glutathione (GSH) levels was determined using the method described by Sedlak and Lindsay [19]. The color intensity of the yellow complex formed as a result of the reaction of 5,5-dithiobis [2-nitrobenzoic acid] with sulfhydryl groups was measured spectrophotometrically at 412 nm and GSH levels were expressed as nmol/g, with the color intensity being directly proportional to the GSH concentration in the medium.

Catalase (CAT) activity in the tissue was assessed according to the method established by Goth, [20]. Tissue incubation with a substrate containing hydrogen peroxide (H_2O_2) resulted in the breakdown of H_2O_2 into H_2O and O_2 through catalase activity. The addition of ammonium molybdate to the medium terminated the reaction. The color change during this process was measured spectrophotometrically against a blank at 405 nm, and catalase enzyme activity was expressed as kU/g protein.

Glutathione peroxidase (GSH-Px) activity in the tissue was determined using the method described by Lawrence and Burk [21]. The yellow color complex formed by the samples with DTNB solution was measured on a spectrophotometer at 412 nm, with GSH-Px enzyme activity expressed as IU/g protein.

Table 1. *Forward and reverse sequences of studied genes*

Genes	Forward and Reverse Primer Sequences	Product length	References
ACTB	F: 5'-TGACAGGATGCAGAAGGAGA-3'	104	[12]
	R: 5'-TAGAGCCACCAATCCACACA-3'		
TNF-α	F: 5'-ACTGAACTTCGGGGTGATCG-3'	153	[13]
	R: 5'-GCTTGGTGGTTTGCTACGAC-3'		
COX-2	F: 5'-TGTATGCTACCATCTGGCTTCGG-3'	94	[14]
	R: 5'-GTTTGGAACAGTCGCTCGTCATC-3'		
TGFβ-1	F: 5'-ATTCCTGGCGTTACCTTGG-3'	117	[15]
	R: 5'-CCTGTATTCCGTCTCCTTGG-3'		
IL-10	F: 5'-TTGAACCACCCGGCATCTAC-3'	91	[16]
	R: 5'-CCAAGGAGTTGCTCCCGTTA-3'		

Statistical Analysis

Before performing the statistical analysis, the parameters were assessed for parametric test assumptions. The Shapiro-Wilk test was used to assess the assumption of normality, and the Levene test was used for homogeneity of variances. Differences in ELISA parameters, histopathological and immunohistochemical findings between groups were determined using the Kruskal-Wallis test. In instances where a significant difference was observed, the multiple Dunn test was used as a post-hoc test. Differences in stress parameters between groups were evaluated with one-way analysis of variance (ANOVA). When a significant difference was revealed, the Tukey test was performed as a post-hoc test. The Spearman correlation coefficient was used to determine the strength and direction of relationships between ELISA and stress parameters. All statistical analyses were performed using the IBM SPSS 23.0 statistical software, and the significance threshold was set at $P<0.05$.

The 2-ΔΔCt method was used to calculate the relative expression levels of target genes in the study and the change in gene expression levels in the groups was presented as fold change compared to the control group [22].

Results

Macroscopically, the kidneys in the control and sham groups appeared normal. However, as the duration of obstruction increased in the UUO groups, the color of these kidneys became lighter, and their volume increased. Particularly on the 6th and 7th days, a significant enlargement of the renal pelvis and the development of hydronephrosis were evident *(Fig. 1)*.

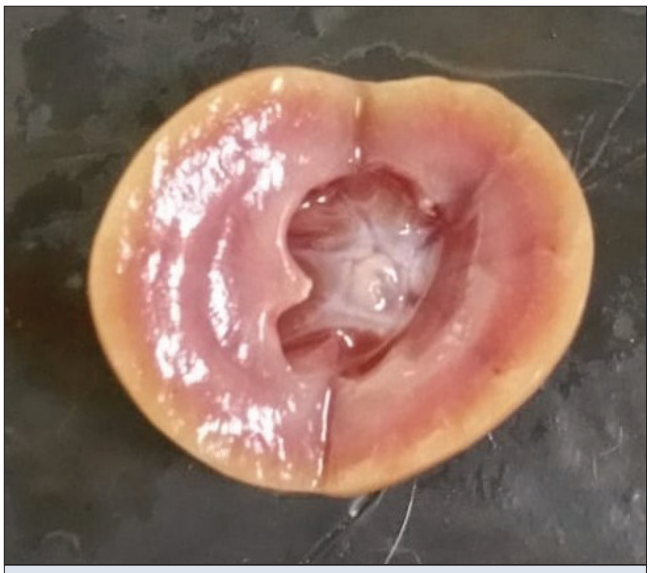

Fig 1. Enlargement of the renal pelvis on the 6th day

Microscopically, the histopathological findings and their scoring are in *Table 2*. Kidneys in the control and sham groups maintained a normal histological structure *(Fig. 2-A)*. In contrast, those subjected to experimental UUO showed statistically significant cystic dilated tubules with mild severity on the 1st day, progressing to moderate severity by the 6th day *(Fig. 2-B,C)*. The vascular hyperemia became less pronounced from the 2nd day onwards *(Fig. 2-D)*. Mononuclear cell infiltration and proteinaceous filtrate in the tubules were evident on the 3rd day *(Fig. 2-E)*. Notably, degenerative changes like hydropic degeneration were observed only on the 5th day, with a statistically significant difference in severity *(Fig. 2-F)*.

Table 3 presents the levels of anti-RELA antibody expression in the groups. Anti-RELA antibody expression

Table 2. *Histopathological findings and scoring observed in the groups*

Groups	Cystic Dilated Tubules		Hyperemia		Hydropic Degeneration		Mononuclear Cell Infiltration		Proteinase Filtrate in Tubules	
	Arithmetic Mean	Standard Error	Arithmetic Mean	Standard Error	Arithmetic Mean	Standard Error	Arithmetic Mean	Standard Error	Arithmetic Mean	Standard Error
Control	0.14 ^{A}	0.14	0.00 ^{A}	0.00	0.00 ^{A}	0.00	0.00 ^{A}	0.00	0.00 ^{A}	0.00
Sham	0.00 ^{A}	0.00	0.00 ^{A}	0.00	0.00 ^{A}	0.00	0.00 ^{A}	0.00	0.00 ^{A}	0.00
Day 1	1.00 ^{B}	0.22	0.25 ^{AB}	0.16	0.14 ^{A}	0.14	0.00 ^{A}	0.00	0.14 ^{A}	0.14
Day 2	1.00 ^{BC}	0.31	1.00 ^{B}	0.27	0.33 ^{A}	0.21	0.29 ^{A}	0.18	0.29 ^{A}	0.18
Day 3	1.29 ^{BCD}	0.18	1.00 ^{B}	0.31	0.29 ^{A}	0.18	1.00 ^{B}	0.22	1.14 ^{BC}	0.34
Day 4	1.71 ^{BCD}	0.29	1.00 ^{B}	0.22	0.71 ^{A}	0.29	1.29 ^{B}	0.29	1.00 ^{BC}	0.22
Day 5	1.71 ^{BCD}	0.18	0.88 ^{B}	0.23	1.57 ^{B}	0.20	1.14 ^{B}	0.14	1.14 ^{BC}	0.14
Day 6	2.14 ^{D}	0.14	0.38 ^{AB}	0.26	1.71 ^{B}	0.18	1.57 ^{B}	0.20	1.14 ^{BC}	0.14
Day 7	2.00 ^{CD}	0.22	0.43 ^{AB}	0.20	1.71 ^{B}	0.18	1.14 ^{B}	0.14	1.29 ^{C}	0.18
P	<0.001		<0.001		<0.001		<0.001		<0.001	

^{A,B,C} *Different capital letters in rows indicate statistical significance between groups (P<0.05)*

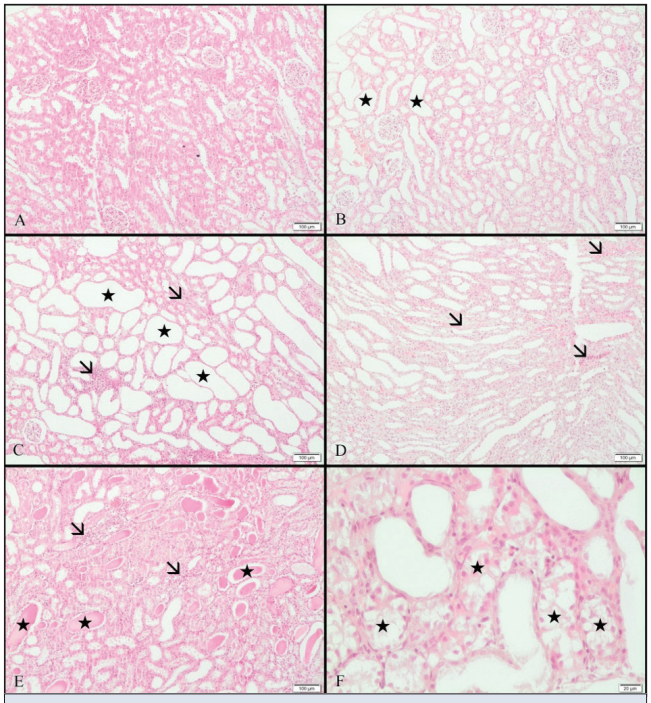

Fig 2. Histopathological changes in the kidneys, H&E. **A-** Control group; normal kidney tissue; **B-** Day 1; slightly cystic dilated tubules *(stars);* **C-** Day 6; cystic dilated tubules *(stars)* and mononuclear cell infiltration *(arrows);* **D-** Day 2; mild hyperemia of the kidneys *(arrows);* **E-** Day 3; proteinaceous filtrate *(stars)* and mononuclear cell infiltration *(arrows)* in tubules; **F-** Day 5; hydropic degeneration (stars) in tubular epithelium

Table 3. *Anti-RELA antibody expression levels in groups*

Groups	Arithmetic Mean	Standard Error
Control	0.14 ^{A}	0.14
Sham	0.00 ^{A}	0.00
Day 1	0.00 ^{A}	0.00
Day 2	0.14 ^{A}	0.14
Day 3	0.14 ^{A}	0.14
Day 4	0.00 ^{A}	0.00
Day 5	0.86 ^{B}	0.14
Day 6	1.14 ^{B}	0.14
Day 7	1.00 ^{B}	0.00
p	<0.001	

^{A,B,C} *Different capital letters in rows indicate statistical significance between groups (P<0.05)*

was negative in the control and sham groups *(Fig. 3-A,B)*. The first statistically significant positivity was detected in the glomeruli and intertubular regions on the 5th day *(Fig 3-C,D)*.

Fig. 4 summarizes the levels of oxidative stress and antioxidant activity markers obtained as a result of the study. The MDA level was significantly elevated on the 2nd day, as well as on the 6th and 7th days, when compared to

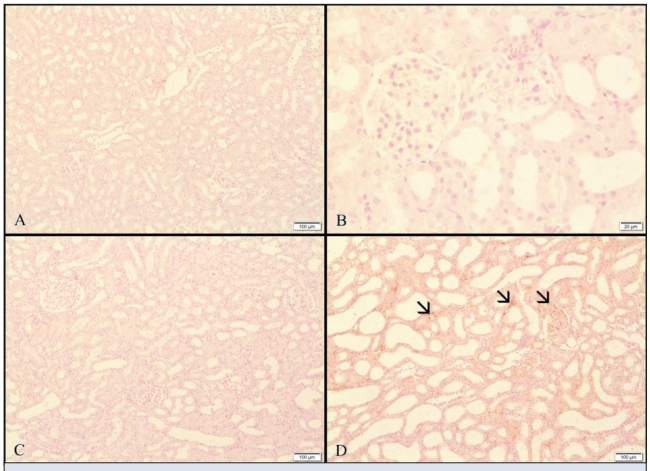

Fig 3. Immunohistochemical staining for Anti-RELA antibody. **A-** Control group; Anti-RELA antibody expression negative; **B-** Day 1; Anti-RELA antibody expression negative; **C-** Day 3; Anti-RELA antibody expression negative; **D-** Day 5; slight Anti-RELA antibody expression in glomeruli and intertubular regions

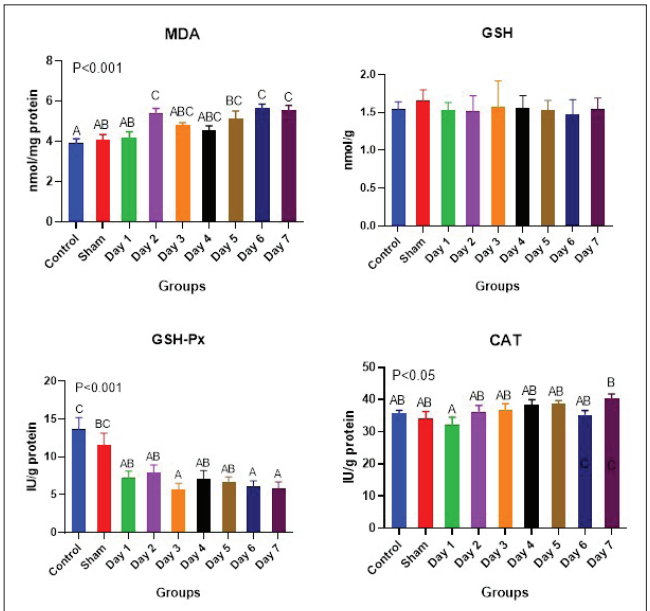

Fig 4. MDA, GSH, GSH-Px, and CAT levels in groups

the control and sham groups. GSH-Px level consistently showed a statistically lower value compared to the control group from the first day. After the 3rd day, it was also lower than the sham group. No significant differences were observed in terms of GSH and CAT compared to the control and sham groups.

Expression levels of the studied genes were significanly changed in different days of experiment. While *TNFα* gene expression levels were consistently higher than in the control and sham groups after the 2nd day, *COX-2* gene expression levels were decrased from day1 to day 5 compared to control (P<0.05). On the other hand, *TGF-β1* gene expression levels were incresed from day 3 (P<0.05). Notably, IL-10 gene expression was significantly higher on the 6th and 7th days compared to the control *(Fig. 5)*.

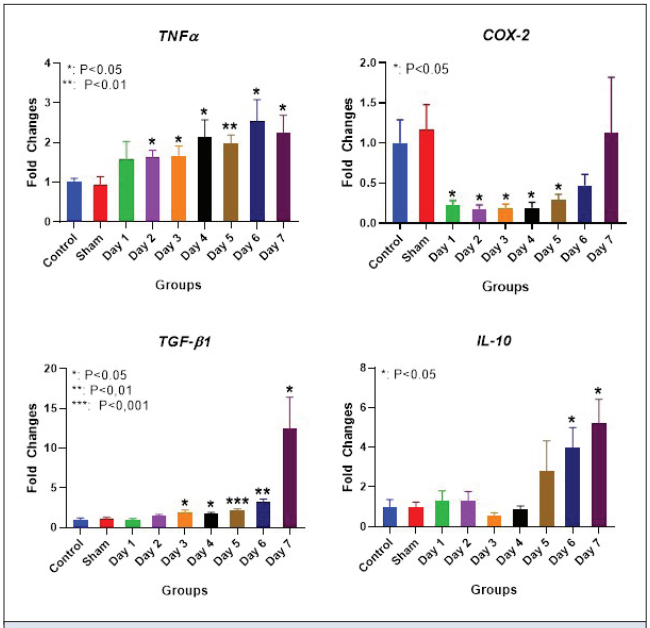

Fig 5. Expression levels of TNFα, COX-2, TGF-β1, and IL-10 genes

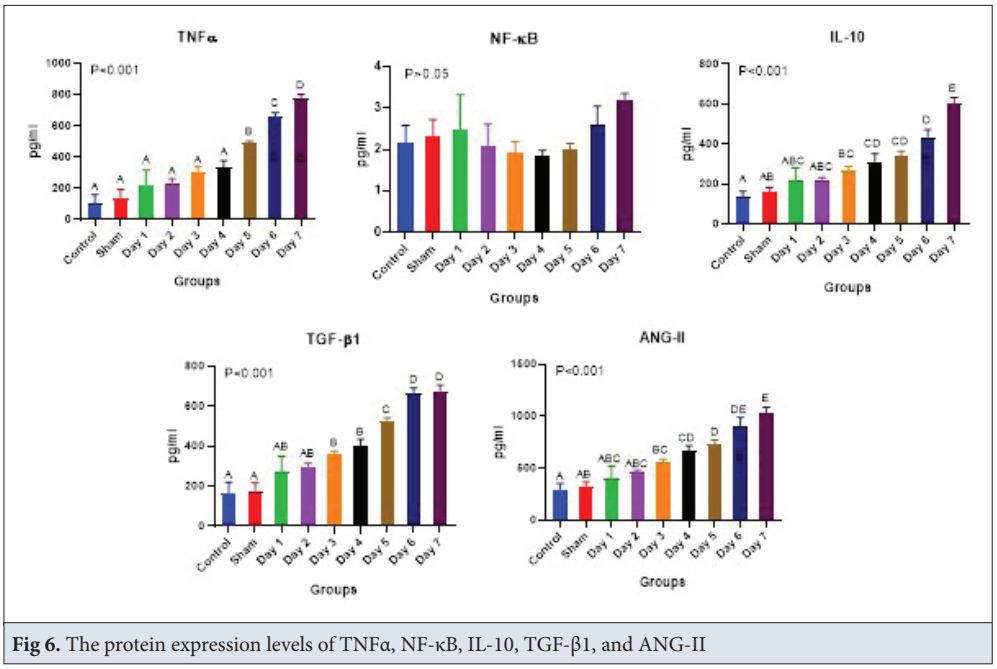

Fig 6. The protein expression levels of TNFα, NF-κB, IL-10, TGF-β1, and ANG-II

Table 4. *Correlation between biochemical parameters and proteins*

Parameters	IL-10	NF-κB	TGF-β1	TNF-α	ANG-II	MDA	GSH	GSH-Px	CAT
IL-10	1	0.353**	0.927***	0.935***	0.970***	-0.476***	0.128	0.485***	-0.090
NF-κB		1	0.259	0.381**	0.338*	-0.118	0.049	0.271*	0.016
TGF-β1			1	0.954***	0.955***	-0.596***	0.189	0.523***	-0.257
TNF-α				1	0.940***	-0.575***	0.183	0.518***	-0.166
ANG-II					1	-0.514***	0.142	0.512***	-0.127
MDA						1	-0.259	-0.397**	0.215
GSH							1	0.073	0.005
GSH-Px								1	-0.062
CAT									1

**P<0.05; **P<0.01; ***P<0.001*

Fig. 6 presents the protein expression levels of the groups. There was no significant change in NF-κB protein levels between the groups. However, ANG-II and IL-10 protein expression levels commenced an increase on day 1 compared to the control and sham groups, becoming significantly elevated compared to the control group on the 3rd day and compared to the sham group on the 4th day. TGF-β1 protein expression level began to rise on the first day, with a significant increase on the 3rd day compared to both the control and sham groups. TNF-α protein expression level started to increase on the 1st day, becoming significant on the 5th day compared to the control and sham groups. *Table 4* illustrates the correlation between biochemical parameters and proteins

Discussion

The UUO model is highly effective, inducing multiple pathophysiological changes within a week of obstruction [1,6,23]. Hassan et al.[9] reported observations in rats subjected to the UUO model over two weeks, including interstitial edema, cystic dilated tubules, atrophy in the glomeruli, thickening of the Bowman capsule, and mononuclear cell infiltration. Similarly, our study revealed distinct histopathological changes in the UUO model, with cystic dilated tubules on the 1st day, hyperemia on the 2nd day, mononuclear cell infiltration, and proteinaceous filtrate in the tubules on the 3rd day, and statistically significant hydropic degeneration in the tubule epithelium on the 5th day.

In acute UUO, it has been reported that mononuclear cell infiltration, particularly by macrophages, follows a biphasic increase starting four hours post-obstruction. The first phase involves a continuous increase in mononuclear cell infiltration over the initial 24 hours, followed by a second phase where the infiltration rises to ten times the normal level within three days and continues to increase for up to 14 days [23]. In the current study, mononuclear cell infiltration began on the 2nd day, reached statistical significance on the 3rd day, and maintained similar levels until the 7th day.

Oxidative stress has been identified as a crucial player in the pathogenesis of UUO, with many markers like MDA showing increased levels. Additionally, major protective antioxidant enzymes such as GSH, GSH-Px, and CAT have been found to decrease [9,24]. Similarly, in the current study, MDA levels increased on the 2nd day and on the 6th and 7th days, while GSH-Px levels consistently remained low from the 1st day. However, no significant changes were detected in terms of CAT and GSH compared to the control and sham groups over the 7 days.

Klahr [25] indicated that ANG-II levels increased with oxidative stress in the UUO model. Consistent with this, the present study found a significant increase in ANG-II levels. The protein expression of ANG-II displayed significant increases on the 3rd day compared to the control, on the 4th day compared to the sham, and continued to rise significantly on the 6th day compared to the preceding days.

The heightened expression of ANG-II can trigger NF-κB activation, inducing both direct and indirect oxidative stress. NF-κB can also be activated by various cytokines such as TNF-α and oxidative stress [1,4,6]. However, in our study, no significant change was observed in the NF-κB protein level. Previous immunohistochemical studies have demonstrated the presence of activated NF-κB complexes in various cells within obstructed kidneys, including glomerular, tubulointerstitial, and infiltrated cells [1,6,26].

The NF-κB transcription factor family comprises p50 (NF-kB1), p52 (NF-kB2), p65 (RelA), c-Rel, and RelB [27]. In our study, the first statistically significant anti-RELA expression positivity was detected in the glomeruli and intertubular regions on the 5th day during immunohistochemical examination.

TGF-β1 is a cytokine that plays a crucial role in tissue damage and inflammation in obstructive nephropathy [24,28]. In the present study, TGF-β1 gene expression was significantly higher than in the control and sham groups every day from the 3rd to the 7th day. The protein expression level of TGF-β1 started increasing on the 1st day, with a significant elevation on the 3rd day compared to both the control and sham groups. Subsequently, on the 5th, 6th, and 7th days, protein expression levels continued to rise significantly compared to the preceding days. TGF-β1 is recognized as a potent chemoattractant, and a noteworthy correlation has been identified between the number of interstitial macrophages and cortical TGF-β1 expression in the UUO model [1]. In our study, TGF-β1 exhibited positive correlations with TNF-α, ANG II, and GSH-Px, while a negative correlation was observed with MDA.

It is well-documented that renal TNF-α levels increase in the early stages of UUO [1,26]. Prud'homme et al.[29] reported a significant increase in plasma levels of TNF-α and IL-10 in the UUO model, reaching a peak at 28 days. The upregulation of TNF-α is associated with severe renal inflammation [30,31]. In our study, TNF-α gene expression was consistently higher than in the control and sham groups every day after the 2nd day. The increase in protein expression levels of TNF-α in the control and sham groups was significant on the 5th day. Furthermore, in the subsequent days, protein expression levels continued to rise significantly compared to the previous days.

IL-10 plays a crucial role in suppressing inflammatory processes by inhibiting the activation of inflammatory pathways [32]. In the renal context, IL-10 has demonstrated

efficacy in suppressing the progression of both acute and chronic kidney injury *in vivo* [32,33]. Jin et al.[32] proposed in their study that IL-10 deficiency exacerbated inflammation and fibrosis in obstructive kidney disease. This exacerbation was attributed to the loss of IL-10's inhibitory effects on inflammatory pathways, including the TGF-β1 signaling pathway and the NF-κB signaling pathway. Consequently, IL-10 emerges as a critical negative regulator of renal inflammation and fibrosis [32,33]. In our study, IL-10 gene expression was significantly higher on the 6th and 7th days compared to the control, sham, and other groups. Additionally, the IL-10 protein expression level showed a significant increase compared to the control group on the 3rd day and the sham group on the 4th day.

In vitro evidence suggests that renal COX-2 expression increases in response to pressure, potentially due to endogenous ROS production during ureteral obstruction [2]. However, in our study, COX-2 gene expression was statistically lower between the 1st and 5th days compared to the control and sham groups.

The UUO model closely mirrors obstructive nephropathy, a condition often encountered in clinical settings due to factors such as benign prostatic hypertrophy or kidney stones, resulting in acute and chronic urinary obstruction [28,31]. A comprehensive understanding of kidney's response to such obstructions holds the potential to refine our comprehension of kidney diseases' pathogenesis and facilitate the development of innovative treatments to mitigate kidney damage [8]. For instance, in our study and consistent with prior research, an increase was observed in oxidative stress marker levels during UUO, coupled with a concurrent decrease in enzymes safeguarding the kidney against oxidative stress. Oxidative stress is associated with many factors that trigger the development of kidney diseases, and eliminating oxidative stress in the acute period is an alternative strategy for treating subsequent chronic kidney diseases.

Anti-TNF-α therapy is a well-established intervention for numerous immunoinflammatory diseases, such as rheumatoid arthritis [34]. Investigating the impact of anti-TNF-α therapy on the pathogenesis of UUO against TNF-α, which elevates even in the early stages of UUO, as indicated by our study, warrants further exploration.

IL-10, with its pivotal role in suppressing inflammatory processes, demonstrated significant increases in protein expression on the 3rd day of our study. This suggests that IL-10 may hold therapeutic potential for chronic kidney diseases, a notion supported by similar results reported by Jin et al.[32].

In conclusion, this study day by day documented the molecular, biochemical, and histopathological changes occurring during the first 7 days of the acute period in rats subjected to UUO. These results are poised to guide future investigations into the pathogenesis of UUO and contribute valuable information to ongoing research aimed at developing treatments for UUO.

Declarations

Availability of Data and Materials: The data given in this study may be obtained from the corresponding auther (T. Kutlu) on reasonable request.

Financial Support: This study was financially supported by the Scientific Research Projects Fund of Hatay Mustafa Kemal University (Project number: 22.GAP.025).

Ethical Approval: This study was done with Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee's permission numbered 2022/03-02.

Conflict of Interest: The authors declared that there is no conflict of interest

Declaration of Generative Artificial Intelligence (AI): The authors declare that the article and/or tables and figures were not written/created by AI and AI-assisted technologies.

Author's Contributions: Conceptualization, T.K., H.Ö., Z.Y. and M.G; funding acquisition, T.K., H.Ö., Z.Y., U.K. and M.G ; methodology, T.K and Z.Y.; resources, T.K., H.Ö., Z.Y., U.K. and M.G.; investigation, T.K., H.Ö., Z.Y., U.K. and M.G.; formal analysis, T.K., H.Ö., U.K. and M.G.; writing-original draft preparation, T.K.; writing-review & editing, T.K., H.Ö., Z.Y., U.K. and M.G.; visualization, T.K.. All authors have read and agreed to the published version of the manuscript.

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Research Article

Comparative Evaluation of Table Egg Quality of Local and Pure Breed Laying Hens in Response to Storage Period Length

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Abstract

This study was made to investigate the fresh and stored egg quality characteristics of local and pure-breed layer chickens. The eggs were randomly collected from a commercial farm that raised different free-range layer flocks such as local Atak-S, commercial Nick Brown, pure-breed Sussex laying hen, and a local cross-breed hen, all of 50 weeks of age. In total, 240 eggs, 60 eggs from each genotype, were further divided into three groups as: fresh eggs, 15 and 28-day storage period, and were stored at 14-16°C and 45-50% humidity conditions. The eggs were analyzed on the basis of internal and external quality, such as egg length, egg width, color characteristics, yolk height, albumin height, yolk color, and Haugh unit. Genotype had a significant effect on shell weight ($P<0.001$), shell thickness ($P<0.001$), albumen index ($P<0.001$), yolk index ($P<0.001$), and HU value ($P<0.003$). The effects of the storage period on albumen index, yolk index, and HU value were found to be significant, respectively ($P<0.001$, $P<0.001$, $P<0.001$). Significant differences existed for the shape index, L*, a*, b*, E, and C* values among the genotype groups ($P<0.001$). In conclusion, eggs of the local, pure-breed, and cross-breed layer chickens showed differences from the eggs of commercial hybrid hens both for external and internal quality. Eggs of commercial Nick Brown and Sussex breed seems slightly better in longer storage conditions.

Keywords: Atak-S, Sussex, Free-range, Storage period, Egg quality

Introduction

Poultry eggs are the most economical source of animal protein and calories for people across the world. Protecting the quality, freshness, and nutritious value of eggs throughout the whole marketing chain is very crucial to human nutrition and food security. The egg quality of poultry is influenced by both genetic and environmental factors such as storage length, animal feed, storage temperature, housing condition [1-4], etc. Good management practices of environmental factors and layer genotypes, such as pure breed or local poultry, can help prevent egg quality, such as shell color, egg weight, shell weight, and yolk weight [5].

Commercial layer genotypes have been selected for generations according to their production performance and quality of eggs, especially for cage housing [6]. In recent years, consumer interest in eggs from natural or backyard systems has led to a constantly decreasing trend in the number of hens housed in the conventional cage system in favor of non-caged or free-range housing systems. But all commercial chickens are not entirely convenient for cage-free or free-range egg production, so using pure or local poultry breeds in these systems is becoming popular for table egg production at first [7]. Consequently, some commercial layers were developed for free-range egg production [8]. As a result of consumer interest in native product, local layer or pure breed hens have been of interest most because they can efficiently produce eggs under adverse environmental conditions and contribute to prevent the animal welfare and biodiversity [9]. Higher interest of consumers for eggs produced in non-cage systems and some welfare problems in these housing systems, leading to an increase in the need for information to find out the best genotype for non-cage free-range systems. Knowledge on the egg quality of pure-bred or local hens during the productive period is required for sustainable egg production.

In practice, it is required to store table eggs less or more according to marketing conditions. During this stage, protecting the freshness and quality of eggs is of utmost

importance. Some components of albumen and yolk may alter and tend to deteriorate egg quality during egg storage. The main factors directly associated with egg deterioration are temperature and relative humidity conditions, besides egg handling and storage time affecting egg quality and shelf life [10]. The higher the storage temperature, the faster the egg quality decreases. Egg weight, albumen height, and HU decreased significantly, while albumen pH increased with the extension of storage time [11]. The storage method had a significant effect on most of the egg quality traits, and eggs stored at 4°C were of good quality and were classified as extra-class eggs even after 28 days [12]. But in most cases, eggs stored in non-refrigerated conditions and the deterioration rate of different kinds of poultry eggs might be different due to their egg quality properties. It was reported that eggs stored at room temperature should be consumed in 2 weeks or refrigerated until eight weeks [13]. According to the Turkish Food Codex on Egg and Egg Products [14], it is not necessary to cool the egg until the 18th day after the ovulation date. However, and then, it should be stored between +8 and +5°C starting from the 18th day. In Türkiye, 95.7% of all table eggs are produced in closed barn systems as conventional cages, unfurnished cages, furnished cages, and non-cage systems, while 4.3% of all table eggs are produced in organic and non-organic free-range conditions [15].

Currently, there has been growing consumer interest in purchasing eggs produced by local or pure-breed hens such as Atak-S and Sussex kept in closed barns and extensive rearing systems [16,17]. In general, local or pure-breed layers are characterized by a small number of egg production, resulting in a lower profit for producers and a higher cost of eggs for the consumer compared to conventional eggs produced by commercial genotypes [18]. However, the benefits of raising these genotypes for egg production have not been assessed in terms of consumer expectations such as egg quality, food safety, economic sustainability, welfare, and the environment. It is not clear whether the egg quality of local or pure-breed chicken can keep up with that of commercial laying hens. Because consumers increasingly pay attention to not only egg weight and shell quality but also their taste, freshness, yolk and shell color, nutritional value, etc. There is also a need to investigate these layer genotypes' internal and external egg quality characteristics in different storage conditions. Physical egg quality parameters, such as shell strength, shell color, Haugh unit, and yolk color, can affect consumer perceptions and might be different. Therefore, this study was conducted to investigate the effects of the length of storage period on the physical quality characteristics of eggs of different genotypes of layer chickens as local, pure, and cross-breed hens compared to commercial laying hens.

Material and Methods

Ethical Statement

This study does not require ethical permission.

Location

Each flock was housed on the same farm separately in Bursa, Türkiye, through the laying period according to standard procedures and Turkish legislation to protect laying hens [19,20].

Animals and Eggs

The eggs used in this study were collected from a commercial farm raised four in different layer flocks: commercial Nick Brown, pure-breed Sussex laying hen, local Atak-S, and a local cross-breed hen. Atak-S hens were developed by the Republic of Türkiye Ministry of Agriculture and Forestry and have been raised commonly in free-range egg production in Türkiye [21]. The cross-breed hens originated from Araucana chickens and produced a mix color of blue, green, and white eggs. Sussex and Atak-S hens are representative of layer hens laying brown eggs that range from cream to light brown in color. Eggs from the commercial Nick Brown are completely dark brown. The eggshell of the cross-breed had a green/blue surface on the eggs *(Fig. 1)*.

Fig 1. Shell color variations of eggs of different genotype of layer chickens

Management

A standard layer diet for the hens was used between 22 and 45 weeks of age (17.86% crude protein, 2.750 metabolizable energy kcal/kg). Subsequently, a second-phase diet was used between 45 and the end of the laying period (16.45% CP, 2800 ME kcal/kg). Hens were housed in a closed

deep litter barn with no windows and free access to the open range area. Less than 30% of the outdoor condition was covered by natural grass. The stocking density was nine hens per m^2, and 250 cm^2 of litter area in a closed barn, and 4 m^2 per hen on the range area, as stated in the Turkish legislation for the protection of laying hens [21]. All houses were equipped with perches, a feeding trough, and a nipple for water supply. Each house had individual nest boxes (1 nest box per 8 hens). Nest space and perch length (15 cm per hen) per hen followed the Turkish legislation for the protection of laying hens. The lighting regime was 16 h per 24 h period from 28 weeks to 72 weeks of age.

Sampling/Data Collection

To evaluate the physical characteristics, eggs were collected from layers of each genotype and divided into three groups: fresh, 15-days storage lengths, and 28-days storage lengths. Fresh eggs were evaluated after 24 h of storage at around 14-16°C and 45-50% humidity. This temperature interval corresponds to the EU regulation EC 589/2008 [8,22] and Turkish regulation [14], since it advises consumers to keep eggs refrigerated after purchase. Eggs in the 15th and 28th days of storage period groups were stored at similar conditions until analysis. Egg analysis was carried out in the Egg Quality Laboratory of the Department of Animal Science at Faculty of Veterinary Medicine, Bursa Uludag University. The egg quality evaluation included egg weight, egg length, egg width, shell color (L*, a*, b*) traits, shell thickness, shell weight, yolk height, thick albumen height, and yolk color. Egg shell color characteristics, egg width, and egg length were measured only on all fresh eggs collected from the flocks, while shell weight, shell thickness, and other internal quality characteristics were measured every period.

In total, 60 eggs from a whole day's production per each genotype were randomly collected, excluding the defective eggs as double-yolk, without shell. Eggs in all groups were weighed with a precision digital scale (0.01 precision). After weighing, the width (along the equatorial axis) and length (along the longitudinal axis) of the eggs were measured with a caliper to 0.1 mm. The egg shape index was calculated as egg width/egg length x 100. The shell colour was measured by a colorimeter (PCE-XXM 20, PCE Instruments Ltd) using the CIE L*a*b*scale [23]. The calorimeter was calibrated on a predefined white plate under daylight [24].

L*-lightness (ranges from 0 for an extremely black and to 100 for a perfectly white);

a*-chromaticity in the red-green axis (red; if it is positive up to 100, green; if it is negative up to -100);

b*-chromaticity in the yellow-blue axis (yellow; if it is positive up to 100, blue; if it is negative up to -100);

C-chroma (the distance of the color point to the L*-axis);

E value is calculated as the square root of the sum of the squares of L*, a* and b* values. The color intensity/saturation index (chroma value) is the square root of the sum of the squares of a* and b* values [25-27].

After the completed measurement of external quality traits, the eggshell was broken along the equatorial axis, and the yolk and albumen were put on a flat glass plate surface. The height of thick albumen and yolk height were measured using a tripod micrometer (Mitutoya, 200 mm). The albumen length and albumen width were determined by using a digital caliper with ± 0.01 mm precision (Mitutoyo, 300 mm, Neuss, Germany). The color intensity of the yolk was determined by visual comparison to a yolk color fan [28]. Colour scales ranged from 1 (pale yellow) to 15 (intense orange). The yolk was manually separated from the albumen and weighed. The albumen weight was calculated as the difference between the egg weight and the sum of shell weight and yolk weight. To measure shell thickness, pieces from three different points of each eggshell with intact membranes were measured with a micrometer to 0.01 mm. The egg shells were washed with water and set to dry at room temperature for 48 h, after which the shell weight was recorded. The egg yolk index, albumen index, and Haugh unit were calculated using the formulas given by, DSM egg quality manual [28], Funk [29], Bender [30], and Haugh [31], espectively.

$$Yolk\ Index = \frac{Yolk\ Height}{Yolk\ width}\ x\ 100$$

$$Albumen\ Index = \frac{Thick\ Albumen\ Height}{Thick\ Albumen\ Width + Thick\ Albumen\ Length/2}\ x\ 100$$

$$Haugh\ Unit = 100\ Log\ (Albumen\ Height + 7.57) - (1.7 x Egg\ Weight^{0.37})$$

where, albumen height (H) in mm, egg weight (W) in grams.

Statistical Tests

The collected data were statistically analyzed using SPSS 24.0 statistical package [32]. The effects of genotype on shape index and egg color traits were tested using one-way analysis of variance. The significance of differences between the average values of breeds and storage length for all other traits was evaluated using two-way analysis of variance (ANOVA) with Tukey's post-hoc test [33]. In all statistical tests used, differences at $P<0.05$ were considered as significant.

Results

External quality traits of fresh eggs in different genotypes of layer chickens are presented in *Table 1*. There were significant differences for all external quality parameters of the eggs from different genotypes as shape index, L*, a*, b*, E and Chroma values, respectively ($P<0.001$).

Egg weight, egg shell weight, and egg shell thickness values in the groups were shown in *Table 2*. There were

Table 1. *External egg quality characteristics of daily eggs obtained from different genotype of layer chickens (Mean ± SEM)*

Genotype	Shape Index	L*	a*	b*	ΔE*	C*
Atak-S	73.96±0.58^{bc}	68.15±1.0^{b}	32.10±1.45^{a}	20.43±0.76^{b}	86.55±1.33^{b}	52.71±1.64^{a}
Nick Brown	76.78±0.35^{a}	49.92±0.17^{c}	48.38±1.67^{b}	34.83±1.34^{a}	69.47±0.88^{c}	48.12±1.19^{a}
Sussex	75.09±0.42^{ab}	71.93±1.48^{b}	35.36±2.61^{b}	20.36±0.74^{b}	84.11±1.18^{b}	41.55±2.28^{b}
Cross Breed	73.19±0.62^{c}	93.16±0.67^{a}	-25.77±1.91^{c}	7.96±0.42^{c}	97.60±0.39^{a}	27.26±1.82^{c}
P-value	0.001	0.001	0.001	0.001	0.001	0.001

L: Brightness, a*: redness, b*: yellowness, ΔE*: color difference, C*: color saturation index*
a-c: Different letters within the columns indicate significantly important differences

Table 2. *Effect of genotype and length of storage period on egg weight, shell weight and shell thickness in the groups (Mean ± SEM)*

Groups		Egg Weight (g)	Shell Weight (g)	Shell Thickness (µm)
Genotype (G)	Atak-S	62.45±0.90	5.96±0.12^{b}	0.34±0.007^{ab}
	Nick Brown	61.12±0.89	7.12±0.14^{a}	0.36±0.006^{a}
	Sussex	62.59±0.91	5.81±0.13^{b}	0.32±0.007^{b}
	Cross Breed	61.01±0.89	5.23±0.14^{c}	0.33±0.006^{b}
Storage Period (SP)	0	65.41±0.67^{a}	6.14±0.10	0.36±0.005^{a}
	15	60.69±0.67^{b}	5.94±0.10	0.34±0.005^{b}
	8	59.28±0.95^{b}	5.97±0.14	0.30±0.007^{c}
Genotype x Storage Period	A-0	65.31±1.34	5.94±0.20	0.35±0.010
	A-15	61.30±1.34	5.97±0.20	0.35±0.010
	A-28	60.74±1.89	5.97±0.29	0.31±0.014
	NB-0	62.06±1.34	6.94±0.20	0.41±0.010
	NB-15	60.98±1.34	7.11±0.20	0.33±0.010
	NB-28	60.34±1.89	7.52±0.29	0.32±0.014
	S-0	68.02±1.34	6.14±0.20	0.34±0.010
	S-15	60.59±1.34	5.23±0.20	0.32±0.010
	S-28	59.16±1.89	6.31±0.29	0.31±0.014
	CB-0	66.25±1.34	5.56±0.20	0.34±0.010
	CB-15	59.90±1.34	5.48±0.20	0.34±0.010
	CB-28	56.88±1.89	4.08±0.29	0.27±0.014
ANOVA	G	n.s	0.001	0.001
	SP	0.001	n.s	0.001
	GxSP	n.s	0.002	n.s

G: Genotype, SP: Storage Period, A: Atak-S, NB: Nick Brown, S: Sussex, CL: Cross-breed
a-c: Different letters within the same columns (genotype and storage period) indicate different values

no significant differences for the average egg weight of different layer genotypes while storage length had a significant effect on the egg weight of the laying hens (P<0.001). Genotype had a significant effect on egg shell weight (P<0.001) and shell thickness (P<0.001) while the effects of storage length on shell thickness were found to be significantly important (P<0.001). Genotype x storage length interaction for shell weight was found to be significantly important, as well.

The effects of genotype and length of storage on internal egg quality traits were presented in *Table 3*. Genotype and length of storage had a significant effect on albumen weight (P<0.05), albumen index (P<0.001 and P<0.001), yolk index (P<0.001 and P<0.001) and HU (P<0.003 and P<0.001). Yolk weight was affected by genotype significantly (P<0.05). Genotype x storage length interaction for all internal parameters was found to be non-significantly important.

Table 3. Effects of genotype and length of egg storage on internal egg quality traits (Mean ± SEM)

Groups		Albumen Weight (g)	Yolk Weight (g)	Albumen Index	Yolk Index	Yolk Color	HU
Genotype	Atak-S	38.86±0.56^{ab}	17.63±0.39^{a}	6.44±0.37^{b}	39.83±0.55^{b}	10.43±0.29	73.73±1.65^{b}
	Nick Brown	37.90±0.55^{bc}	16.10±0.43^{c}	10.01±0.36^{a}	42.40±0.53^{a}	10.77±0.28	81.09±1.66^{a}
	Sussex	39.19±0.52^{a}	17.59±0.44^{a}	6.18±0.37^{b}	38.65±0.55^{b}	10.57±0.27	75.51±1.65^{ab}
	Cross Breed	37.24±0.50^{c}	18.54±0.41^{a}	5.65±0.38^{b}	40.37±0.54^{b}	10.07±0.28	71.97±1.66^{b}
Storage Period	0	41.63±0.62^{a}	17.64±0.36	10.74±0.28^{a}	45.24±0.41^{a}	10,88±0,21	88.23±1.25^{a}
	15	37.39±0.61^{b}	17.36±0.38	5.05±0.28^{b}	38.50±0.41^{b}	10.30±0.21	70.76±1.25^{b}
	28	35.89±0.60^{b}	17.42±0.39	4.02±0.40^{b}	34.09±0.59^{c}	10.20±0.30	59.95±1.76^{c}
Genotype x Storage Period	A-0	41.89±1.09	17.48±0.60	9.26±0.56	45.20±0.83	11.00±0.42	87.52±2.49
	A-15	37.73±0.90	17.60±0.59	4.62±0.56	37.24±0.83	10.90±0.42	68.29±2.49
	A-28	36.96±0.89	17.81±0.61	5.43±0.79	34.24±1.17	9.40±0.59	57.05±3.53
	NB-0	39.19±0.91	15.93±0.59	8.00±0.56	47.73±0.83	10.70±0.42	97.07±2,49
	NB-15	37.97±1.01	15.90±0.54	5.36±0.56	40.03±0.83	10.40±0.42	74.57±2.49
	NB-28	36.35±0.99	16.47±0.58	3.34±0.79	36.47±1.17	11.20±0.59	62.20±3.53
	S-0	43.72±0.98	18.16±0.56	7.57±0.56	42.63±0.83	10.80±0.42	83.09±2.49
	S-15	37.76±0.92	17.60±0.59	5.81±0.56	37.60±0.83	10.10±0.42	73.84±2.49
	S-28	35.82±0.93	17.03±0.58	4.11±0.79	32.77±1.17	10.80±0.59	63.74±3.53
	CB-0	41.69±0.88	19.00±0.61	8.13±0.56	45.38±0.83	11.00±0.42	85.23±2.49
	CB-15	35.58±0.88	18.34±0.62	4.41±0.56	39.10±0.83	9.80±0.42	66.34±2.49
	CB-28	34.50±0.99	18.30±0.62	3.18±0.79	32.86±1.17	9.40±0.59	56.79±3.53
ANOVA	G	0.05	0.05	0.001	0.001	n.s	0.003
	SP	0.05	n.s	0.001	0.001	0.08	0.001
	GxSP	n.s	n.s	n.s	n.s	n.s	n.s

G: Genotype, SP: Storage Period, A: Atak-S, NB: Nick Brown, S: Sussex, CB: Cros-breed
a-c: Different letters within the same columns (genotype and storage period) indicate different values

Discussion

Egg weight is an essential trait for egg quality, and it mainly depends on the hen genotype. In general, native poultry breeds lay smaller eggs than commercial hybrid strains [34,35]. Local and exotic hens had smaller eggs than commercial hybrid hens [36]. Sözcü et al.[37] compared the performance of two Turkish local laying hens, and they reported that the eggs from Turkish Atak-S hens tended to be heavier than eggs from Turkish Atabey hens. Dual-purpose hens, which are used for both egg-laying and meat, have lower quality eggs and egg weights if we compare them to commercial layer hens [38]. In this current study, there were no significant differences in the daily fresh egg weight of different genotypes. According to EU Commission regulations [22] eggs of all genotypes used in this study can be classified as medium eggs and their weight is found to be very close to the bottom level of large eggs (large weight ≥63 g). This was important because egg quality may be negatively affected by higher egg weight due to the synthesis of calcium for eggshell mass and the synthesis of protein in egg albumen. Weight loss during egg storage progressively increased with the length of the storage period. But the most distinctive losses were determined during the 15-day storage period. There were no significant differences for the egg weight between 15 and 28 days of the storage period. Along with the length of the storage period, egg weight loss can be affected by hen breed, breeder's age, room temperature, and room humidity etc.[39,40].

There were significant differences in the shell weight of the eggs among the genotype groups. Commercial Nick Brown had significantly greater egg shell weight than the other genotype groups. Genotype x storage period interaction for eggshell weight revealed that length of storage had a significant effect on eggs of Sussex and local cross-breed hens, while no significant differences were detected for eggs of Atak-S and Nick Brown hens. This means egg loss in Nick Brown and Atak-S was found to be lower than Susses and Cross-breed hens with the length of egg storage. In this study, the eggs of commercial Nick Brown hen had significantly the greatest shape index

value. Eggs of Sussex layer breed and Atak-S genotypes had similar shape index values. The shape index value of all breeds was between acceptable values of 72-76 [41] for A-grade eggs and eggs of Nick Brown was a bit higher than the acceptable standards. Although there was not much more impact of shape index on consumer interest in table eggs, the unnatural shape and poor shell quality of breeder hen eggs are not desired because of the higher risk of cracked eggs and poor hatchability [42]. In a study, it was reported that Italian dual-purpose purebred Ermellinata di Rovigo showed the lowest shape index compared to commercial layer hens [43]. In general, egg shell weight does not increase after 50 weeks of age while egg weight still increases after this age. This disbalance between egg and shell weight development results in an increased risk of weak shell quality and more broken/cracked eggs.

The most visible feature of table eggs and one of the most sensitive issues for consumers is their eggshell color and thickness which is an indicator of the shell quality of eggs. In this study, the shell thickness of the eggs was significantly affected by genotype and storage period ($P<0.001$). The shell thickness value of eggs in commercial Nick Brown and fresh eggs was found to be greater than the others. In agreement with our results, Grasshorn et al.[10] reported that eggshell thickness was negatively influenced by storage duration. Sözcü et al.[37] reported that Turkish Atak-S hens had a stronger eggshell structure than the eggs of Turkish Atabey hens. The shell thickness of eggs is mainly determined by the proportion of calcium deposition and the length of egg shell formation in the uterus [6]. Time spend in uterus and oviposition time also has an effect on the thickness of the eggshell. We did not use the same eggs in every period of storage to determine the egg quality. Individual differences for egg shell thickness might be a reason for differences in terms of egg storage.

Although eggshell color does not affect the shell quality, flavor, cooking characteristics, nutritional value, or shell thickness of eggs in general, especially in pure breed poultry, the eggshell color is characteristic of a specific poultry breed [44]. In this study, the eggshell colors of the hens were naturally different from each other *(Fig. 1)*. The color variability of table eggs, brown and white, is attractive for consumers, particularly if the differences are as extreme as green or blue [45]. The cross-breed hens have a perfectly white eggshell color with a 93.16 L* value. Atak-S and Nick Brown followed the cross-breed hen for the L* value. Commercial Nick Brown that normally laying a dark brown egg had the lowest egg shell color lightness. It was found that the L* values of the eggs in all breeds were in agreement with their eggshell color as darker or lighter. Like in an agreement with eggshell color, the "a" values of eggs from Nick Brown were found to be darker (redness) than the other genotypes. Eggs of cross-line hens had significantly more extreme a* color trait values than the other eggs. As with other color characteristics, the E value in eggs of cross-line hens and the chroma values in eggs of Atak-S hens and Nick Brown hens were found to be significantly greater. Sussex püre-breed and Atak-S genotype had similar egg color characteristics as L*, b*, E, and chroma color values. Drabık et al.[44] showed that the proportion of particular mineral elements in eggs was correlated with the L*, a*, and b* color space coordinates of egg shells. In that study, it was reported that there was a relationship between shell color and egg albumen quality. The color of eggshell is a result of pigments deposited on the shell of hens and L*, a*, b* values of shell colour were varously correlated with pigment contents [46].

In this study, layer genotype has significantly affected both the albumen weight and yolk weight of the eggs ($P<0.05$). Similarly, Nolte et al.[2] reported that the yolk and albumen percentages of the eggs were significantly influenced by the layer genotype. The eggs of Sussex pure-breed had the greatest albumen weight value, while the eggs of cross-breed hens had the largest yolk weight. Several authors reported that native breeds lay eggs with a higher percentage of yolk and a larger amount of albumen than commercial hybrid strains [34,47,48]. In a study, the albumen weight of the eggs of two commercial layer chickens (Hy-line white and brown) was found to be significantly greater than that of native Ermellinata di Rovigo and Robusta Maculata hens [35]. The albumen weight of the eggs was also significantly affected by the length of storage ($P<0.05$), while the albumen index of the eggs was significantly affected by both genotype and storage period ($P<0.001$). In agreement with our study, Kralik et al.[49] reported that the storage period significantly influenced the albumen height of the table eggs. A higher albumen height of eggs indicates denser albumen, and it directly increases the value of the Haugh unit.

Yolk color and yolk weight are commonly used for comparison to yolk quality. In this study, yolk weight and yolk index were significantly affected by genotype, while the storage period affected only the yolk index of the eggs. Similar to the findings of Giampietro-Ganeco et al.[50] the yolk index decreased with increasing storage time. The yolk index value of the fresh eggs should meet the standard reference value as a good yolk index of 0.45 reported by Mertens et al.[51]. According to the DSM egg quality manual, we can consider all eggs as fresh because eggs with a yolk index between 0.29 and 0.38 are identified as fresh [28]. But the eggs with prolonged storage periods and in all genotype groups were under the standard reference value for yolk index as extra fresh eggs (0.38). The eggs stored at room temperature might have been showing significant differences in the yolk index value

from the 2nd week on [13]. A freshly laid egg yolk is round and firm. The strength of the yolk membrane weakens with yolk ages and allowing water to be absorbed from the white. In our study, there were no significant differences in the yolk color of eggs in all genotype and storage period groups. But Nolte et al. [2] reported that the yolk color of the eggs was influenced by the effects of genotype. The yolk color value of the eggs in all interactive groups varied from 9.40 to 11.20. Our results were found to be much greater than the findings of Son et al.[52] for yolk color (from 7.50 to 8.40) in eggs of laying hens. The hen itself, the environment, correct husbandry practices, and good quality feed are very important to deliver an attractively pigmented yolk.

The haugh unit is the most widely used measure of the freshness of eggs and reflects the quality of eggs all over the World [11,53]. Albumen height, yolk height, HU, and yolk pH are good indicators of storage time, and it can be assumed that it is important to consider that eggs were fresh and of good quality [10,13]. In this study, the Haugh unit values were significantly affected by the genotype and length of the egg storage period. Commercial Nick Brown had significantly greater HU, while Atak-S, Sussex, and cross-breed had similar HU values. In agreement with the findings of Federn et al.[13], Kralik et al.[49] and Petek and Abdourhamane [54] the HU values calculated in our study quickly decreased with the length of storage period. Average HU values of fresh eggs from all genotypes were found to be acceptable for the AA grade standard of table eggs at a score of 72 or above [55] . Eggs of Nick Brown and Sussex at 15 days of storage also met the standard value for HU. Son et al.[52] reported that HU varied from 74.9 to 77.9 in laying hens. Genotype had a significant effect on Haugh units, and commercial laying hens, in general, had a higher HU value than pure-breed or local layers. In another study, Rizzi [35] showed that the eggs of Hy-line white showed a greater HU value than the eggs of Hy-line Brown, local Robusta Maculata, and Ermellinata di Rovigo breeds. Kejela et al.[38] reported that the Haugh unit of the eggs of the native chickens was 74.91 and 82.55, while for Sasso chickens were 86.50 and 87.04, and Bovans brown were 94.60 and 86.29, respectively.

The external and internal egg quality characteristics of poultry eggs are affected by a variety of factors such as genotype, nutrition, housing conditions, animal health, stage of laying period, environmental condition, length of egg storage, and storage conditions. Besides these factors, some external quality parameters such as egg size, eggshell thickness, shape index, eggshell color, and thickness might have an effect on internal egg quality. In general, the free-range eggs had superior egg quality parameters compared to the eggs from colony cages [1,56]. If we will implement cross or pure-breed laying hens in commercial egg production, it is necessary to identify these birds according to other production criteria such as egg weight and egg weight increase rate, shell strength, dry matter of egg albumen or yolk ratio. In our study, daily fresh eggs in all genotypes exhibit high-quality measurement according to their HU values, and eggs produced by Nick Brown and Sussex met an A-graded egg standard even until the 15 d of storage. Therefore, the laying hen genotype should be taken into account when determining the maximum storage time in room or refrigeration conditions. It should be best to consume the eggs as fresh as possible because the persistence of egg quality tends to decline due to the length of the egg storage period. The eggs of the commercial Nick Brown genotype seems slightly superior to those of other group eggs, while Sussex and Atak-S had similar external quality characteristics. Further studies, including production traits, economics, and the influence of consumer interest, are required for more profitable and sustainable egg production when choosing the laying hen genotype for non-cage systems, especially for free-range housing.

Declarations

Availability of Data and Materials: Datasets used in this experiment are available from the corresponding author (MP) on request.

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Research Article

From Deadly to Life-Saving Effects: Antimicrobial and Antibiofilm Effects of *Tarantula cubensis* Extract on Bacterial and Fungal Pathogens

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Abstract

Every year, many people around the world lose their lives due to antibiotic resistance therapy. These resistances pose serious health threats to both individuals and societies, making even simple services difficult to treat. Antibiotic resistance not only affects medical practices, but also causes a huge economic burden on health systems. In this study, *Tarantula cubensis* spider venom was investigated to produce new antimicrobial agents effective against Gram (+) and Gram (-) bacteria and *Candida* species. Antimicrobial and antibiofilm activity of *T. cubensis* extract against microorganisms were tested using minimum inhibition, resazurin and time killing curve methods. MIC values of *T. cubensis* extract were determined as 14.3-0.45 mg/mL by broth dilution method and 14.3-3.58 mg/mL by resazurin method for the investigated pathogens. Time-kill curve studies confirmed the growth retardant, bacteriostatic/ fungistatic and bactericidal/ fungicidal activity of *T. cubensis* extract. Antibiofilm studies also showed that *T. cubensis* extract significantly inhibited and blocked biofilm formed by pathogens (melittin 8-82%, apamin 1.8-78%). *T. cubensis* was effective in inhibiting biofilm formation (from 99.98% to 75.68%) and eradicating biofilm (from 32.97% to 4.67%) at the highest concentration. *T. cubensis* extract was found to have high antibacterial and antibiofilm properties. Such natural poisons offer a promising and powerful solution to control microbial populations, combat pathogens, and protect human and animal health.

Keywords: *Tarantula cubensis*, Theranekron, Antimicrobial activity, Antibiofilm activity, Time-killing curves

Introduction

Antimicrobial resistance (AMR) has become a persistent global public health issue, with an estimated 10 million deaths annually worldwide by 2050 [1]. When bacteria, fungi, viruses, parasites, and other microbes grow to the point that they eventually develop resistance to the antimicrobial drugs, such antibiotics, that are used to treat such illnesses, it is known as antimicrobial resistance (AMR) [2]. AMR has become one of the biggest worldwide issues of the twenty-first century because of the speed at which the rate of AMR infections is increasing and the lack of new antimicrobial drugs being developed to address this problem [3]. The effects of excessive or improper use of antibiotics in a variety of settings, particularly clinical treatment, agriculture, animal health, and the food chain, may be one of the primary causes of the current problem [4].

AMR is sometimes referred to as the "Silent Pandemic" and is an issue that requires prompt attention, better management, and should not be put off until later [5]. In response to AMR, a number of countries and international health organizations have taken action to solve the issue. The "One Health Approach" was created to make sure that each agency collaborates with other agencies and stays within its area of competence in order to reduce the possible impacts of antimicrobial resistance. The World Organization of Animal Health (OIE) and the Food and Agriculture Organization of the United Nations (FAO) are two organizations that must work together globally to implement this strategy [6]. Furthermore, the World Health Organization (WHO) created the Global Action Plan for controlling AMR (GAP-AMR) and the Global Antimicrobial Resistance and Use Surveillance System (GLASS) in order to fulfill the goals of the GAP-AMR program [7].

Because of the problem of antibiotic resistance, cationic amphipathic peptides, or "antimicrobial peptides," are naturally occurring antibacterial chemicals that are used as one of the preferred antimicrobial groups in the management of infectious illnesses. It has been shown that AMPs have generated attention as additional or alternative antibiotics due to their potency, rapid action, and capacity to eliminate both Gram-positive and Gram-negative bacteria [8]. Theranekron D6 (TD6), sold as an alcoholic extract of the spider *Tarantula cubensis*, is widely used in veterinary medicine for the treatment of animal diseases such as dermatitis, arthritis, inflammatory hoof diseases (panaricium, foot rot), phlegmon, ulcers, abscesses, injuries, purulent lesions and necrosis. In proliferative and necrotic tissues, TD6 demonstrates demarcative, regenerative, antiphlogistic, and resorptive actions. To speed up the healing process, TD6 promotes epithelialization [9]. It is believed to activate the body's defensive mechanisms and naturally reduce inflammation in any proliferative lesion, while its exact mode of action is unknown [10]. According to some reports, it creates a demarcation line around the necrotic tissue as soon as surgery is completed, stops the spread of catabolic enzymes from the necrotic tissue to the surrounding areas and prevents inflammation, all while shielding the ligaments from additional tissue structure degradation [11]. The aim of this study was to investigate the antimicrobial, antibiofilm and biofilm-destroying effects of Theranekron D6 (TD6), an alcoholic extract of *T. cubensis* spider, on pathogens of two Gram-positive bacteria *(Staphylococcus aureus*, and *Enterococcus faecalis),* two Gram-negative bacteria *(Escherichia coli*, and *Proteus vulgaris)* and two *Candida* fungal species *(Candida albicans*, and *Candida parapsilosis).*

Material and Methods

Ethical Statement

Ethics committee approval is not required for this study.

Tarantula cubensis Venom Extract

Theranekron® (alcoholic extract of *T. cubensis)* was purchased from Richter Pharma AG (Wels, Austria) and obtained from the local veterinary clinic with the permission of the veterinarian (Kütahya, Türkiye). As described from Richter Pharma AG, it is prepared by processing the whole spider and diluting it in alcohol. It is commercially available in 50 mL bottles, contains *T. cubensis* D6 and contains 286 mg in 1 mL of ethanol.

Resazurin Preparation

0.01 g Resazurin was dissolved in 50 mL sterile distilled water and sterilized with a 0.22 µm filter and stored at 4°C for 2 weeks after preparation [12].

Bacterial Strains

S. aureus American Type Culture Collection (ATCC) (29213), *E. faecalis* (ATCC 29212), *E. coli* (ATCC 25922), *P. vulgaris* (ATCC 6386), *C. albicans* (ATCC 10231), and *C. parapsilosis* (ATCC 22019) strains were used in this study. These standard strains stored at -80°C were inoculated on tryptic soy agar (TSA, Neogen®, Lansing, MI, USA) and *Candida* species were inoculated on sabouraud dextrose agar (SDA, Neogen®, Lansing, MI, USA) and Candida species were incubated for 48 h and other pathogens for 24 h at 37°C. All strains used in the experiment were treated with sterile 0.9% NaCl solution to achieve a 0.5 McFarland standard using a DEN-1 densitometer [13].

Antimicrobial Activity of *Tarantula cubensis* Extract on Pathogens

Antimicrobial and antifungal activity of *T. cubensis* extract was determined by disk diffusion, Minimum Inhibitory Concentration (MIC) methods. MIC values were also evaluated by Resazurin method and ELISA method.

Application of the Disk Diffusion Method

Mueller Hinton Agar (MHA, Neogen®, Lansing, MI, USA) medium was used for antimicrobial activity and SDA medium was used for antifungal activity of *T. cubensis* extract by disk diffusion method. 0.5 McFarland standard, *C. albicans* and *C. parapsilosis* were inoculated on SDA medium, *S. aureus*, *E. faecalis*, *E. coli* and P. *vulgaris* suspensions were inoculated on MHA medium and spread all over the medium with a swab and 4 sterile antimicrobial susceptibility test discs (Bioanalyse, Turkey) were placed on the medium. Volumes of 25, 20, 15, and 10 µL of *T. cubensis* extract were added to each disk. The petri dishes were allowed to dry for 10-15 min and incubated at 37°C for 24 h for bacteria and 48 h for *Candida* spp. After incubation, it was checked whether inhibitory zone diameter was formed or not and the zone diameters were measured and recorded [14].

Minimum Inhibitor Concentration (MIC) Determination

RPMI-1640 broth (Becton Dickinson, Heidelberg, Germany) for *C. albicans* and *C. parapsilosis* and 100 µL of cation added Mueller Hinton broth (MHB, Merck, Darmstadt, Germany) for *S. aureus, E. faecalis, E. coli* and *P. vulgaris* were added to all wells of 96 microplates to determine the MIC values of antimicrobial agents by liquid microdilution method. 100 µL of *T. cubensis* extract (286 mg/mL) was added to the first well and serial dilutions were made in duplicate (28.6-0.06 mg/mL). Finally, 100 µL of bacteria/candida suspension at 0.5 McFarland turbidity was added to each of these wells. To control bacterial

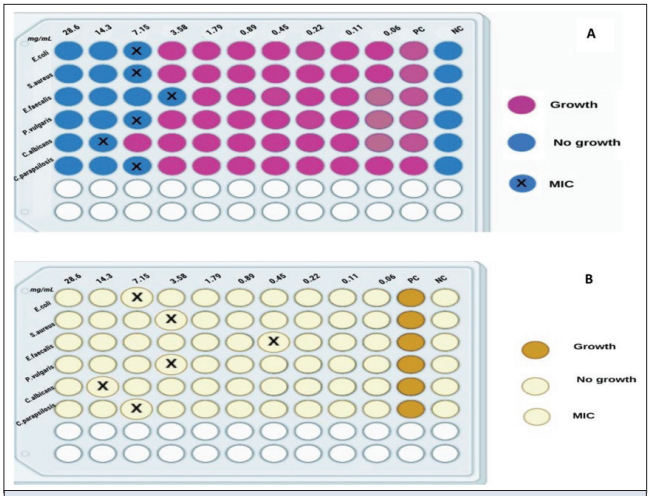

Fig 1. Minimum inhibitor concentration (MIC) determination. **A:** Resasurin method; **B:** Liquid microdilution method

growth, only bacteria (positive control) was added to one last row of the microplate and only medium (negative control) was added to one well. The microplate was incubated in an oven at 37°C for 18-20 h *(Candida* spp. for 48 h). The lowest concentration at which no growth was observed after incubation was considered as the MIC value. After incubation, the absorbance at 600 nm (A600) was determined for each well with a spectrophotometer (Thermo Scientific, Multiskan SkyHigh Microplate Spectrophotometer). Growth inhibition percentages for each microorganism at different *T. cubensis* extract concentrations were calculated as follows: Percent inhibition (%) = [1- (Sample OD/Control OD)] X100

To determine the MIC value by the resazurin method, after incubation, 10 µL of 0.01% resazurin solution was pipetted into all wells and the plates were incubated again at 37°C for 24 h. Any color change from purple to pink was considered positive *(Fig. 1)* [12].

Determination of Time Dependent Kill Curves (Kinetic Kill Curves)

Time-dependent killing level was performed to demonstrate the duration of action of *T. cubensis* extract. *T. cubensis* extract concentrations were prepared at 2X MIC for all microorganisms. To ependorfs, 500 µL of 0.5 McFarland turbid suspension of microorganisms and 500 µL of *T. cubensis* extract concentration were added. Incubated at 37°C. At 0 h, 1 h, 3 h, 6 h, 8 h and 24 h, 50 µL of each tube was added to 450 physiological saline (0.9%) and 100 µL were spread onto TSA/SDA medium. After incubation at 37°C for 24 h (48 h for *Candida* spp.), the colonies formed in the petri dishes were counted and the number of bacteria per mL (cfu/mL) was calculated taking into account the dilution factor. The bactericidal/fungicidal effect's concentration- and time-dependent variation was measured, and its log10 value was examined. Based on the results, a time-death graph was created, with the logarithmic value of the number of bacteria displayed on the "y" axis and time displayed on the "x" axis [15]. After 24 h of incubation, ≥3 $\log_{10}$ and <3 $\log_{10}$ reductions in the total number of CFU/mL relative to the control were considered bactericidal/fungicidal and bacteriostatic/fungistatic activity, respectively. Moreover, after at least 6 h, regrowth was defined as ≥2 $\log_{10}$ rise in viable CFU/mL count [16].

Tarantula cubensis Extract Effect on Biofilm Formation and Eradication

The effect of *T. cubensis* extract on biofilm formation of microorganisms was investigated using microdilution method [17]. RPMI-1640 broth for *C. albicans* and *C. parapsilosis* and 100 µL of Luria Bertani Broth (LB, HiMedia, India) containing 1% glucose for *S. aureus, E. faecalis*, *E. coli* and *P. vulgaris* were added to all wells. Serial dilutions of the prepared *T. cubensis* extract into the first well were prepared from 28.6-0.06 mg/mL to a final volume of 100 µL per well. Each plate was inoculated with 100 uL of 0.5 McFarland bacterial suspension. The positive control with bacteria only and the well with medium only were used as negative controls. After incubation at 37°C for 18-24 h, the medium was carefully aspirated and the biofilms formed were washed three times with 200 µL sterile phosphate buffer saline (PBS) to remove floating bacteria. Staining was then performed with 200 µL of 0.1% crystal violet for 30 min. Excess stain was washed off using PBS and the plates were allowed to dry. The dye bound to the biofilm in each well was dissolved in 200 µL of 33% glacial acetic acid for Gr(+) bacteria and 95% ethanol for Gr(-) bacteria for 10 min and absorbance was checked at 600 nm (Thermo Scientific Multiskan FC). The percentage of biofilm formation was calculated as follows [18]: % Biofilm formation = (OD 600 of treated biofilm/OD 600 of non-treated biofilm) X 100

Elimination of biofilm formation was examined using the minimum biofilm elimination concentration assay. RPMI-1640 broth for *C. albicans* and *C. parapsilosis* and 100 uL LB containing 1% glucose for *S. aureus, E. faecalis, E. coli* and *P. vulgaris* were added to all wells. Each plate was inoculated with 100 uL of 0.5 McFarland bacterial suspension. The positive control containing only bacteria and the well containing only medium were used as negative controls. The plates were incubated at 37°C for 18-24 h for biofilm development. The culture was then washed carefully once with sterile PBS. These biofilms were then exposed to 200 µL of *T. cubensis* extract at various concentrations ranging from 28.6-0.06 mg/mL and incubated again at 37°C for 18-24 h. After incubation, adherent cells were washed 3 times with sterile PBS. They were then stained with 200 µL of 0.1% crystal violet for 30

min. Excess stain was washed off using PBS and the plates were allowed to dry. The dye bound to the biofilm in each well was dissolved in 200 µL of 33% glacial acetic acid for Gr (+) bacteria and 95% ethanol for Gr (-) bacteria for 10 min and the absorbance was checked at 600 nm (Thermo Scientific Multiskan FC) and calculated as follows [17]. % Biofilm eradication = [1-(OD 600 of treated biofilm/OD 600 of non-treated biofilm)] X 100. Biofilm inhibition was defined as concentrations indicating 50% and 90% eradication.

Results

Antimicrobial Activity of *Tarantula cubensis* Extract by Disk Diffusion Method

In this study, the antimicrobial and antifungal activities of *T. cubensis* extract were investigated. Antimicrobial and antifungal activities were determined against all test microorganisms with inhibition zones ranging from 24.0-8.0 mm for bacteria and 22.0-8.0 mm for *Candida* spp. *(Table 1)*.

Minimum Inhibitory Concentration of *Tarantula cubensis* Extract

The MIC activities of *T. cubensis* extract on the analyzed microorganisms are presented in *Table 2*. While the MIC values of *T. cubensis* extract were between 14.3-0.45 mg/mL, 14.3-3.58 mg/mL concentrations were determined by the resazurin method. The lowest MIC value was 0.45 mg/mL on *E. faecalis* strain and 3.58 mg/mL *(Table 2)* on *E. faecalis* strain by resazurin method *(Fig. 1)*.

Table 1. *Antimicrobial activity of Tarantula cubensis extract*

Test Microorganisms	Inhibition Zones (mm)*			
	Extract Quantities			
	25 µL	20 µL	15 µL	10 µL
Staphylococcus aureus	18	10	8	8
Enterococcus faecalis	20	19	11	8
Escherichia coli	24	21	18	14
Proteus vulgaris	12	10	9	6**
Candida albicans	16	13	10	8
Candida parapsilosis	22	18	14	10

** Values include disk diameters (6 mm), ** Zones are not observed*

Table 2. *Minimum inhibitory concentration of Tarantula cubensis extract*

Test Microorganisms	MIC (mg/mL)	Resazurin (mg/mL)
Staphylococcus aureus	3.58	7.15
Enterococcus faecalis	0.45	3.58
Escherichia coli	7.15	7.15
Proteus vulgaris	3.58	7.15
Candida albicans	14.3	14.3
Candida parapsilosis	7.15	7.15

MIC: minimum inhibitory concentration

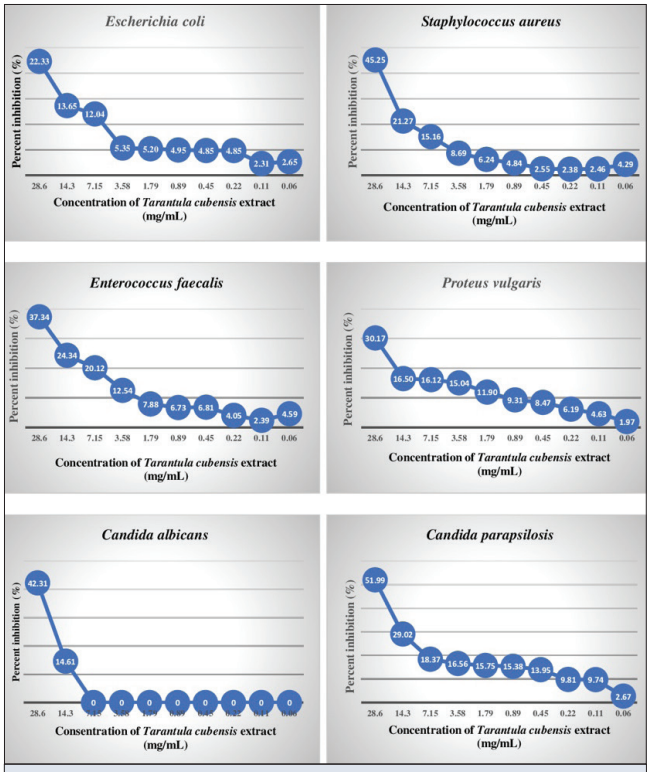

Fig 2. Growth inhibition percentages for each microorganism at different *T. cubensis* extract concentrations

Percent Inhibition of *T. cubensis* Extract

In order to evaluate the effect of *T. cubensis* extract on the inhibition of bacteria, variable amounts (28.6-0.06 mg/mL) were evaluated. The results reveal that the inhibition of bacterial cells is directly linked to the amount of *T. cubensis* extract consumed. It was noticed that the percentage of bacterial inhibition ranged from 1.97% to 51.99% for the variable concentration of *T. cubensis* extract in the range of 28.6-0.06 mg/mL. In general, we can say that the inhibition efficiency is directly proportional to the respective concentrations of *T. cubensis* extract *(Fig. 2)*.

Results for Time-Dependent Kill Curves

According to the analysis of time-dependent killing curves, *T. cubensis* extract was found to be bactericidal/fungicidal against all pathogens we examined. For *E. coli*, *E. faecalis*, and *P. vulgaris*, the positive control and *T. cubensis* group have a similar baseline level at 0 h (~6 log CFU/mL), while the negative control shows no growth. At 1 h, the *T. cubensis* group slightly suppresses growth compared to the positive control, but the difference is not very pronounced. Between 3 and 24 h, *T. cubensis*

Table 3. *Number of viable colonies in the time dependent killing test of the examined pathogens during the evaluation hours of Tarantula cubensis extract (log_{10})*

Test Microorganisms	log_{10} Number of Live Colonies (CFU/mL)					
	Hour					
	0	1	3	6	8	24
Staphylococcus aureus	4.95*	4.93*	4.87*	4.77*	4.47*	**4.59****
Positive control	5.95	6.08	6.19	6.26	6.69	7.81
Enterococcus faecalis	4.7*	4.81*	4.9*	4.89*	4.8*	4.97*
Positive control	5.6	5.85	5.89	6.06	6.54	7.19
Escherichia coli	4.82*	4.79*	4.79*	4.66*	4.22*	5.16*
Positive control	6.08	6.13	6.24	6.39	6.66	7.46
Proteus vulgaris	4.58*	4.85*	4.66*	4.3*	**4.18****	5.01*
Positive control	6.19	6.56	6.77	6.8	7	7.37
Candida albicans	4.25*	4.64*	4.6*	4.43*	4.39*	**4.25****
Positive control	6.03	6.15	6.56	6.6	6.89	7.53
Candida parapsilosis	4.39*	4.51*	4.48*	4.12*	3.97*	**3.6****
Positive control	5.98	6.27	6.4	6.61	5.71	7.01
Negative control	0	0	0	0	0	0

** Bacteriostatic/fungistatic activity; ** Bactericidal/fungicidal activity*

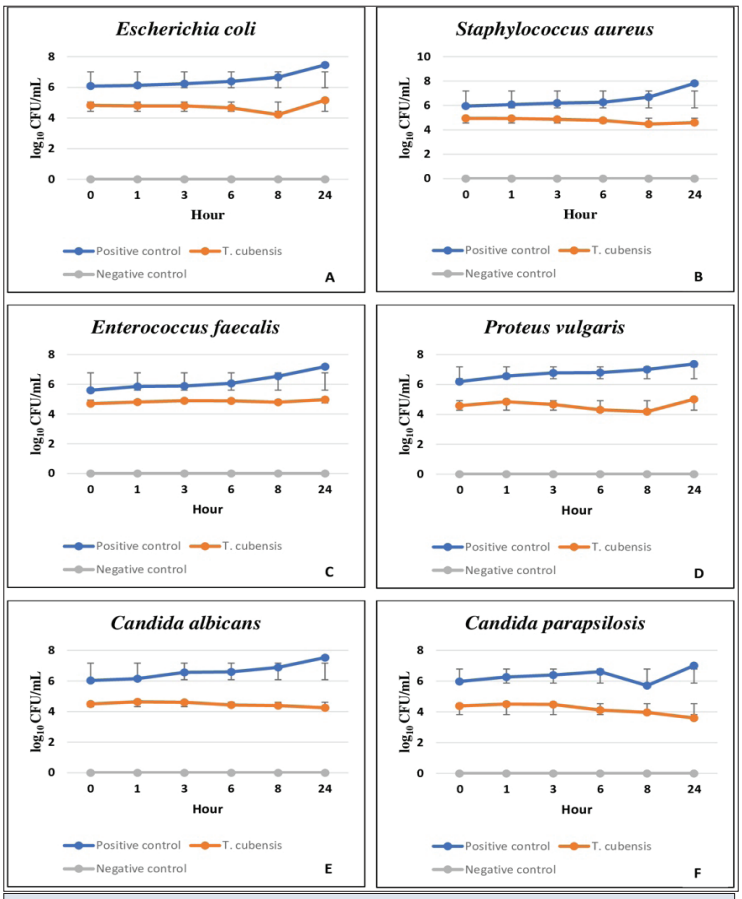

Fig 3. A: *Escherichia coli,* **B:** *Staphylococcus aureus,* **C:** *Enterococcus faecalis,* **D:** *Proteus vulgaris,* **E:** *Candida albicans,* **F:** *Candida parapsilosis.* X-axis represents time to kill and Y-axis represents logarithmic time(log) growth phase. Error bars indicate standard deviation

Table 4. *Effect of different concentrations of T. cubensis extract on biofilm formation and eradication of pathogens (%)*

Microorganisms	Concentrations (mg/mL)																	
	28.6		14.3		7.15		3.58		1.79		0.89		0.45		0.22		0.11		0.06	
	F	E	F	E	F	E	F	E	F	E	F	E	F	E	F	E	F	E	F	E
Staphylococcus aureus	82.41	22.61	81.66	13.94	79.47	11.01	76.83	6.55	76.41	7.11	76.41	5.57	75.65	0	61.86	0	60.49	0	54.0	0
Enterococcus faecium	93.48	29.65	87.62	19.21	59.83	18.85	55.79	5.12	55.43	4.01	54.83	0	54.08	0	50.44	0	50.44	0	42.93	0
Escherichia coli	75.68	32.97	72.45	28.95	68.83	28.52	68.62	22.97	68.60	20.63	66.11	21.20	60.35	17.04	66.11	11.51	57.22	7.13	55.12	0
Proteus vulgaris	99.98	4.67	87.62	19.21	59.83	18.85	99.73	0	99.68	0	99.60	0	99.27	0	99.11	0	97.60	0	88.06	0
Candida albicans	99.96	7.13	99.95	0	92.81	0	83.20	0	82.25	0	79.33	0	73.84	0	72.47	0	64.53	0	54.03	0
Candida parapsilosis	99.96	21.70	99.68	8.45	99.49	3.28	87.08	0	86.59	0	82.14	0	79.89	0	74.87	0	56.42	0	54.58	0

F: formation; E: eradication

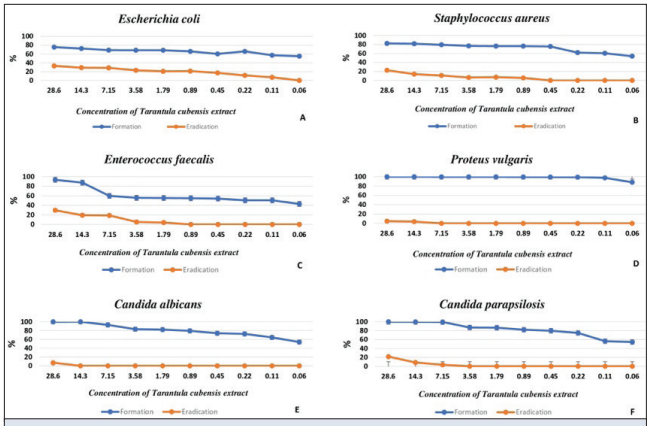

Fig 4. A: Effect of different concentrations of *T. cubensis* extract on biofilm formation and eradication of pathogens (%). *Escherichia coli,* **B:** *Staphylococcus aureus,* **C:** *Enterococcus faecalis,* **D:** *Proteus vulgaris,* **E:** *Candida albicans,* **F:** *Candida parapsilosis*

continues to suppress growth compared to the positive control, but not completely. The growth rate slows down, but does not increase as much as in the positive control. For *S. aureus*, *C. albicans*, and *C. parapsilosis*, the positive control and *T. cubensis* group have a similar baseline level (~6-6.5 log CFU/mL) at 0 h, while the negative control shows no growth. At 1 h, *T. cubensis* starts to suppress growth earlier and significantly compared to the positive control. Between 3 and 24 h, *T. cubensis* significantly suppresses growth compared to the positive control, and the difference persists. The effect of *T. cubensis* is stronger in these species. The change in colony number as a result of the time-dependent killing test is shown in *Table 3* and the time-dependent killing graph of the isolate studied is shown in *Fig. 3*.

Antibiofilm Activities

T. cubensis extract was found to significantly affect biofilm formation and eradication on the tested bacteria. *T. cubensis* extract was found to be more effective on biofilm formation from 99.98% to 75.68% at the highest concentration *(Table 4)*. Although a significant reduction in biofilm formation was detected, it gave a low percentage (32.97% to 4.67%) in biofilm eradication. Significant reduction in biofilm formation was observed at all concentrations, while biofilm eradication was not observed at all concentrations *(Fig 4, Table 4)*. *T. cubensis*, extract showed the greatest effect on *P. vulgaris* in biofilm formation and on *E. coli* in biofilm inhibition *(Fig 4, Table 4)*.

Discussion

Infectious diseases continue to be a major global health problem, contributing 41% of the disease burden as defined by Disability-Adjusted Life Years worldwide [21]. One of the primary reasons of this issue is the widespread occurrence of acquired bacterial resistance to antibiotics, which poses a major danger to public health worldwide

today in the form of pandemics and epidemics of antibiotic resistance. Due to the problem of resistance to antibiotics, there is an urgent need to develop new, potent antimicrobial strategies based on natural agents with different mechanisms of action, which serve as sources of antibacterial and antifungal activity [22]. The antimicrobial and antibiofilm effects of Theranekron D6, an alcoholic extract of the *T. cubensis* spider were investigated.

Nature is known to offer many bioactive compounds in the form of animal venoms, algae and plant extracts that have been used in traditional medicine for decades. Animal venoms and secretions are a pharmaceutically promising wealth of molecules [23]. Studies have observed that many animal poisons and secretions such as frogs, bees, scorpions and ants show antimicrobial effects [15,24,25].

Many studies have shown the antimicrobial activity of many antimicrobial peptides obtained from spider species, which are venomous animals. Al-Kalifawi et al.[26] investigated Al-Ankabut's home (spider web) extract as an antimicrobial biomaterial against multidrug resistance bacteria and found an inhibition zone of 12 mm for *Enterobacter* spp. 8 mm for *E. coli*, 10 mm for *Proteus* spp., and 16 mm for *S. aureus*. The venom of *Lycosa coelestis* has also been reported to have antimicrobial activity on *S. aureus* and *E. coli* [27]. In animal studies of *T. cubensis* extract, it has been reported that it has a therapeutic effect on oral lesions [28], and clinically accelerates uterine involution and completes uterine involution by supporting the resolution and elimination of infection in the uterus [29]. By disk diffusion method, we detected a zone of 24.0-8.0 mm for bacteria and 22.0-8.0 mm for *Candida* spp. The MIC concentration was 14.3-0.45 mg/mL by liquid dilution method and 14.3-3.58 mg/mL by resazurin method.

Bacteria can adapt to different conditions thanks to biofilms, which aid in their survival. They include persister cells, which are well-known for having a high level of antibiotic resistance [19]. When bacteria are in a planktonic stage, they are susceptible to antibiotics, but because of the durability of biofilms, they become resistant to them [20]. Thus, the effectiveness of *T. cubensis* extract against pathogen biofilms was assessed in this work. Tests were conducted on the effects of *T. cubensis* extract on the prevention of biofilm formation as well as the elimination of pre-formed biofilm. The natural peptide isolated from the venom of the spider *Lycosa erythrognatha (L. erythrognatha)* showed a strong reduction of antibiofilm properties (67%) as well as antifungal activity [30]. *L. coelestis* effectively inhibited biofilm formation on *E. coli* and disruption of mature biofilms [27]. *L. erythrognatha* showed remarkable efficacy with >30% inhibition of biofilms on Methicillin-resistant *Staphylococcus aureus* (MRSA) and was reported to be superior to vancomycin in terms of rapid bactericidal and anti-biofilm effects [31]. In our study, we found that *T. cubensis* extract showed inhibition from 99.98% to 75.68% and a significant reduction in biofilm formation from 32.97% to 4.67%.

L. erythrognatha spider toxin has been reported to show high bactericidal activity against MRSA cells and can eliminate a high bacterial load after only 1 h of exposure and completely eliminate the bacterial load within 3 h [31]. It was observed that the number of visible colonies of the Chinese wolf spider Lycosa sinensis on *E. coli* decreased significantly in the first 5 min [32]. In the time-dependent killing method of *Lycosa singoriensis* on *E. coli*, significant microbial reduction was reported in the first 10 min [33]. In our study, we found significant microbial reduction from the beginning of the time-dependent killing method on all pathogens.

In this study, the growth of various microorganisms over time was observed, and bacteriostatic, fungistatic, bactericidal, and fungicidal effects were noted. *E. coli* and *S. aureus* showed clear bactericidal effects at 24 h, while *P. vulgaris* exhibited bactericidal activity at 8 h. *C. parapsilosis* displayed fungicidal activity at 24 h, but no significant reduction was observed for other microorganisms, particularly *E. faecalis* and *C. albicans*, indicating bacteriostatic or fungistatic effects. Overall, antimicrobial and antifungal activities were effective on certain microorganisms at specific time points, with varying results depending on the organism.

The current research reveals a real solution for antimicrobial resistance using natural *T. cubensis* extract. The antimicrobial and antibiofilm effects of *T. cubensis* extract on bacterial and fungal pathogens support the use of this natural compound as a potential therapeutic agent, paving the way for future research and therapeutic applications. These findings suggest that tarantula venom and similar biological resources may play an important role in developing infection control and treatment strategies.

Only standard laboratory strains were used in the study and no comparative evaluation was made with clinical isolates. This is considered as a limitation of the study.

In conclusion, the antimicrobial and antibiofilm effects of *T. cubensis* extract on bacterial and fungal pathogens are remarkable for their ability to inhibit biofilm formation as well as the action of these components against microbes. These properties allow the development of new treatment methods, especially in the fight against antibiotic-resistant microorganisms. Identification of active compounds in *T. cubensis* extract and further investigation of the mechanisms of these compounds may help to take important steps towards clinical applications. In addition, it can be said that these studies have great potential in

terms of reducing the risk of infection and increasing the effectiveness of existing treatment methods, especially in individuals with weak immune systems. Future research should further investigate the pharmacological properties and application potential of tarantula venom.

Declarations

Availability of Data and Materials: Materials and data sets from the study are available upon request from the corresponding author.

Funding Support: No funding was provided for this study.

Competing Interest: The authors declare that they have no conflict of interest.

Ethical Approval: Ethics committee approval is not required for this study.

Declaration of Generative Artificial Intelligence: The author of the current study declare that the article and/or tables and figures were not written/created by AI and AI-assisted technologies.

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Research Article

Genome-wide and RNA-Seq Highlight Genetic Characteristics of Rumpless Signals in Piao Chicken

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Abstract

Piao chicken is a unique rumpless chicken breed in China. Understanding the genetic relationship between rumpless chicken and other specific selection target breeds in the process of differentiation of the rumpless signal genes is helpful to reveal the genetic basis of rumplessnesss. In this study, transparent staining was performed on the bones of 16 tailed chickens and 16 Piao chickens to observe the dynamic bone formation process of embryos at different developmental stages. Microarray data of 988 chickens, resequencing results of 30 Piao chickens and 30 tailed chickens and 10 transcriptome samples were used for analysis. The results showed that tailbone development had tail buds before 4 to 6 days of incubation, and the difference was not significant between tailed chicken and Piao chicken, and the tail buds gradually degenerated in the later stage of Piao chicken. Most genes showed obvious inhibited expression in 8 d of embyro. We also found that exon skip was the most common type, and the PSI values of the *ADGRL3* and *PROM1* genes were differentially expressed between tailed chicken and Piao chicken of embryonic development constantly, which may be a special role that has not been recognized before. The network attributes of Piao chicken is tighter and the linkage between genes is stronger, especially the wnt signaling pathway and notch signaling pathway were mentioned.

Keywords: Piao chicken, Rumpless chicken, Dynamic transcriptional characteristics, Co-expression network, Signaling pathway

Introduction

There are hundreds of domestic chicken breeds worldwide, including indigenous, commercial, and cockfighting chicken, were developed by artificial selection for different purposes [1]. The rich genetic diversity and extensive genetic basis of these breeds provided excellent materials [2,3] in the sustainable utilization and conservation of these genetic resources [4,5]. “Rumpless” is when a genetic mutation causes an animal to lose a tail and have a stunted tail bud [6]. The rumpless chicken has the loss of the tail omnium and the tail vertebrae, which the first two bones behind the ischium have the shape of vertebrae, and the last one is irregularly shaped [7]. This difference in the tip of the tail shows how different species terminate their spines [8]. Previous studies have reported that the mutation region of rumplessness in embryo of rumpless Araucana chicken at different stages is located the 2.14 Mb chromosome region of chromosome 2, and two unique genes *IRX1* and *IRX2* are central for developmental prepatterning. Although there were no mutations in their coding region sequences, the protein expression levels

of *IRX1* and *IRX2* were significantly different between tailed and rumpless chicken [3]. Wang et al.[9] revealed that *IRX4*, *IL18*, *HSPB2* and *CRYAB* genes are implicated in tail development, and showed that rumplessness might be selected along with high-yield traits in Piao chicken. The three regions with the strongest repeatable selection signal are CHR2:85.52-86.07 Mb, CHR4:75.42-75.48 Mb, and CHR24:6.14-6.25 Mb. Hongshan chicken have rumpless, however, the number of caudal vertebrae in rumpless Hongshan chickens was normal, so rumplessness in Hongshan chicken was not related to the development of the caudal vertebrae. rumplessness in Hongshan was due to abnormal development of tail feather rather than abnormal development of caudal vertebrae [10]. Wang's reorting allowed us to reduce the search area to 71.8-72 Mb on the Z chromosome, which appeared a strong candidate pseudogene *LOC431648* involving in wnt/β-catenin signaling pathway to regulate feather development in chicken [11]. By measuring two tail length variables (central and maximum) of Japanese indigenous chickens, it was revealed that the shape of the tail feathers varies with the growth stage [12]. The research team previously identified 17 selected regions related to rumplessness by comparing the genomes of tailed chicken and rumpless chicken, which are mainly distributed on chromosomes 2, 10 and 15, and are mainly involved in neural development, skeletal development, ganglia development and other processes. At the same time, the QTLs in the candidate regions were found to be related to egg weight, carcass weight, pecking feather, tibia character, spleen weight, body weight and growth traits. It is noteworthy that *IRX4* (CHR2:86065381-86068294) and *IRX2* (2:8,6624614-86631727) genes were annotated near the interval 86100001-86140000 and 86190001-86230000 on 2 chromosome [13].

In the selection process of other breeds, is there any overlap between mutant genes in their genomes and those of rumpless chickens? What are the transcriptional characteristics of these overlapping genes between chicken and rumpless chickens? So far, the genetic mechanism of rumplessness in Chinese Piao chicken not been clarified [14]. The purpose of this research was to observe the key period of bone development of tailed chicken and Piao chicken by staining the embryonic bones and making transparent specimens to observe the overall skeleton histomorphology and the position relationship between bones and muscles. Based on the microarray data from public database GEO, the differentiation relationship of tail length traits among different selected breeds was compared [15]. Furthermore, we found the transcriptional expression characteristics of rumplessness in embryo development, which left the selective differentiation signal during the long process of chicken breed selection. The dynamic changes of gene expression regulation network and the functional classification of differential gene sets were compared between tailed chicken and rumpless chicken during development.

Material and Methods

Ethical Statement

All experimental procedures were approved by the Animal Protection and Use Committee of Inner Mongolia Agricultural University and strictly followed animal welfare and ethical guidelines ([2020]085).

Sample Collection and SNP Calling

The downloaded microarray data is used for analysis SNP and population structure analysis. Neighbor-joining tree was contructed using PHYLIP software (vision 3.69) [16] based on quality-controlled data *(Fig. 1)*. A total of 988 samples came from report of Zhang et al.[15] were regrouped and screened in this study, which were divided into local, commercial, game and wild types according to different uses. We calculated the pairwise F_{ST} in 4 and the top 5% loci were selected and the genes in the genome were mapped. Data on the genomes of tailed and rumpless chicken came from Hu's doctoral dissertation [13], including whole-genome re-sequencing results of 30 Piao chicken and 30 tailed [13]. In this experiment, compared the influence of rumplessnesss on the formation of different breeds by overlapping analysis between candidate genes of Hu [13] and differentiated genes of different breeds.

Egg Hatching

The egg incubator tank filled with water, temperature and humidity of incubators were adjusted 2 h in advance before placing fertilized eggs (Camellia chicken and Piao chicken). The temperature for chick hatching period and incubation eggs was 37.5-39.5ºC. The relative humidity during egg incubation was 65-70%, while during the hatching period was 55-60%. The round blunt end of the fertilized egg facing up. The egg incubator was ventilated

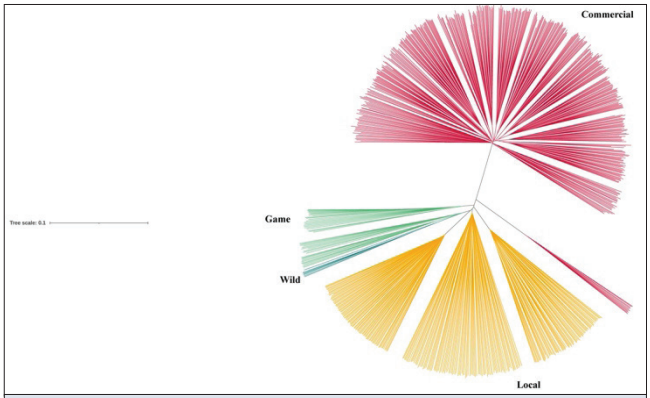

Fig 1. Neighbor-joining tree of G, W, L, and C chicken

for 10 min every day, and the eggs were turned 15 times every day during egg incubation [17].

Sample Collected of Chicken Embryos

We carefully removed the egg from the incubator, rounded end up, tip down, and placed it on an egg seat. Use tweezers to poke a small hole in the eggshell and remove the eggshell and chorionic membrane. When eggs are 1-3 d, they cannot be sampled because embryos have not been formed so samples are taken from 4 d. The embryo is bled to death by carefully cutting the blood vessels around the embryo (pre-embryo) or umbilical cord using small scissors. The embryos are removed with a small spoon and placed in a petri dish. The egg yolk is rinsed off with saline and the amniotic membrane is removed to reveal the embryo. Embryos are small before 7 d and can be directly put into formalin fixative. After 7 d, chicken embryos were and internal organs were removed and rinsed with distilled water to make soft tissue and vertebrae of chicken embryos more transparent. After 10 d, better penetrate the liquid and observe the shape of the coccyx, we removed the internal organs of the chicken embryos after death, removed the skin, and gently cut or pricked the muscles around the coccyx with a knife.

The chicken embryos were clamped on both sides with wire mesh, fixed with formalin (5-6 d, 4% formalin/12 h fixed; 7-8 d,10% formalin/12 h fixed; 9-21 d, 10% formalin/24 h fixed), washed with running water, dehydrated by ethanol solution with concentration ≥70%.

Pre-transparent Treatment

The completely dehydrated chicken embryos were put into 1-10% KOH transparent until the chicken embryo muscle tissue transparent. The transparent time was determined by the size of the chicken embryo (4 d, 1% KOH, 8 min 10 s; 5 d, 1% KOH, 22 min 36 s; 6 d, 1% KOH, 51 min 36 s; 7 d, 2% KOH, 2 h; 8 d, 3% KOH, 24 h; 9 d, 4% KOH, 24 h; 10 d, 5% KOH, 12 h).

Bone Staining Method

Cartilage staining solution (Alcian blue, 0.15% alcian blue ethanol (70%) solution: glacial acetic 70% ethanol =1 : 1 : 18, mixed evenly); Toluidine blue configuration (distilled water: toluidine blue staining solution =100 : 0.05, mixed evenly). Hard bone staining solution: Preparation of cyruxin teleost staining solution: 200 mL solution of 0.01 g alizarin red mixed with of 1% potassium hydroxide to make a dark purple stain.

Methods for cartilage staining [18]: embryos of chicken were immersed in ali xin blue cartilage solution for two days or immersed in toluidine blue cartilage solution for two days. Methods for decolorizing: remove excess color from the soft tissues other than the bones, the embryos of chicken were placed in absolute ethanol, 50% ethanol, and water for one day each. Hard bone staining: embryos of chicken completed the above procedure were immersed in the configured alizarin red bony staining solution for one day.

Decoloration and Transparency Steps

In this study, 25%, 50%, 75%, 100% gradient glycerol and 1% potassium hydroxide mixtures were used to remove excess staining solution (25%, 50%, 75%, 100% gradient glycerol refers to glycerol and potassium hydroxide volume ratio of glycerol: potassium hydroxide 1:3; Glycerol: potassium hydroxide is 1:1; Glycerol: potassium hydroxide 3:1; Glycerol: potassium hydroxide is 1:0). Next, 25%, 50%, 75% and 100% gradient glycerol were successively soaked in each gradient for 1 d, during which the specimen was constantly shaken to make the liquid exchange more thorough and achieve the desired transparent effect [18].

RNA-seq and Alternative Splice Processing

Please refer to Hu's paper for RNA-seq of gene expression in different embryonic stage of PB and RB [13]. In short, caudal bone samples from days 8, 11, 14, 16, and 21 of the PB and RB embryo were sequenced. The differentially expressed genes were screened by RNA-SEQ analysis (|Log2FoldChange| ≥1, P value <0.05, Q value <0.05).

In this study, rMATS 4.1.2 software [19] was used to identify five types of alternative splicing, namely skipped exon (SE), alternative 5'splice site (A5SS), alternative 3'splice site (A3SS), mutually exclusive exons (MXE) and retained intron (RI). PSI values (lncLevels) are used to filter differential alternative splicing (P value <0.05, Q value <0.05). When a gene has multiple splices, select the one with the most significant difference. The closer PSI value is to 1, the less probability of alternative splicing occurs. The closer PSI value is to 0 conversely, the more likely alternative splicing occurs [20].

Weighted Gene Co-expression Network Analysis and Network Visualization

To reveal the developmental transcriptional expression network of differentiated gene sets in tailed and rumpless chicken, weighted gene co-expression network analysis (WGCNA) [21] was used for network analysis in this study. The soft thresholding power of PB group was selected 18, R^2 was first greater than 0.64 of topology. RB group was selected 30, R^2 was first greater than 0.45 of topology. The minimum module size was set to 20. Cytoscape3.6.1 software [22] for analyzing network visualization with unsigned network. Finally, hub genes in modules were screened through the degree of gene connectivity.

Analysis of KEGG Pathway

KEGG pathway was analyzed by KOBAS online tool *(http://kobas.cbi.pku.edu.cn/genelist/). Gallus gallus* species, gene symbol, and KEGG pathway were selected. According to

the P value, the top 20 were screened for the display of the bar chart.

Results

Comparative Genomic Analysis of Rumpless Chicken and Multiple Chicken Breeds

Phylogenetic tree analysis was performed on chip data from 988 chickens. According to *Fig. 1,* the tree scale is 0.1. The phylogenetic tree showed that the four types of chicken were divided into four branches, and the commercial breed showed a long distance from the game variety, the wild type, and the local variety, with obvious population differences.

The first 5% of fixation index (F_{ST}) sites were screened for genomic regions that had high population differences between them, as shown in the *Fig. 2,* the degree of differentiation among breeds were C *vs.* G, C *vs.* W, L *vs.* C, L *vs.* W, G *vs.* W, G *vs.* L in sequence, which hit 783, 114, 42, 792, 866 and 744 genes too. The number of highly differentiated loci overlapped genes in CG, CW, LC, LW, GW, LG, and 258 rumpless signal genes was 50, 46, 23, 17, 5 and 4 respectively, which were stripped of duplicates, 76 genes *(Fig. 3)* (Among them, the 258 rumpless signal genes of tailed chicken and rumpless chicken came from our team previous research [13]).

Dynamic Histomorphology and Transparent Staining of Bone in Tailed and Rumpless Chicken

In terms of appearance and histomorphology, there was no significant difference in tail development between tailed chicken and rumpless chicken from 1 d to 4 d, and the tail buds became more prominent from 5 d. The tail buds of tailed chicken were sharp, but those of rumpless chicken were round and blunt. With the increase of age, the tail bud of tailed chicken continued to grow and became, but the tail of embryo of rumpless chicken did not grow significantly on 9 d, and after 10 d, the tail almost did not grow and only had a small bulge *(Fig. 4)*.

The transparent staining method can observe the natural distribution and position of cartilage and hard bone in the body as a stereoscopic visual effect of bone development. Both soft tissue and cartilage were blue after staining the cartilage. Soft tissue decolorized by gradient ethanol is

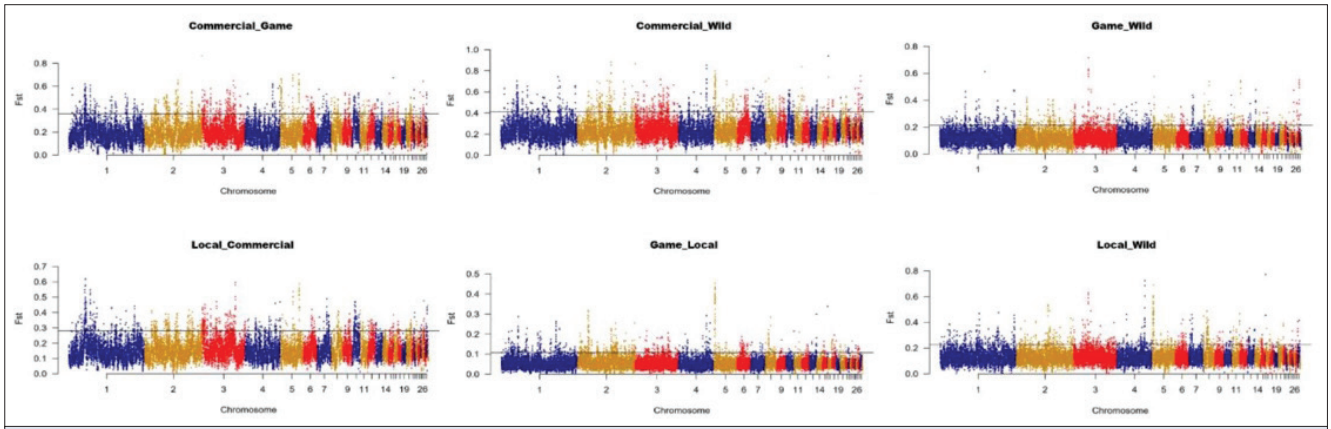

Fig 2. Distribution of F_{ST} among different C, G, W and L breeds. The dividing line represents the first 5% of differentiation sites

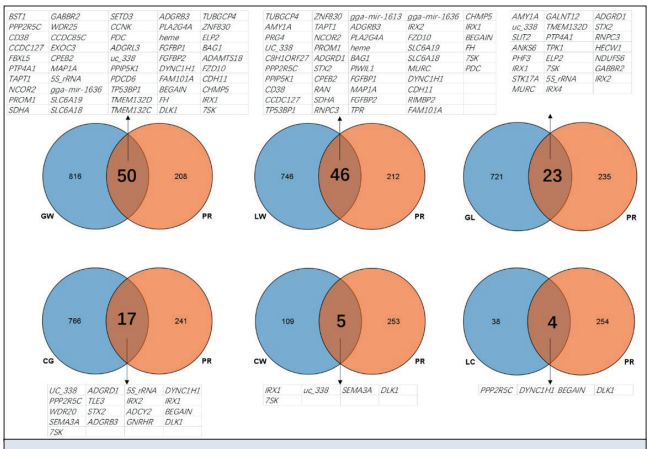

Fig 3. The overlapping genes between rempless chicken differentiation genes and CG, CW, LC, LW, GW, LG breeds. PR represents the gene of significant differentiation of rumpless chicken

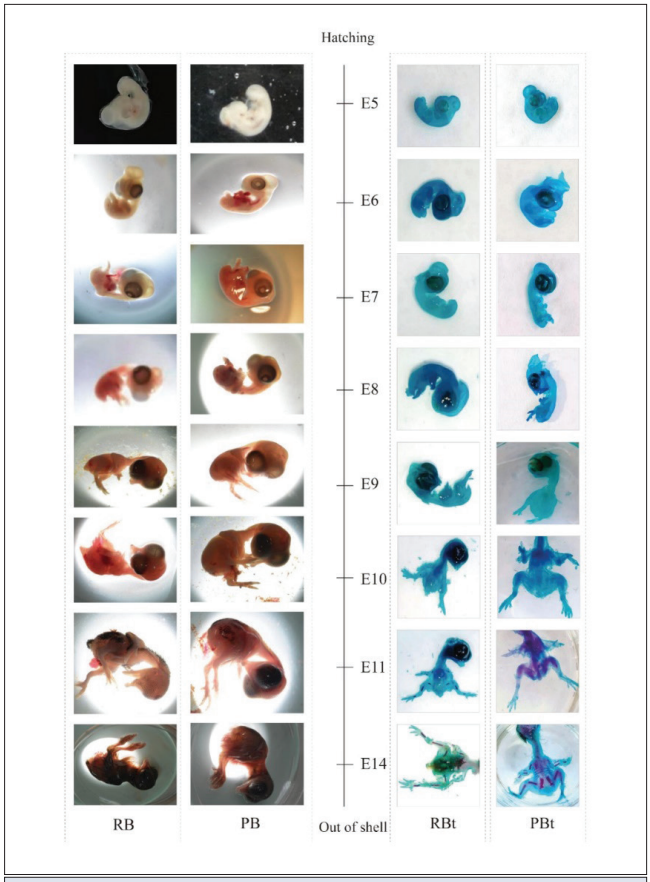

Fig 4. Histomorphology and bone hyalinization of embryo samples from rumpless Piao chicken (PB) and tailed chicken (RB). PB: rumpless Piao chicken, RB: tailed chicken. PBt: transparence dyeing results of rumpless Piao chicken; RBt: Transparence dyeing results of tailed chicken. E: Embryonic day age

transparent and light blue. Hyalinization results in blue cartilage, deep red hard bone, and light blue jelly outside the bone. The results of cartilage staining showed that the bone development of tailed chicken and rumpless chicken embryos showed blue staining on the spindle bone from incubation 6 d, and then blue staining on the long bones of limbs, ribs, phalanges, wing roots, and tailbone. The caudal bone of rumpless chick embryo was very short and did not develop in late incubation. Bone staining showed that spotty-like red staining appeared in the main vertebra and long bones of limbs from the 7 d of incubation, and then the red staining gradually increased. Red staining appeared in the bones of phalanx, ischium, ribs and wing root, but no red staining appeared in the coccyx, indicating that the coccyx was not ossification. These results indicated that during embryo development of rumpless chicken, the development of coccyx was normal in the early stage of embryo (before the 4 d to 6 d stage of incubation), and degenerated or stopped in the late stage. This rumplessness may be related to the inhibition of genes related to bone development. The following analysis will focus on genome and transcriptome data after 8 d (Scoop sample on 6 d could not extract complete RNA, so it was abandoned).

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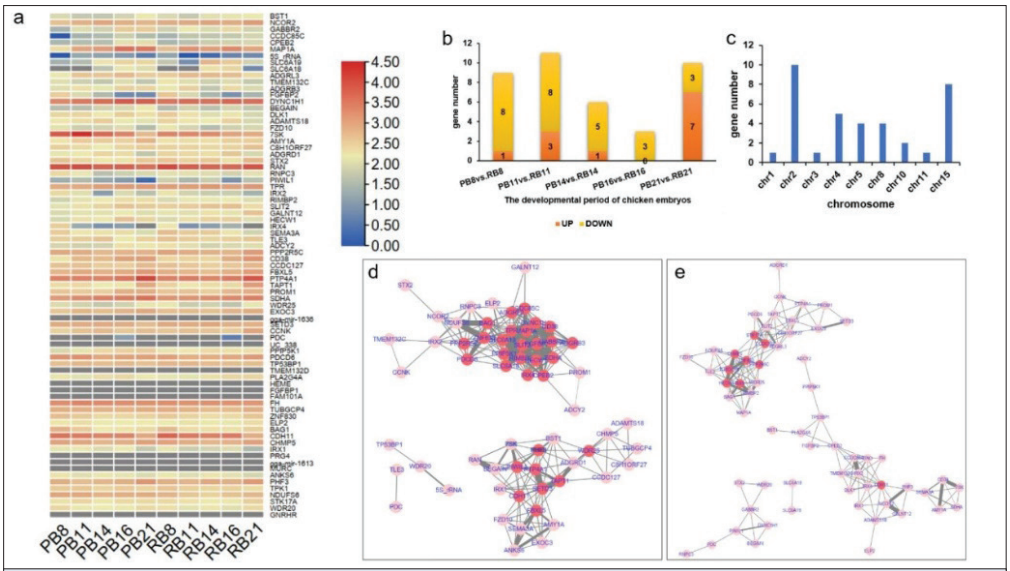

Fig 5. a) Expression distribution of differentiation signal gene sets (76 genes) of rumplessnesss in different breeds at different developmental stages of tail chicken and rumpless chicken (P<0.05, adjP<0.05, |logFC|>1. All genes from *BST1* to *ADCY2* were differentially expressed); **b)** Differential expression and distribution of differentiation signal gene sets (76 genes) of rumplessnesss in different developmental stages of tailed and rumpless chicken (P<0.05, adjP<0.05, |logFC|>1. Up represents up-regulated genes and down represents down-regulated genes); **c)** Distribution of 36 DEGs in chromosomes; **d)** Differentiation signal gene sets (76 genes) in the co-expression network of rumpless Piao chicken; **e)** Differentiation signal gene sets (76 genes) in the co-expression network of tailed chicken

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Developmental Transcriptome Analysis Reveals Rumpless Dynamic Expression of Rumpless Signaling Gene Set

According to bone transparent staining, blue staining was found from the 6 d, but the RNA acquisition of the 6 d of the failed, which may be due to sample degradation and other reasons, so the data of the 8 d was obtained. The differential expression of 76 strongly differentiated genes between tailed chicken and rumpless chicken was analyzed by transcriptome sequencing at embryonic stage 8, 11, 14, 16, 21 d respectively. These 76 genes indicated the characteristics of tail in the selection process of different chicken breeds.

As shown in *Fig. 5-a*, log10 (FPKM+1) transformation of differentially expressed genes (DEGs) was carried out and heat map was drawn. Among them, 36 genes located between heat map *BST1* and *ADCY2* were all DEGs, and 2, 4, and 15 were abundant. The up-regulation distribution of these DEGs at different developmental stages showed that 12 of them were higher and 1 of them were lower in Piao chicken than in tailed chicken at 8 d of embryo. At 11 d of embryo, there were 13 higher and 3 lower in Piao chicken than in tailed chicken. At 14 d of embryo, there were 14 higher and 1 lower expression in Piao chicken than in tailed chicken. At 16 d of embryo, there were 6 higher and 0 lower expression in Piao chicken than in tailed chicken. At 21 d of embryo, there were 6 higher and 10 lower expression in Piao chicken than in tailed chicken *(Fig. 5-b)*.

Table 1. *Differentially expressed genes with high network connectivity in embryos of tailed chicken and rumpless chicken*

Genes	Degree	Modules	Average Shortest PathLength	Betweenness Centrality	Neighborhood Connectivity
SLC6A19	22	PB-turquoise	1.406	0.093	15.591
FGFBP2	20	PB-turquoise	1.469	0.050	16.500
TPR	20	PB-turquoise	1.469	0.046	16.400
MAP1A	19	PB-turquoise	1.500	0.017	17.211
DYNC1H1	19	PB-turquoise	1.500	0.017	17.211
RIMBP2	19	PB-turquoise	1.500	0.017	17.211
SLIT2	19	PB-turquoise	1.500	0.017	17.211
GABBR2	18	PB-turquoise	1.688	0.022	16.444
SLC6A18	18	PB-turquoise	1.563	0.037	16.778
CCDC85C	17	PB-turquoise	1.594	0.053	16.765
HECW1	11	RB-turquoise	2.192	0.028	9.182
RAN	10	RB-turquoise	2.231	0.019	9.600
IRX2	9	RB-grey	2.040	0.139	7.000
IRX4	9	RB-grey	2.240	0.064	6.889
RIMBP2	9	RB-turquoise	2.308	0.012	10.000
ADGRL3	9	RB-turquoise	1.808	0.080	8.667
C8H1ORF27	9	RB-turquoise	2.115	0.104	7.222
CCDC85C	8	RB-grey	2.080	0.096	7.375
CPEB2	7	RB-grey	2.400	0.383	5.714
TMEM132C	7	RB-grey	2.120	0.073	8.143

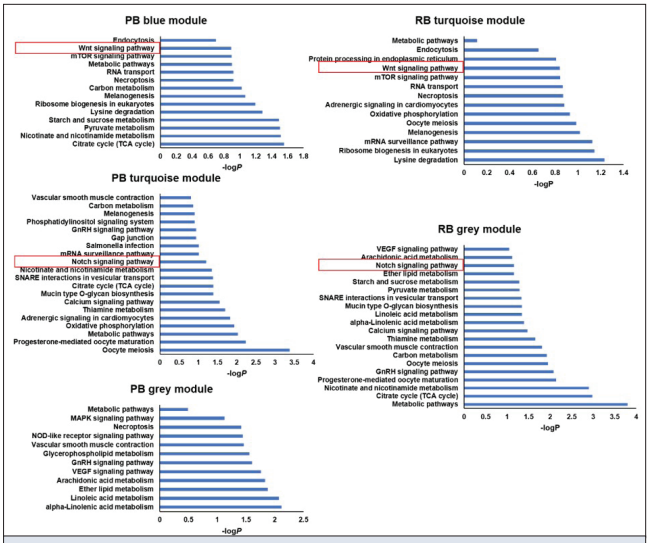

Fig 6. Enrichment of signal pathways of different gene co-expression modules in tailed and rumpless chicken

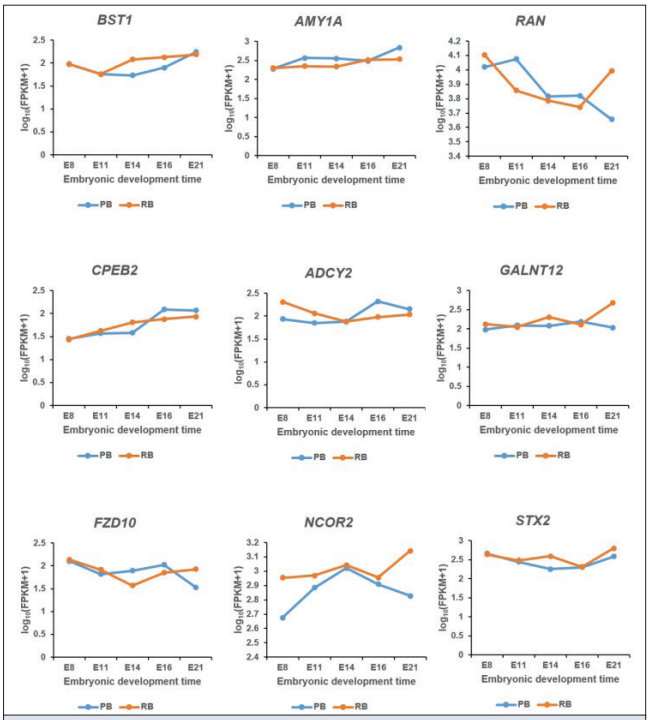

Fig 7. Dynamic FPKM temporal expression of differentially expressed genes in common signaling pathways in tailed and rumpless chicken

WGCNA Reveals Co-expression Network of Rumpless Signal Gene

The global network construction results of WGCNA show that the Piao chicken β value is 18 and the network topology fit index is 0.64. It is divided into three co-expression modules, namely turquoise, blue and grey. There are 12 and 8 hub genes with degree greater than 10 in turquoise and blue modules respectively *(Fig. 5-c)*. The β value of tailed chicken was 30, and the network topology fit index is 0.45, which was divided into two modules turquoise and grey. Genes with degree greater than 10 were mainly distributed in turquoise (8 genes) *(Fig. 5-d)*. The expression of these differentiation signals of rumplessness was more closely interacted with each other and the weight between genes was greater than that in tailed chicken.

As shown in *Table 1*, we sorted out the network attribute parameters of the top 10 genes with differentially expressed genes and large degree among the network genes of tailed chicken and rumpless chicken. The network connectivity of the rumpless chicken is larger than that of the tailed chicken. The first three hub genes were *SLC6A19*, *FGFBP2* and *TPR* of rumpless chicken network, which were 22, 20 and 20 genes that had very similar expression patterns. The first three hub genes were *IRX2*, *IRX4* and *RIMBP2* of tailed chicken network, which were 9, 9 and 9 genes that had very similar expression patterns. The *IRX2* and *IRX4* genes related to rumplessnesss reported in previous literatures were included *(Table 1)*.

KEGG (Kyoto Encyclopedia of Genes and Genomes) Analysis Delineate the Signaling Pathway Relevant to Posterior Patterning

KOBAS (KEGG Orthology Based Annotation System) signal pathway enrichment analysis was performed for different modules of genes in tailed chicken and rumpless chicken respectively *(http://kobas.cbi.pku.edu.cn/)*. The result is shown in *Fig. 6*, except for PB enrichment in 6 pathways (glycerophospholipid metabolism, NOD-like receptor signaling pathway, MAPK signaling pathway, salmonella infection, gap junction, phosphatidylinositol signaling system). RB is specifically enriched in protein processing in endoplasmic reticulum. The other 31 pathways were common enrichment pathways, all of these include: nicotinate and nicotinamide metabolism, metabolic pathways *(BST1)*; starch and sucrose metabolism, metabolic pathways *(AMY1A)*; ribosome biogenesis in eukaryotes, RNA transport *(RAN)*; melanogenesis, mTOR signaling pathway, wnt signaling pathway *(FZD10)*; notch signaling pathway *(NCOR2)*; oocyte meiosis, progesterone-mediated oocyte maturation *(CPEB2)*; progesterone-mediated oocyte maturation, adrenergic signaling in cardiomyocytes, calcium signaling pathway *(ADCY2)*; mucin type O-glycan biosynthesis *(GALNT12)*; SNARE interactions in vesicular transport *(STX2)*. Red boxes indicate some typical signaling pathways related to traits reported by previous studies.

The genes that were not differentially expressed at 8 to 11 d and were present after 14 d included the *BST1*, *CPEB2*, *FZD10*, *STX2*, *AMY1A*, *GALNT12*, *RAN*. *FZD10* and *RAN* were differentially expressed only on 21 d. The difference between 8 d and 11 d was *ADCY2*, and only the difference between 8 d was *NCOR2* *(Fig. 7)*.

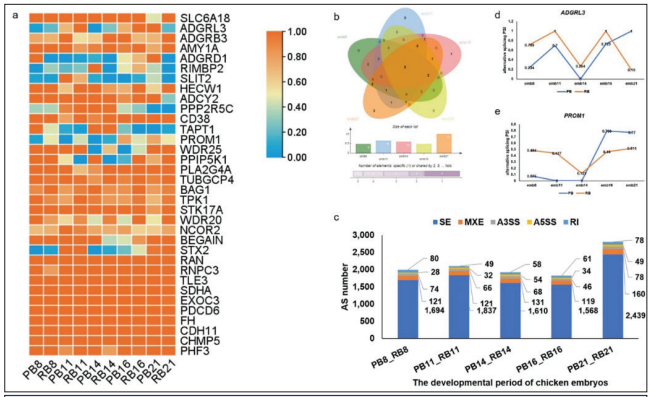

Fig 8. a) Differential variable splice number expression heat map (PSI) of transcriptome at different developmental stages; **b)** Venn analysis of SE at different developmental stages; **c)** Differentially expressed alternative splicing and differentiation factor genes at each stage; **d)** The dynamic distribution of PSI of *ADGRL3* at each embryonic development stage; **e)** The dynamic distribution of PSI of *PROM1* at each embryonic development stage

Alternative Splicing of Differentiation Signal Gene Sets for Rumplessnesss in Embryo Development Differences Between Tailed Chicken and Rumpless Chicken

Transcriptome sequencing was used to analyze the global distribution of differential alternative splicing in tailed chicken and rumpless chicken, and it was found that there more alternative splicing in late embryo, especially on 21 d. The exon skip (SE) splicing form was the most prominent among all the splicing forms. With the development process, 1 694, 1 837, 1 610, 1 568 and 2 439 SE were identified respectively *(Fig. 8-c)*. Next, the expression of splicing PSI on SE of 34 genes *(Fig. 8-a)* among 76 differentiated genes that had differential alternative splicing between tailed chicken and rumpless chicken breeds was compared, which can reveal the pre-transcriptional splicing characteristics of differentiation signal gene sets during embryonic development (due to multiple splicing in a gene, only the most significant PSI was screened in this study). We obtained 9 genes that were differentially expressed at a certain stage of embryo and underwent differential alternative splicing: *SLC6A18*, *ADGRL3*, *ADGRB3*, *AMY1A*, *ADGRD1*, *RIMBP2*, *SLIT2*, *HECW1* and *ADCY2 (Fig. 8-a)*. In addition, *ADGRL3* and *PROM1* were differentially expressed in all 5 periods *(Fig. 8-b,d,e)*.

Discussion

In this study, we investigated the differences of the whole genome among different breeds, especially the overlapping genes with rumplessness candidate genes involved in selection reported by focused on the dynamic transcriptome expression of these overlapping genes during embryonic development of tailed chicken and rumpless chickens. As reported that caudal vertebral bodies are formed from different forms of cartilage that remodel to bone at distinct stages [24]. First, the eggs of tailed chicken (Camellia chicken) and rumpless chicken (Piao chicken) were incubated in this study. After death, the chicken embryos were stained with alcian blue and alizarin red [23,24] for cartilage and hard bone, and transparent specimens were made. This study observed the changes of caudal embryo development, it was found that the caudal bud appeared sharp protrusion from the 4 d of tailed chicken hatching. After that, the tail buds grew relatively with the increase of body size. However, round and blunt tail buds appeared from the 4 d of rumpless chicken, and after the 9 d, tail buds stopped growing and became round and blunt more obvious. Zwilling as early as 1942 proposed that dominant rumpless embryos have a reduced tail at the end of the 4 day [25]. Some studies suggest that the tail bud of chicks begins to produce mitotic inhibitors on the 3 d of hatching [26], and lasts until the 4 d to 5 d of hatching, when the tail bud reaches the maximum length, and then a 3-step remodeling process is carried out: 1.Differential growth between the tip of the tail and the more anterior regions; 2. The anterior regions become incorporated into the caudal portions of the trunk. 3. Cell at the tip of the tail die, And then you end up retaining the proximal part to form the final tail [27,28].The results of this study showed that during embryo development, tailbone development was no significantly difference in the early stage of embryo between tailed chicken and Piao chicken (hatching 4-6 d), and degenerated or stopped in the late stage of Piao chicken, which is a visualization of the developmental characteristics of the tail bone of a rumpless chicken.

Next, we compared the genomes of large chicken species with different uses to try to find out the differences and relationships between them and the tailless chicken. While the results of this study do not prove mutated genes in each genome versus the tailless genome, this interesting comparison of overlapping genes could reflect genome-level differences between results showed that the differentiation degree of several chicken breeds was obvious, and there were some overlapping genes with these cultivars [29], especially commercial *vs.* game chicken and commercial *vs.* wild, which had more overlapping genes with tailless selection signal, with 50 and 46 genes, respectively. This suggests that some of the rumplessness signal genes reported by Hu [13] are involved in the difference between other varieties. These results demonstrated there is rich genetic diversity and extensive genetic basis of the provided excellent molecular materials for further heterosis and high-yield breeding. This helps to explain the history and divergence of these breeds and to facilitate genetic breeding.

Gene expression variation is a key underlying factor influencing phenotypic can occur via pre- transcriptional

and transcriptional expression [30]. In this study, we also focused on the dynamic transcriptional patterns of these genes during embryonic development of tailed chicken and Piao chicken. Among the 76 rumpless signaling gene set, 36 genes were differentially expressed, and most of them were concentrated on chromosome 2, 4 and 15. They were distributed at different embryonic stages, and most of them were differentially expressed at 11 and 21 days. There are research reports that through genome-wide association and linkage analyses, the candidate region was fine-mapped to 798.5 kb (chromosome 2: 86.9 to 87.7 Mb). Whole-genome sequencing analyses identified a single variant, a 4.2 kb deletion, which was completely associated with the rumpless phenotype [31].

Co-expression gene clusters of development are paradigms for the study of gene regulation [32]. We used WGCNA to identify regulatory networks of these signaling genes during embryonic development in tailed and rumpless chicken. A key characteristic of scale-free networks is a small number of highly interconnected hub genes (hubs). Because hubs are more likely than nohubs to be necessary for the integrity of the network and the survival of an organism, the identification of so-called "hubs genes" is of great significance. Therefore, we identified highly co-expression hub genes in each co-expression network. The centrality of nodes is measured by three parameters: shortest path length, betweenness centrality and degree. The shortest path length is the probability of functional correlation between gene/protein and gene/protein, the larger it is, the stronger the gene/protein regulation function to other genes/proteins [33]. Degree is used to measure the centrality of a node. A node with a higher degree reflects the higher connectivity between two sub-networks, but the connectivity within the sub-network is not high. Often acts as a communication gene/protein between two modules. Degree attribute indicates the degree of connection between a gene/protein and other genes/proteins. The higher the connection degree, the more critical the hub gene/protein is in the regulation of biological organism. The major evolutionary maintains the association of the hub and expression genes in clusters. These genes were differentially expressed at different stages of embryo development between tailed and rumpless chicken, and their distribution was not balanced. These genes were co-expressed and interacted more strongly in Piao chicken than in tailed chicken. The first three hub genes were *SLC6A19*, *FGFBP2* and *TPR*. The first three hub genes were *IRX*2, *IRX*4 and *RIMBP2*. The Iroquois gene is expressed in clusters in most vertebrates and misexpression of *IRX1* and *IRX2* within the tailbud precedes all observed genetic and morphological changes. The *IRXA* cluster contains *IRX1*, *IRX2* and *IRX4,* which have similar expression patterns [32-34]. In this study, *IRX2* appeared in the comparison results of genome differences of multiple breeds, and *IRX2* appeared in the core of gene co-expression network of tailed chicken, and the connectivity of gene co-expression network of tailless chicken was results indicate one of the characteristics of *IRX2* gene expression during embryonic development between tailed chicken and Piao chicken.

The results of functional enrichment of these differentially expressed genes showed that *ADCY2* and *NCOR2* genes were inhibited on the 8 d of embryo. On 11 d, the repressed gene was *ADCY2*. Notch signaling pathway, oocyte meiosis, progesterone-mediated oocyte maturation, adrenergic signaling in cardiomyocytes, calcium signaling pathway was affected. Most genes began to be differentially expressed after 14 d, which significantly affected the development of coccyx. The genes that were inhibited on 14 d were *BST1*, *CPEB2*, *STX2*, and *GALNT12*, which affected signal pathway include nicotinate and nicotinamide metabolism, metabolic pathways, oocyte meiosis, progesterone-mediated oocyte maturation, SNARE interactions in vesicular transport, mucin type O-glycan biosynthesis. The genes that were inhibited on 21 d were *FZD10* and *RAN*, affecting melanogenesis, mTOR signaling pathway and wnt signaling pathway. It has been reported that notch and wnt signaling pathways play an important role in regulating segmental formation and tail bone termination and extension [35]. According to Rashid's review, a prevalent pleiotropic effect of mutations that cause fused caudal vertebral bodies is tail truncation, and at least half of the mutated genes are located in the notch/wnt pathway, leading to changes in somite number or size of short-tailed birds [36]. Notch signaling pathway can mediate embryonic development and tissue renewal and is highly conserved during evolution. Studies have shown that it regulates chondrocyte, osteoblast and osteoclast differentiation [37]. It is expressed in the early stage of osteoblast differentiation, leading to bone formation inhibition and bone loss [37]. When the wnt signaling pathway extends in the tail, it can coordinate with the concentration of RA and Fgf signaling pathways to form a gradient balance and regulate the formation of body segments [38]. Notch signaling pathway is activated when wnt3a/Fgf8 reaches the gradient equilibrium point. However, when RA concentration is too high, wnt3a/Fgf8 is inhibited, which in turn inhibits notch [39]. In conclusion, notch is expressed in coccyx of both tailed and rumpless chicken in preembryonic stage, and the expression of notch is higher in tailed chicken, and the wnt signaling pathway was inhibited by the low expression of *FZD10*. Since no RNA was detected from 4 to 7 days of embryo, notch expression was inhibited earlier in rumpless chicken, which was also the main reason why PCR verification was not carried out in this study.

In addition, the mechanisms that mediate phenomena such as alternative splicing elusive. Alternative splicing is the core mode of gene regulation in higher eukaryotes, which can affect plant and animal growth and development [40], signal transduction [41] and regulatory responses under biological/abiotic stress [42,43], play a key role in regulating osteoblast function and bone formation [44]. In this study, it was found that the SE splicing was the most prominent alternative splicing occurred more at embryo 21 d than at the early stage. Among the 76 rumpless signaling gene set, only 34 genes underwent differential alternative splicing, among which the PSI of *ADGRL3* and *PROM1* were significantly different at each embryo time, again indicating the existence of transcriptional differential characteristics of some differentiation factors. Therefore, different exon splicing forms represented by *ADGRL3* and *PROM1* may be one of the factors leading to the differences of these genes. In conclusion, this study highlight genetic charateristics of transcriptome and genome in tailed chicken and Piao chicken.

In conclusion, genomic differences in different breeds and genetic characteristics of bone in embryo indicate the molecular mechanism of tailbone development in a Piao chicken by comprehensive analysis of the histomorphology, genome and transcriptome of embryo skeleton, which may enhance useful resources for rumplessness breeding, also in-fluence the selection process of other breeds.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding authors (YYS & YC) on reasonable request.

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Ethical Statement: All experimental procedures were approved by the Animal Protection and Use Committee of Inner Mongolia Agricultural University and strictly followed animal welfare and ethical guidelines ([2020]085).

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Author Contributions: WMQ performed the experiments, analysed the results, and drafted the manuscript. YC and ZXL assisted in the experimental design and summarized the experimental results. YYS and WMQ conceived and designed the study, revised the manuscript and funded the study. All authors have read and agreed to the published version of the manuscript.

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Research Article

Establishment and Application of Dual RPA-Basic and RPA-LFD Detection Method for *Pasteurella multocida* and *Actinobacillus pleuropneumoniae*

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Abstract

Based on recombinase polymerase amplification (RPA) detection technology (combined with (lateral flow dipstick, LFD)), it is aimed to establish a dual recombinase polymerase amplification method for the rapid identification of *Pasteurella multocida* (Pm) and *Actinobacillus pleuropneumoniae* (APP). The conserved fragments of Pm *kmt1* gene and APP *ApxIV* gene were selected for the amplification of target fragments. Eight pairs of primers for Pm and APP, and one probe for KMT1Pn and APP323Pn were designed. A single RPA-Basic primer screening test was performed. The reaction time and temperature of double RPA were optimized. The optimal primers and probe matching systems of dual RPA-LFD were explored. Dual RPA sensitivity and specificity tests were performed. The method was used to detect 60 clinical samples. The results of the primer screening test showed that the primers had the strongest specificity and the highest amplification efficiency for ApxIV2698F/ApxIV3020R and KMT1F/KMT1R. The method had the best amplification efficiency at a reaction temperature of 37ºC and a reaction time of 35 min. The optimal primer ratio of KMT1F/KMT1R and ApxIV2698F/ApxIV3020R was 2 µL : 1.5 µL, and the optimal probe ratio of KMT1Pn and APP323Pn was 0.6 µL : 0.4 µL. The minimum detection limit of dual RPA-Basic and RPA-LFD sensitivity test was 10^{-6} ng/µL. The specific test results showed no cross-reaction with enteropathogenic *Escherichia coli, Salmonella, Glaesserella parasuis, Staphylococcus aureus, Streptococcus suis, Aeromonas hydrophila*. Using 60 clinical samples of suspected Pm and/or APP infection to evaluate the detection system, the detection rate of dual RPA-Basic and RPA-LFD is higher than that of PCR, indicating that they have strong practicability. This study successfully established a dual RPA-Basic and RPA-LFD detection method for Pm and APP, which can be used for the rapid differential diagnosis of Pm and APP mixed infection in clinical.

Keywords: *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, Recombinase polymerase amplification, Lateral flow dipstick, Rapid detection

Introduction

Actinobacillus pleuopneumoniae (APP) belongs to the family *Bartonellaceae*, the genus *Actinobacillus*, has pods and is mostly hemolytic. There are many serotypes of APP, and the serotypes correlate with different courses of disease [1]. Based on differences in capsular antigens, APP can be classified into serotypes 1-15. The researchers found serotypes 16-19 in strains that could not be typed [2-4]. All serotypes of APP can cause disease, but the strength of pathogenicity varies. The main clinical features of APP infection in pigs are hemorrhage, necrosis and fibrinous exudation in lungs [5]. The acute form has a high morbidity and mortality rate, which can be up to 80%~100% [6]. Some sick pigs are often accompanied by chronic pneumonia after recovery, resulting in growth stagnation and long-term bacterial and becoming a source of infection for other pigs, causing large economic losses to the pig industry [7].

Pasteurella multocida (Pm) is a Gram-negative zoonotic

pathogen that can cause disease in pigs, cattle, sheep, chickens, ducks, rabbits and other hosts, causing a variety of animal and human infections [8]. Pm usually exists in the nasal cavity, tonsils, lungs and other parts of pigs, and can cause diseases such as swine plague and infectious atrophic rhinitis of pigs [9]. Swine plague is an acute septicemic infection usually characterized by sepsis and hemorrhagic inflammation of tissues and organs [10]. Pm can cause atrophic rhinitis in pigs with clinical signs of sneezing, nosebleeds, and growth retardation [11]. The serious harm of Pm is also manifested in that pigs are susceptible to secondary infection when the body's defense ability is weakened after infection with other disease, and its incidence is generally not affected by seasonal interference, causing significant economic losses to the pig industry [12].

APP and Pm are both common and important pathogens of porcine respiratory disease syndromes and are often mixed infections, which makes actual production prevention and control face a great challenge [13]. At the same time, the use of overdose or non-sensitive antibiotics has a negative impact on the control of animal diseases. The misuse or abuse of antibiotics can lead to the development of bacterial resistance and increased antibiotic residues in pigs and their products [14]. The establishment of rapid detection methods for App and Pm is the key to the successful control of these diseases. The commonly used bacterial detection methods include bacterial isolation, indirect ELISA, indirect hemagglutination, PCR and immunofluorescence, etc. [15,16]. Bacterial isolation and PCR methods are most commonly used. Bacterial isolation takes at least 2~3 d to produce identification results, which is time-consuming and laborious, not conducive to taking measures for timely treatment, and the sensitivity is not high and the results are inaccurate [17]. Although PCR based detection methods can quickly detect pathogens, they require professional technicians and expensive instruments, and is difficult to realize point-of-care testing (POCT). Serologic and immunologic methods are prone to false negative results [15,16]. Recombinase polymerase amplification (RPA) is a nucleic acid isothermal amplification technology developed by the British company TwistDx Inc. in 2006, which has received widespread attention in recent years [18]. This technology eliminates the limitations of conventional PCR requiring precise thermal cycling. RPA utilizes certain specific proteins and enzymes to achieve a target-specific amplification process for the purpose of detecting specific pathogens [19-21]. Due to these characteristics, RPA does not require higher temperature denaturation, annealing and extension steps in the amplification reaction, thus making the nucleic acid amplification more convenient and faster [22]. Compared with other conventional PCR technology, it can be carried out in the temperature range of 20~45ºC, and the reaction speed is faster. Usually, the detection process only takes 15~25 min [18,23,24]. RPA amplification can be combined with agarose gel electrophoresis, lateral flow chromatography (LFD) and fluorescence signal analysis to reflect the amplification results. Among them, RPA electrophoresis (RPA-Basic) has the advantage of low cost, while RPA-LFD has the advantage of visible results to the naked eye and is one of the more common RPA amplification binding methods [25]. The current study has not established a dual RPA detection method with APP and Pm as the research objects. So this study designed primers and probes for APP and Pm based on genes *ApxIV* and *kmt1*, respectively, and established a dual RPA detection method to realize fast APP and Pm detection, and made a preliminary application to provide a new means for veterinary clinical diagnosis and epidemiological investigations.

Material and Methods

Ethical Statement

All procedures performed in studies involving animals were in accordance with the ethical standards of the Henan Institute of Science and Technology with approval code number: 202009.

Experimental Strains

Pasteurella multocida (Pm, C44-1), *Actinobacillus pleuopneumoniae* (APP, CVCC259), *Streptococcus suis* (CVCC606), Enteropathogenic *Escherichia coli (E. coli*, isolated strain), *Glaesserella parasuis* (GPS, isolated strain), *Listeria monocytogenes (L. monocytogenes*, isolated strain), *Salmonella* (CVCC541), *Staphylococcus aureus (S. aureus*, ATCC49525), *Aeromonas hydrophila (A. hydrophila*, AH-1) are all preserved in our laboratory [26].

Primer Design and Synthesis

The gene sequences of APP *ApxIV* (GenBank accession number: HM021153), *16S rRNA* gene (GenBank accession number: D30030.1), disulfide bound formation protein E *(dsbE)* gene (GenBank accession number: AF458420.1), Pm outer membrane protein *(ompH)* gene (GenBank accession number: U50907.1), lipoprotein E *(plpE)* gene (GenBank accession number: GU108958.1) and hydrolase family protein *(kmt1)* gene (GenBank accession number: MN518176.1) published in GenBank were analyzed and compared, and primers and nfo probes were designed based on the principle of designing RPA primers and nfo probes [16]. The 5' end of the probe was labeled with carboxyl (FAM) and the 3' end was modified with C3-Spacer, and the THF site was placed in the middle of the 5' end of the probe. At least 15 nucleotides were added to the 3' end of the probe after the THF residue. The primers

Table 1. *Probe and primers used for RPA-LFD detection*

Primer/Probe	Sequence (5'→3')	Fragment Size (bp)
KMT1F	GGCTCGTTGTGAGTGGGCTTGTCGGTAGTCT	129
KMT1Rn	Biotin-GTCCAATCAGTTGCGCCGTTGTCAAGGAAG	
KMT1Pn	FAM-TGGCTTGTGGCAAAGAAAAGCACAGTTTTG[THF]TGGGCGGAGTTTGG3spacer	
PLPEF	ATGGCAGTTATGGACAACCTTCATCAGA	169
PLPER	CCAACTCAGTTTACATCACTTAATACGG	
OMPHF	TGGTTTCACATTTGGTGGTGCGTATGTCTT	184
OMPHR	GTGCTGCTGGCGGATTCTGTTCAACTTCTT	
APP2698F	AGCAGTGCTTCTGTCGTTAGAGTCACGCCTTC	323
APP3020R	Dig-CGAGAATAATCGGCTACCCATTTCCCTTCG	
APP323Pn	FAM-CAATTAAGTAGTATACGCAATGTAAAGCAT [THF]ATCCTACCGTTATGC-C3spacer	
APP3F	ATGGCATTATTTGGCACTGACGGTGATGAT	453
APP3R	GGCCATCGACTCAACCATCTTCTCCACCTG	
ApxIV680F	CTGAACATGAGGATTTGTTTCTCGGTGGTG	680
ApxIV680R	CCCATTATTTCCGTCCGGTTTATTCAGGTC	
16S444F	AAGTTCTTTCGGTAGCGAGGAAGGTATCAA	444
16S444R	GATTTACTACGTTAGCTTCGGGCACCAGAC	
dsbE18F	GCTTCCATACTTGCCTTATTCGGTTATCGT	399
dsbE399R	CGCATCCTTCGGTTGATCGGAATAGGTAAG	

and probes were synthesized by Sango Biotech (Shanghai) Co., Ltd *(Table 1)*.

Genomic DNA Extraction

According to the growth characteristics of Pm, APP, SS, GPS, Enteropathogenic *E. coli*, *L. monocytogenes*, *S. aureus*, *A. hydrophila*, *Salmonella*, the bacteria were cultured to the logarithmic stage and genomic DNA was extracted using the Ezup Column Bacterial Genomic DNA Extraction Kit (Absin Shanghai Biotechnology Co., Ltd.). The concentration of DNA was determined using Nanodrop 2000 and DNA was stored at -80ºC for use.

RPA-Basic Reaction

Single RPA-Basic Primer Screening: The mixture of Pm and APP genomic DNA prepared as above was used as the template for RPA amplification, while ddH_2O was set as the negative control. According to the instruction manual of TwistAmpTM Basic kit (TwistDx (UK) Company), a 50 µL reaction system was set up: 2.4 µL each of forward and reverse primers, 2.2 µL of template (DNA), 29.5 µL of reaction buffer, 2.5 µL of magnesium acetate solution, and ionized water were added to 50 µL, and the reaction was carried out in a metal bath at 39ºC for 20 min. The amplification products were appropriately diluted and detected by electrophoresis on 2 % agarose gel to screen the best primer.

Establishment of Dual RPA-Basic Reaction System and Optimization of Reaction Conditions: The above 50 µL reaction system was slightly modified by adding a pair of primer of 4.8 µL, and deionized water was reduced accordingly. Six reaction temperature gradients of 25°C, 30°C, 35°C, 37°C, 39°C and 45°C were set to determine the optimal reaction temperature. The RPA reaction was carried out according to 10 min, 15 min, 20 min, 25 min, 30 min, 35 min, 40 min and 45 min, respectively, to optimize the optimal reaction time. The primer volume ratios were set as (µL): Pm (0, 0) and APP (2.4, 2.4), Pm (0.4, 0.4) and APP (2.4, 2.4), Pm (0.9, 0.9) and APP (2.4, 2.4), Pm (1.3, 1.3) and APP (2.4, 2.4), Pm (1.9, 1.9) and APP (2.4, 2.4), Pm (2.4, 2.4) and APP (2.4, 2.4), Pm (2.4, 2.4) and APP (1.9, 1.9), Pm (2.4, 2.4) and APP (1.4, 1.4), Pm (2.4, 2.4) and APP (0.9, 0.9), and the primer ratios were screened for use in subsequent studies.

Dual RPA-Basic Specificity and Sensitivity Detection: The constructed reaction system was used to detect GPS, Enteropathogenic *E. coli*, *L. monocytogenes, S. aureus, A. hydrophila* and *Salmonella* genomic DNA. Sterile deionized water was used as a negative control to evaluate the specificity of this reaction. The template was successively diluted from 10^{-1} ng/µL to 10^{-8} ng/µL in a 10-fold ratio. The sensitivity of the proposed dual RPA-Basic system was tested and the minimum detection limit of the method was evaluated.

RPA-LFD Reaction

Screening of Dual RPA-LFD Probes: The RPA-nfo amplification reaction was performed according to the recommended reaction conditions for the TwistAmpTM nfo kit (50 µL system) : 29.5 µL reaction buffer, 2.1 µL forward and reverse primers, 0.6 µL probe (10 µM), 2.2 µL template (DNA), and 11 µL nuclease-free water. After the reaction, the results were interpreted using flowmeter chromatographic strips (Ustar Biotechnology (Hangzhou) Co., Ltd.). Specifically, 5 µL was added into a centrifuge tube containing 195µL MGCBB, and after mixing evenly, the sample end of the colloidal gold test strip was inserted into the centrifuge tube for balance, and the results were interpreted within ten minutes. The same reaction conditions were controlled for each pair of Pm or APP RPA-nfo reaction, and positive control and negative control were set for each pair of primers.

Optimization of Dual RPA-LFD Reaction System: In order to improve the sensitivity of RPA-LFD reaction, different primer and probe concentration ratios were set to optimize the RPA-LFD reaction. The reaction components included 40.9 µL A buffer and 2 µL template. The forward and reverse primers and probes of Pm and APP varied according to the ratio gradient of 1 : 1 : 0.3, and a total of 7 experimental groups and negative control group were set up. The above consisted of pre-mixing solution, which was added to 0.2 mL RPA-nfo reaction tube containing lyophilized enzyme powder. Finally, 2.5 µL B buffer was added to the lid of the reaction tube and thoroughly mixed. After mixing, the reaction liquid was thrown (or rapidly centrifuged) to the bottom of the tube, and then the reaction tube was immediately incubated at 39ºC in a constant temperature device for 20 min. After the reaction was over, 5 µL was taken and added into a centrifuge tube containing 195 µL MGCBB, and after mixing evenly, the sample end of the colloidal gold test strip was inserted into the centrifuge tube for balance, and the quality control line and detection line were observed. The results were interpreted within 10 minutes.

Specificity and Sensitivity of Dual RPA-LFD: In order to explore the specificity of RPA, Pm, APP, SS, GPS, Enteropathogenic *E. coli, L. monocytogenes, S. aureus, A. hydrophila*, and *Salmonella* genomic DNA were extracted as detection objects, respectively. The specificity of the method was evaluated by RPA-LFD detection under the optimized double RPA-LFD optimal conditions. The template was diluted at a multiplicity of 10^{-1} ng/µL ~10^{-7} ng/µL. Sensitivity experiments were performed using the established dual RPA-LFD system to evaluate the lowest detection limit of the method.

PCR Detection

The PCR assay (Sango Biotech (Shanghai) Co., Ltd.) was performed with a 25 µL system, and the amplification program was 94°C pre-denaturation for 5 min, denaturation at 94°C for 40 s, annealing at 63°C for 45 s, and extension at 72°C for 90 s, with a total of 35 cycles. The amplified PCR products were verified on a 2% agarose gel.

Clinical Sample Test

To further confirm the effect of using the dual RPA-Basic and RPA-LFD in clinical application, 60 clinical lung tissues of pigs suspected of being infected with Pm and/or APP from Henan and Shandong provinces were collected, and dual RPA methods were performed under the optimal reaction conditions using pMD-18T-kmt1 and pMD-18T-ApxIV recombinant plasmid as a positive control, ddH_2O as a blank control, and total DNA from healthy pig lung tissues as a negative control. The results were compared to those obtained from a conventional PCR assay conducted on DNA extracted from the respective clinical samples.

Statistical Analysis

SPSS 24.0 statistical software was used for statistical analysis, with the operating procedures for testing Actinobacillus pleuropneumoniae in pigs (local standard in Anhui Province) and the diagnostic techniques for porcine brucellosis (agricultural industry standard in the People's Republic of China) as the gold standards. The detection efficiency of RPA-LFD and PCR were calculated separately, and Kappa test was used to analyze the consistency of the two results. Kappa<0.4 indicates low consistency; 0.75≥Kappa≥0.4 indicates moderate consistency; When 1.0 ≥Kappa>0.75, it is considered highly consistent.

Results

Screening of Primers

The study designed a total of 8 primer pairs for Pm *kmt1*, *OmpH*, and *PlpE* genes along with APP *ApxIV*, 16S rRNA, and *dsbE* genes *(Table 1)*. The results showed that APP ApxIV2698F/ApxIV3020R primer pairs and Pm KMT1F/KMT1R primer pairs could obtain clear and single bands at positions 323 bp and 129 bp on electropherograms after RPA amplification reactions, while the other primer pairs were amplified without bands, dragging, or non-specific bands *(Fig. 1-A,B)*. Genomic DNA of SS, GPS, Enteropathogenic *E. coli, L. monocytogenes, S. aureus, A. hydrophila*, and *Salmonella* were used as positive controls (ddH_2O was used as a negative control) for the Pm/APP, Pm, and APP Basic-RPA assay. The results are shown in *Fig. 1-C*, and no bands appeared in all lanes except for the positive control, which showed bright bands. It indicated that the specificity of the APP ApxIV2698F/ApxIV3020R primer pair and the Pm KMT1F/KMT1R primer pair was

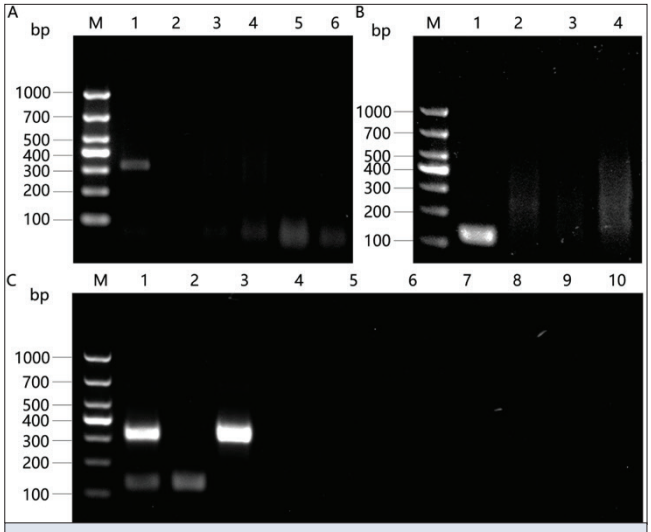

Fig 1. RPA-Basic primer screening results. A. Screening results of APP primer RPA-Basic. M: DL1000 DNA Marker; 1~6 are: primer KMT1F/KMT1R, negative control, primer dsbE18F/dsbE399R, primer ApxIV680F/ApxIV680R, primer 16S444F/16S444R, primer APP3F/APP3R; B. Screening results of Pm primer RPA-Basic. M: DL1000 DNA Marker; 1~4 are: primer KMT1F/KMT1R, primer OmpHF/OmpHR, negative control, primer PlpEF/PlpER; C. Specific results of primer selected by Pm and APP. M: DL1000 DNA Marker; 1~10 are: Pm/APP, Pm, APP, SS, GPS, Enteropathogenic *E. coli*, *L. monocytogenes*, *S. aureus*, *A. hydrophila*, *Salmonella*, negative control

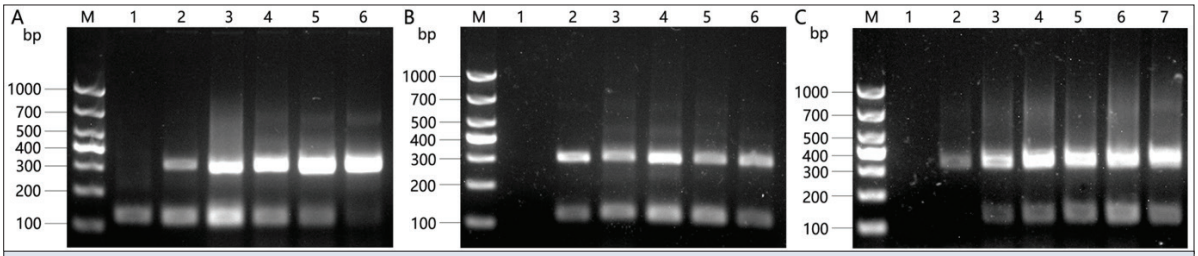

Fig 2. Optimization of dual RPA-Basic amplification conditions. A. RPA-Basic primer ratio screening results. M: DL1000 DNA Marker; The volume ratio of primer 1-6 (μL): Pm (0.2, 0.2) and APP (2.4, 2.4), Pm (0.4, 0.4) and APP (2.4, 2.4), Pm (0.7, 0.7) and APP (2.4, 2.4), Pm (1.2, 1.2) and APP (2.4, 2.4), PM (2.4, 2.4) and APP (2.4, 2.4), Pm (1.7, 1.7) and APP (2.4, 2.4); B. Reaction temperature optimization results. M: DL1000 DNA Marker; The reaction temperatures from 1 to 6 are: 25ºC, 30ºC, 35ºC, 37ºC, 39ºC, 45ºC; C. Reaction time optimization results. M: DL1000 DNA Marker; The reaction time from 1 to 7 are 10 min, 15 min, 20 min, 25 min, 30 min, 35 min, 40 min

good, so ApxIV2698F/ApxIV3020R and KMT1F/KMT1R primer pairs were identified to be used in the subsequent experiments.

Optimization of Dual RPA-Basic Reaction Conditions

The results of RPA-Basic reaction showed that the brightness of electrophoretic bands was relatively consistent when the primer volume ratio was 2.4 μL upstream and downstream of Pm and 0.7 μL upstream and downstream of APP *(Fig. 2-A)*. After repeating the experiments, the optimal volume ratio of Pm and APP primer was finally selected as 2.4 μL: 0.7 μL. The optimal primer volume ratio was used for the screening of RPA-Basic reaction time and temperature. As shown in *Fig. 2-B*, the RPA-Basic amplification products could be specifically detected when the temperature was between 30 and 45°C. Considering the later application of clinical detection, 37°C was chosen as the optimal reaction temperature in this study. As shown in *Fig. 3-C*, the electrophoresis results showed that the RPA-Basic method could detect target product in the range of 15~40 min at the reaction temperature of 37°C, but there was no significant difference in the brightness of electrophoretic bands after 35 min. In order to ensure the timeliness of the detection, 35 min was chosen as the optimal reaction time in this study.

Specificity and Sensitivity of the Dual RPA-Basic Assay

The specificity of the dual RPA-Basic assay was performed using optimized reaction conditions. The results are shown in *Fig. 1-C*, where no bands appeared in all lanes except for the positive control, which appeared as bright bands, indicating that the specificity of this RPA-Basic assay was good. After determining the specificity, the sensitivity of the dual RPA-Basic detection system was studied. The

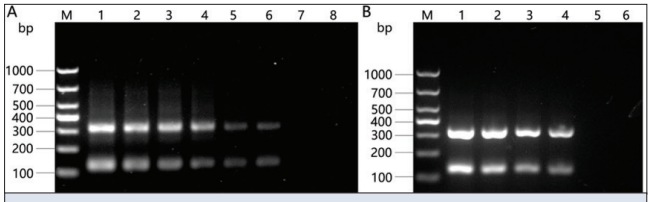

Fig 3. Comparison of the specificity and sensitivity of the dual RPA-Basic assay with the PCR assay. A. Basic-RPA sensitivity test results. M.1000 DNA Ladder; Nucleic acid concentrations in lanes 1-8 are (ng/μL): 10-1, 10-2, 10-3, 10-4, 10-5, 10-6, 10-7, negative control; B. PCR sensitivity test results. M.1000 DNA Ladder; Nucleic acid concentrations in lanes 1-6 were in the order of (ng/μL): 10-1, 10-2, 10-3, 10-4, 10-5, negative control

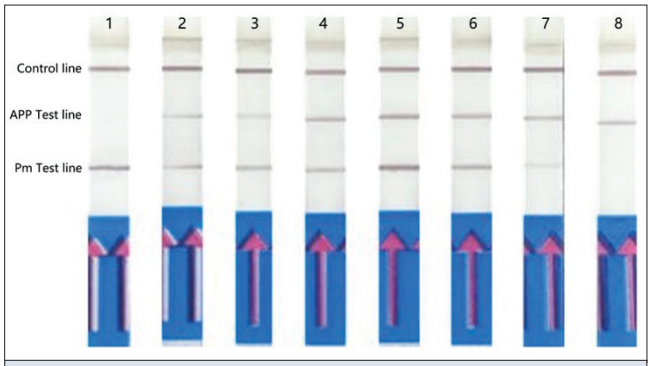

Fig 4. Screening results of dual RPA-LFD detection probes. Lanes 1-9 are: M: DL1000 DNA Marker; primer volume ratios from 1 to 8 are (μL): APP (0, 0, 0) and Pm (2, 2, 0.6), APP (0.7, 0.7, 0.4) and Pm (2, 2, 0.6), APP (1.5, 1.5, 0.4) and Pm (2, 2, 0.6) , APP (2, 2, 0.6) and Pm (1, 1, 0.6), APP (2, 2, 0.6) and Pm (1.5, 1.5, 0.4), APP (2, 2, 0.6) and Pm (1, 1, 0.4), APP (2, 2, 0.6) and Pm (0.5, 0.5, 0.4), APP (2, 2, 0.6) and Pm (0, 0, 0.4)

results are shown in *Fig. 3*, which shows that as the mixed template concentration gradually decreased from 10^{-1} ng/μL, the color of the detection line of APP and Pm also gradually became lighter until it disappeared at 10^{-7} ng/μL template concentration. Tt can be concluded that the RPA-Basic reaction system can detect as low as 10^{-6} ng/μL, which is higher than the lowest detection concentration of PCR (10^{-4} ng/μL).

Screening of Dual RPA-LFD Detection Probes

The corresponding probes were designed according to the primers obtained from RPA-Basic screening. The selected reverse primers for Pm and APP were labeled with biotin and digoxin, respectively, and the designed probes were labeled with FAM fluorescein. The results are shown in *Fig. 4*, and obvious double detection lines can be seen in experimental groups 2, 3, 4, 5, 6 and 7. In order to keep the color depth of APP and Pm detection lines consistent and facilitate the subsequent detection, the combinations of Pm upper and lower primer probe volumes of 2 μL, 2 μL, and 0.6 μL and APP upper and lower primer probe volumes of 1.5 μL, 1.5 μL, and 0.4 μL were comparatively selected for the subsequent experiments.

Specificity and Sensitivity of the Dual RPA-LFD Assay

Using the optimized dual RPA-LFD system with Pm and APP, Pm, APP as positive control, ddH_2O as negative

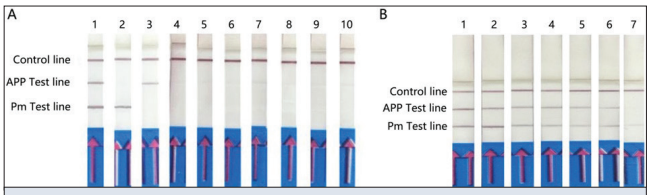

Fig 5. Specificity and sensitivity of RPA-LFD. A. Specificity and sensitivity of RPA-LFD. 1-10 are: Pm/APP positive control, Pm positive control, APP positive control, negative control, SS, GPS, Enteropathogenic *E. coli*, *L. monocytogenes, S. aureus, A. hydrophila, Salmonella*. B. RPA-LFD sensitivity. 1-7 Nucleic acid concentrations are (ng/μL): 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}

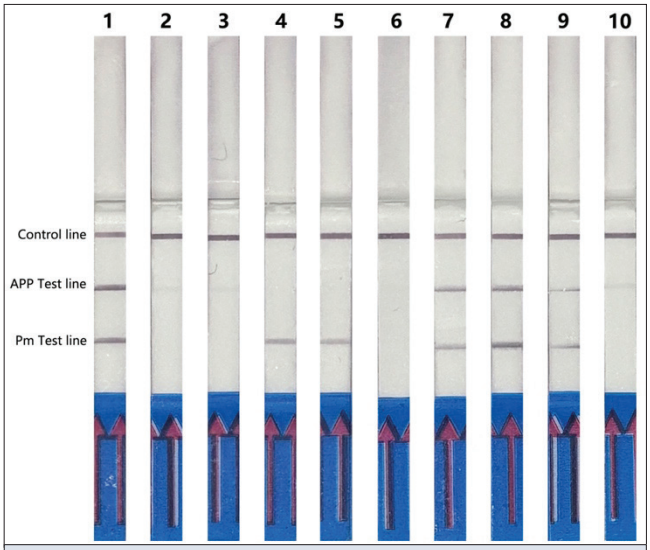

Fig 6. Clinical sample results with the dual PRA-LFD system. Lane 1, Pm and APP positive control, Lane 2, negative control, Lanes 3-10, clinical test samples

control, SS, GPS, Enteropathogenic *E. coli, L. monocytogenes, S. aureus, A. hydrophila*, and *Salmonella* as detection objects for RPA-LFD assay. As shown in *Fig. 5-A*, the double positive and single positive results of Pm and APP were established, and the other groups and negative controls showed only the quality control line, which determined that the specificity of the assay was established. To determine the sensitivity of the dual RPA-LFD, the template was sequentially diluted to seven concentrations ranging from 10^{-1} to 10^{-7} ng/μL in 10-fold ratios. The sensitivity experiments were carried out using the established optimal dual RPA-LFD system, and the results were shown in *Fig. 5-B*. The color of the detection lines of APP and Pm gradually faded from 10^{-1} to 10^{-5} ng/μL, and the detection lines completely disappeared after 10^{-6} ng/μL, and only the quality control line appeared in the negative control. Therefore, the detection limit of the dual RPA-LFD assay established in this study was 10^{-6} ng/μL, which is higher than the lowest detection concentration of PCR (10^{-4} ng/μL).

Clinical Sample Test Results

Sixty clinical samples were tested by PCR, double RPA-Basic and double RPA-LFD, and the results of the three

Table 2. Application of different methods for the detection of actual sample

Detection Methods	Number of Positive Cases			Sensitivity (%)			Specificity (%)			Positive Predictive (%)			Negative Predictive (%)			Kappa		
	Pm	APP	Pm/APP	Pm	APP	Pm/APP	Pm	APP	Pm/APP	Pm	APP	Pm/APP	Pm	APP	Pm/APP	Pm	APP	Pm/APP
PCR	7 (11.7%)	8 (13.3%)	2 (3.3%)	76.9	68.2	66.7	100	100	100	100	100	100	94.3	86.5	94.9	0.795	0.631	0.592
RPA-Basic	9 (15.0%)	15 (25.0%)	4 (6.7%)	90.9	100	100	100	100	100	100	100	100	98.0	100	100	0.936	1.0	1.0
RPA-LFD	9 (15.0%)	15 (25.0%)	4 (6.7%)	90.9	100	100	100	100	100	100	100	100	98.0	100	100	0.936	1.0	1.0

methods were statistically analyzed. Using the established dual LFD-RPA method to detect each serotype strain of Pm and APP, the test lines could be clearly observed *(Fig. 6)*. As shown in *Table 2*, a total of 7 Pm-positive samples, 8 APP-positive samples and 1 Pm/APP double-positive sample were detected by conventional PCR. Dual RPA-LFD detected a total of 9 Pm-positive samples, 15 APP-positive samples and 4 Pm/APP-positive samples. In summary, the dual RPA-LFD method has a higher detection rate than PCR. From the point of the difficulty and time spent on the operation of the three methods, RPA-LFD takes the shortest time, and the operation process is relatively simple. There is no need to learn the operation skills of large-scale instrumentation, and only need to master the basic experimental techniques to complete the whole experiment.

Discussion

In recent years, with the rapid development of pig farming and the increasing degree of intensification of farming, the impact of bacterial diseases on pig farming has been highlighted. The intensive environment of pig farming provides very favorable conditions for pathogens to multiply and infect pig herds, which brings great difficulties in disease prevention and control. APP is a typical bacterium that infects and attacks the lungs through the respiratory tract, and its infection leads to severe irreversible damage to the lungs, resulting in acute death. Respiratory infectious diseases caused by APP have always been one of the most important bacterial infectious diseases plaguing the pig farming industry [27]. Pm is the main representative bacterium of the genus Pasteurella, which can cause a variety of diseases such as atrophic rhinitis and hemorrhagic septicemia in pigs. At present, a variety of diseases caused by Pm have different degrees of occurrence and prevalence in many areas, and the harm to the pig industry is becoming more and more serious. This study selected the highly conserved the conserved fragments of Pm *kmt1* gene and APP *ApxIV* gene as the diagnostic target and developed RPA-Bacic and RPA-LFD detection method with high sensitivity, strong specificity, and wide detection range, which can be conveniently and quickly applied in laboratories, especially in POCT.

The *kmt1* gene is a species-specific gene of Pm and has been used as a target gene for PCR and LAMP methods to detect all subspecies of Pm [28,29]. The *plpE* gene is present in all serotypes of Pm and is a specific conserved gene of the bacterium. The sequence similarity of the *plpE* gene in different serotypes of Pm is more than 92%, which makes it suitable for the detection of Pm pathogen [30]. *OmpH* is a major protein presented on the outer membrane of Pm. Comparison of the *OmpH* sequences of 15 serotypes of Pm shows that this protein is highly conserved (72-100% homology) [31,32]. Both purified natural *OmpH* and whole bacteria can induce high levels of antibodies, and the induced protection rate is comparable [31,32]. In this study, three primers were designed for Pm-conserved *kmt1, plpE,* and *OmpH* genes, and the best primers with the highest amplification efficiency, namely KMTF1/KMTR1 (and corresponding nfo probes), were analyzed and selected by RPA method for subsequent RPA experiments. The *Apx IV* gene can be found in all serotypes of APP, so it can be used as a target gene for detecting APP species-specific [16]. Primers were also designed for APP 16S rRNA and *dsbE* genes in this study, but they were not effective. Finally, this study established dual RPA-Basic and RPA-LFD assays based on the conserved genes *Apx IV* of APP and *kmt1* of Pm.

It can be seen from this study that specific amplification bands can be shown after 15 min from the beginning of the RPA-Basic reaction, and the amplification effect is better at 25~45 min, and the best amplification effect is achieved at 35 min, which indicates the superiority of the RPA technology in terms of the detection time. The amplification bands of the RPA reaction have obvious bands in the range from 25 to 45°C, which shows that the amplification effect is better in this temperature range. When the temperature exceeded 37°C, the amplification bands showed a tendency to weaken, which may be caused

by the higher temperature reducing the enzyme activity in the reaction. In this experiment, the optimal reaction temperature was set at 37ºC and the optimal reaction time was set at 35 min. It was found that the lowest detection limit of Basic-RPA and RPA-LFD reached 10^{-6} ng/μL, which was higher than that of the conventional PCR assay and had good sensitivity. In addition, the primers/probes designed in this study were used to amplify RPA with SS, GPS, Enteropathogenic *E. coli, L. monocytogenes, S. aureus, A. hydrophila*, and *Salmonella*, and the results showed that no specific bands were generated, which verified that the method had good specificity. The RPA assay is convenient, short and simple and can be applied to rapid detection in the front line of farm production. Finally, the dual RPA methods and conventional PCR methods were used to test the DNA extracted from clinical samples. The results show that using industry standards and local standards as the gold standard, dual RPA methods detection of Pm and APP has a specificity of 100% and a sensitivity of 90.9%. PCR technology showed 100% specificity and a sensitivity of 76.9%. The sensitivity of dual RPA methods is higher than that of PCR technology, which demonstrated the reliability and practicality of the dual RPA methods.

In conclusion, based on the exploration and optimization of RPA reaction conditions, the APP/Pm dual RPA-Basic and RPA-LFD rapid detection methods were successfully established. These two methods can complete the detection within 50 min, which is faster than the traditional PCR method. It does not require special instruments and experimental conditions, such as thermal cycler, which is suitable for basic units and on-site testing environments with a lack of instruments. The equipment is portable and simple to operate, without the need for professional operators, and has a high degree of specificity. In conclusion, this study is expected to provide a faster and more reliable detection technology for the implementation of APP and Pm detection and monitoring as well as prevention in farms, customs entry-exit animal disease quarantine laboratories and other institutions, as well as to provide a reference for the development of other animal disease detection technologies.

Declarations

Availability of Data and Materials: The data that support the findings of this study are available on request from the corresponding author (X. X and K. D).

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Competing Interests: The authors declared that there is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): Not applicable.

Ethical Statement: All procedures performed in studies involving animals were in accordance with the ethical standards of the Henan Institute of Science and Technology with approval code number: 202009.

Author Contributions: XX and KD designed the research and project outline. JL, XW, SL and ML carried out the experiments and analysis the data. QZ, KW, LW, YC, MC, CZ, ZT, XL and HZ drafted the manuscript. HZ, XX and KD revised the manuscript. All authors have read and approved the final manuscript.

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Research Article

Antimicrobial Susceptibility, Virulence Characteristics, and Incidence of Class 1 and 2 Integrons in *Salmonella* Infantis Isolated from Clinical Cases in Broilers

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Abstract

Salmonella Infantis is a poultry-adapted *Salmonella* enterica serovar that is increasingly reported in broilers and is also regularly identified among human salmonellosis cases. This study aimed to investigate the presence and distribution of virulence determinants and their antimicrobial susceptibilities of *S.* Infantis isolates obtained from clinical cases of broilers. In addition, selected 6 *S.* Infantis isolates were further characterized using whole-genome sequencing (WGS). The majority of the isolates was resistant to at least two or more antimicrobials. Only two isolates were susceptible to all antimicrobials tested. Higher rates of resistance were observed against ciprofloxacin (96.4%), tetracycline (96.4%), sulfamethoxazole-trimethoprim (76.8%), but low resistance rates to chloramphenicol (8.9%), and ampicillin (8.9%) were detected. WGS analysis revealed the presence of different resistome, but *aac(6')-Iaa* and *tetA* genes in all isolates, and mutations in *gyrA* and *parC* genes playing a role in quinolone resistance. WGS also revealed that all isolates were of sequence type 32 (ST32). Based on the presence of virulence genes, the isolates were characterized into five virulence profile. Among the examined virulence genes, *invA*, *sopB*, *pipD*, *sifA*, *stn*, *spaN*, *slyA*, and *hilA* were present in all isolates. Only one isolate had all virulence genes examined. The findings of this study provide valuable information on *S.* Infantis strains isolated from clinical cases of broilers and current antimicrobial resistance levels and virulence determinants. High resistance rates and the widespread occurrence of many virulence genes reveals that the isolates have significant pathogenic potential and pose a threat to public health.

Keywords: Antimicrobial susceptibility, Integron, *Salmonella* Infantis, Virulence, Whole genome sequencing

Introduction

Non-Typhoidal *Salmonella enterica* (NTS) is a foodborne pathogen that causes human gastroenteritis [1], with 93 million cases of gastroenteritis and 155.000 deaths; among them, approximately 85% of the cases are associated with the consumption of contaminated food [2]. In 2022, 65.208 human salmonellosis cases were reported by 27 EU countries, corresponding to an EU notification rate of 15.3 cases per 100.000 population. The three most commonly reported *Salmonella* serovars in 2022 were *S.* Enteritidis (54.6%), *S.* Typhimurium (12.1%) and monophasic *S.* Typhimurium (1,4,[5],12:i:-) (10.4%), representing 77.1% of the 47.122 confirmed human cases. The fourth and fifth serovar were *S.* Infantis (2.3%) and *S.* Newport (1.1%) [3]. NTS infections are frequently characterized with acute onset of diarrhea, abdominal cramps, and fever, which is usually self-limiting, resolving between 1 and 7 days without treatment, depending on the host status. However, NTS infections could cause severe clinical manifestations in individuals, including immune-compromised patients, infants, and older adults, may develop bacteremia or invasive infections such as meningitis, osteomyelitis, endovascular infections, and septic arthritis. In this case, antimicrobial therapy might be needed [1].

Approximately more than 2600 *Salmonella* serovars were identified according to the White–Kauffmann-Le Minor [4], of which *S.* Infantis has emerged as the fourth most common serotype causing human salmonellosis in Europe [3], with 1868 *S.* Infantis related human infection cases [5]. Based on the data of the Ministry of Health in

Türkiye between 2012 and 2016, it was reported *S.* Infantis was among the top three serotypes and the frequency of three serotypes isolated from human clinical samples were reported to be 57.3-74.1% for *S.* Enteritidis, 3.0-8.5% for *S.* Typhimurium and 4.0-6.7% for *S.* Infantis [6].

In Türkiye, National *Salmonella* Control Program revealed that 24.5% of broiler flocks were being colonized with *Salmonellae*, among which *S.* Infantis (17.7%) was the most common serotype [6]. In this report, *Salmonella* contamination in broiler carcass samples was determined as 47%, and 72.6% of *Salmonella* isolates were serotyped as *S.* Infantis, followed by *S.* Kentucky (8.6%) and *S.* Enteritidis (7.7%) [6].

The emergence of *Salmonella* antimicrobial resistance (AMR) is a growing public health concern, particularly resistance against the critically important antimicrobials such as fluoroquinolones and third-generation cephalosporins [7]. During the last decade, *S.* Infantis isolated from various sources including animals and humans has been frequently identified as having multidrug resistance (MDR). In 2014, conjugative megaplasmids pESI (also termed "pESI-like", 280 to 300 kb) that harbour virulence, fitness, and MDR genes were first described in Israel [8], and subsequently in Italy, Switzerland, Hungary, Russia, the United States, Latin America, Japan, and in Türkiye [9].

Additionally, some *S.* Infantis strains with pESI-like plasmid have been reported to carry a colistin-resistance gene *(mcr-1),* since colistin is considered as a last resort antibiotic for the treatment of infections caused MDR Gram negative bacteria [10].

Integrons, as one of the mobile genetic elements (MGEs), play a signicant role in the spread of antimicrobial resistance genes (ARGs) among bacteria. Integrons can capture one or more cassette genes and disseminate ARGs via transmissible plasmids and insertion sequences (ISs). Integrons are categorized into three classes according to the sequences of the *intI* gene, and the most prevalent class of integrons is class 1, frequently reported in Gram-negative bacteria [11].

The pathogenicity of *Salmonella* strains has been related to numerous virulence genes, located at different sites of bacteriel genome, plasmids, and prophages. Clusters of chromosomal virulence genes, termed *Salmonella* pathogenicity islands (SPIs), play important roles in adhesion, invasion, intracellular survival, systemic infection, fimbrial expression, antibiotic resistance, toxin production, and Mg^{2+} and iron uptake [12]. For example, genes in SPI-1 (such as *invA*, *orgA*, *prgH*, *sipB*, and *spaN)* encode a type 3 secretion system 1 (T3SS-1) that allows *Salmonella* to invade phagocytic and non-phagocytic cells. Genes such as *spiA* in SPI-2 encode a type 3 secretion system 2 (T3SS-2) that enables *Salmonella* to survive and multiply in host cells [13]. Plasmids also carry virulence genes that contribute to *Salmonella* pathogenicity. Of these, *spvB* is responsible for colonization of deeper tissues [14]. Therefore, virulence genes profile analysis is beneficial for estimating bacterial potential pathogenicity.

This study was designed to determine the antimicrobial resistance phenotypes, virulence gene profile, prevalence of integron 1 and 2 of *S.* Infantis isolated from internal organs of diseased broilers. In addition, selected six strains were further characterized using whole genome sequencing (WGS).

Materials and Methods

S. Infantis Isolates

In this study, 56 *S.* Infantis isolated from the internal organs (liver, heart, spleen) and joint fluid samples of diseased broilers brought to the Microbiology Laboratory of the Department of Microbiology, Faculty of Veterinary Medicine, Aydın Adnan Menderes University between 2021-2023 for diagnosis purposes were used as the material of the study. The isolation method and serotyping was performed according to the ISO 6579-1:2017 and Kauffmann-White scheme, respectively.

DNA Extraction

Genomic DNA for PCR analyses was extracted using boiling method, and the isolated DNA was stored at -20°C until use.

Molecular Confirmation

The isolates phenotypically identified as *Salmonellae*, were molecularly confirmed by amplifying the *invA* gene, which is genus specific [15]. The *fljB* gene was used as a marker for molecular identification of *S.* Infantis, using previously reported species spesific primers [16].

Antimicrobial Susceptibility Testing

Antimicrobial susceptibilities of the isolates were performed and evaluated according to Clinical Labrotory Standards Institue (CLSI, 2022) criteria using the disc diffusion method. Following discs were used: gentamicin (CN, 10 µg), imipenem (IPM, 10 µg), ceftazidime (CAZ, 30 µg), cefepime (FEB, 30 µg), aztreonam (ATM, 30 µg), ampicillin (AM, 10 µg), amoxycillin-clavulanic acid (AMC, 10/20 µg), sulfamethoxazole-trimethoprim (SXT, 1.25/23.75 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg) and tetracycline (TE, 30 µg). *E. coli* ATCC 25922 was used as a control strain for antimicrobial susceptibility testing. The isolates that were resistant to at least one antimicrobial in three or more antimicrobial categories were defined as multi-drug resistant (MDR) [17].

Detection of Virulence Genes by PCR

The eleven virulence genes *(invA*, *sopB*, *sopE*, *sifA*, *spvC*, *pipD*, *spaN*, *stn*, *slyA*, *hilA,* and *spvR)* were examined as previously reported [18].

Detection of Integrons

Class I and II integrons were investigated as previously reported by Bass et al.[19] and Goldstein et al.[20].

A dendrogram was also constructed based on antimicrobial susceptibility, virulence and integron profiles of the isolates. The bands for each isolate were counted using the zero-one manual method, the data was then entered into the following site: *http://insilico.ehu.es/dice_upgma/*, dendrograms were plotted.

Whole-Genome Sequencing

For whole-genome sequencing, 6 isolates were selected based on antimicrobial resistance phenotype, virulence and integron profile. The genomic DNA of selected *S.* Infantis strains was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quantity and quality of the extracted DNA were measured using a Qubit 3.0 (Thermo Fisher Scientific, Waltham, MA, USA). The sequencing libraries of genomic DNA were prepared with the Illumina Nextera XT DNA Library Preparation Kit (Illumina Inc, San Diego, CA, USA) and the paired-end (2x150 bp) sequencing was run on the NovaSeq 6000 platform (Illumina, San Diego, CA, USA).

Quality Control, Trimming, Assembling and Annotation

After trimming low-quality reads and removing adapter sequences using Trimmomatic v 0.36 [21], the quality of both raw reads and trimmed reads was assessed using FastQC v 0.11.9 *(http://www.bioinformatics.babraham.ac.uk/projects/fastqc/;* accessed on 19 August 2024). The de novo genome assembly was conducted using the SPAdes algorithm (v 3.14.1) by applying the default parameters [22]. The quality of assembly was evaluated using QUAST v.5.0.0 [23], and contigs longer than >200 bp were included in further analysis. Gene predictions and annotations were performed using the National Center for Biotechnology Information's (NCBI) Prokaryotic Genome Automatic Annotation Pipeline (PGAAP) [24].

WGS-Based Characterization of *S.* Infantis Strains

The MLST type and the presence of acquired antimicrobial resistance genes of the strains were searched using the bioinformatic tools available at the Center for Genomic Epidemiology (CGE) platform *(http://www.genomicepidemiology.org/).* Mutations in the topoisomerase II (*gyr*A) and topoisomerase IV *(parC)* genes that mediate fluoroquinolone resistance in the strains were also analyzed using CGE platform.

Phylogenetic Analysis

For phylogenetic analysis, whole genome sequences of 33 *S.* Infantis isolates of chicken and human origin from different countries were retrieved from the PATRIC *S.* Infantis database. The tree was generated with maximum likelihood estimation methods. The evolutionary relationship between the *S.* Infantis strains was inferred by the aligned core-genomes using the program RAxML in PATRIC.

Results

Antimicrobial Resistance, Virulence and Integron Profiles

The isolates revealed various rate of resistance to cipro-floxacin (n=54, 96.4%), tetracycline (n=54, 96.4%), sulfamethoxazole-trimethoprim (n=43, 76.8%), chloramphenicol (n=5, 8.9%), and ampicillin (n=5, 8.9%), but, all isolates were susceptible to amoxycillin-clavulanic acid, imipenem, ceftazidime, cefepime, and aztreonam. Additionally, two isolates were susceptible to all antimicrobials tested.

Of virulence genes examined, *invA*, *sopB*, *pipD*, *sifA*, *stn*, *spaN*, *slyA*, *hilA* were detected in all *S. Infantis* isolates, but *sopE*, *spvC*, *spvR* were detected in 17.6%, 8.9% and 5.4% of the isolates, respectively. Only one isolate was found to have all virulence genes.

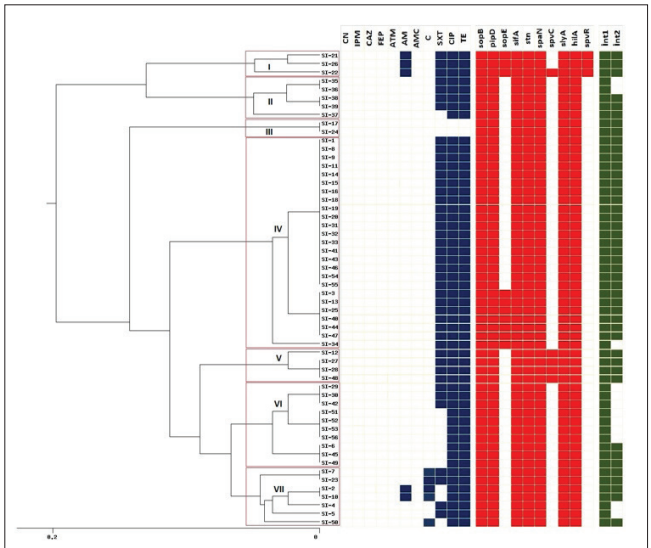

Fig 1. Dendrogram constructed based on antimicrobial susceptibility, virulence and integron profiles. Antimicrobial resistance, virulence and integrons are indicated blue, red and green squares, respectively. CN; gentamicin, IPM; imipenem, CAZ; ceftazidime, FEB; cefepime, ATM; aztreonam; AMP; ampicillin, AMC; amoxycillin-clavulanic acid, C; chloramphenicol, SXT; trimethoprim-sulfamethaxole, CIP; ciprofloxacin, TE; tetracycline

Table 1. *The sequence statistics and genome features of whole-genome sequencing analysis*

Characteristics	Data for the Isolates					
	ADU_VET1	ADU_VET2	ADU_VET3	ADU_VET4	ADU_VET5	ADU_VET6
No of contig	742	92	104	91	93	83
Genome size (bp)	5448326	5104776	4916112	4899282	4910037	4915377
Largest contig	123553	1195269	1195275	1179801	1171637	1195274
N50*	36616	203915	217644	203915	217644	217644
N90**	6623	56592	75578	52688	60144	67113
L50***	47	5	5	6	5	5
L90****	178	24	18	25	20	19
GC percent	51.56	52.11	52.15	52.17	52.16	52
Accession number	JBEOLT010000000	JBEOLU000000000	JBEOLV000000000	JBEOLW000000000	JBEOLX000000000	JBEOLY000000000

** A value that is equal to or greater than 50% of the total length of all the contigs; **A value that is equal to or greater than 90% of the total length of all the contigs; ***Smallest number of contigs whose length sum makes up half of genome size; **** Smallest number of contigs whose length sum makes up 90% of genome size*

Table 2. *Genetic characteristics of S. Infantis isolates*

Characteritics	ADU_VET1	ADU_VET2	ADU_VET3	ADU_VET4	ADU_VET5	ADU_VET6
Sequence Type (ST)	32	32	32	32	32	32
Incompatibility type	Col, IncFIB	IncFIB	IncFIB	IncFIB	IncFIB	IncFIB
Resistance phenotype	AMP, C, TE, CIP	C, SXT, TE, CIP	SXT, TE, CIP	SXT, TE, CIP	AM, SXT, TE, CIP	AM, SXT, TE, CIP
Antimicrobial resistance genes	*aac(6')-Iaa, blaTEM-1B, floR, qnrS1, tetA*	*aac(6')-Iaa, aph(6)-Id, aph(3")-Ib, aadA1, floR, sul1, sul2, tetA, dfrA14*	*aac(6')-Iaa, aadA1, sul1, tetA, dfrA14*	*aac(6')-Iaa, aadA1, sul1, tetA, dfrA14*	*aac(6')-Iaa, aadA2, aph(3')-Ia, aadA1, bla-TEM-1B, sul1, sul3, tetA, dfrA12*	*aac(6')-Iaa, aadA1, aph(3')-Ia, sul1, tetA, dfrA14*
Amino Acid Substitutions						
QRDR						
gyrA	(S83Y) TCC*TAC	(S83Y) TCC*TAC	(S83Y) TCC*TAC	(S83Y) TCC*TAC	(S83Y) TCC*TAC	(S83Y) TCC*TAC
parC	(T57S) ACC*AGC	(T57S) ACC*AGC	(T57S) ACC*AGC	(T57S) ACC*AGC	(T57S) ACC*AGC	(T57S) ACC*AGC

All isolates harbored integron genes, alone or in combination. In general, both class 1 and 2 integrons were detected in 76.8% (n=43) of the isolates, class 1 integron was only detected in 13 isolates (23.2%) alone. Dendrogram constructed based on antimicrobial susceptibility, virulence and integron profiles of the isolates was given in *Fig. 1*. Accordingly, *S.* Infantis isolates were assigned in seven categories.

Sequencing Statistics

The median length of genome assembly of the isolates was 4.92 Mbp with an average GC% of 52±0.215. The average N_{50} of the assembled contigs was 1.83 Mbp. Detailed sequence statistics and genome features are summarized in *Table 1*.

In Slico Detection of Acquired and Mutation Mediated Resistance

Based on the WGS analyses, 15 different types of acquired resistance genes found against various classes of antimicrobials including beta-lactams *(bla_{TEM-1B})*, aminoglycosides *(aac(6')-Iaa, aph(6)-Id, aph(3")-Ib, aadA1, aadA2,* and *aph(3')-Ia),* amphenicols *(floR),* sulphonamides *(sul1, sul2, sul3),* diaminopyrimidines *(dfr12, dfr14),* quinolones *(qnrS1),* and tetracycline *(tetA).* Amino acid substitutions in quinolone resistance-determining region (QRDR) of *gyr*A and *par*C genes were identified in all representative isolates *(Table 2).*

Sequence Type and Phylogenetic Analysis

WGS data indicated that all *S.* Infantis isolates belonged to ST32. For phylogenetic comparison, genomes of 33 *S.* Infantis strains of human and chicken present in the PATRIC were selected *(Fig. 2).* ADU_VET strains except ADU_VET1 were formed a separate cluster with ERUVET19 (isolated from chicken meat), indicating very high similarity. ADU_VET1 was formed a separate small cluster with Japan human isolates. But, other Türkiye isolate, Chicken/TR01 isolate, was closely related to human clinical isolates from Brazil and Bolivia, and a chicken isolate from Austria.

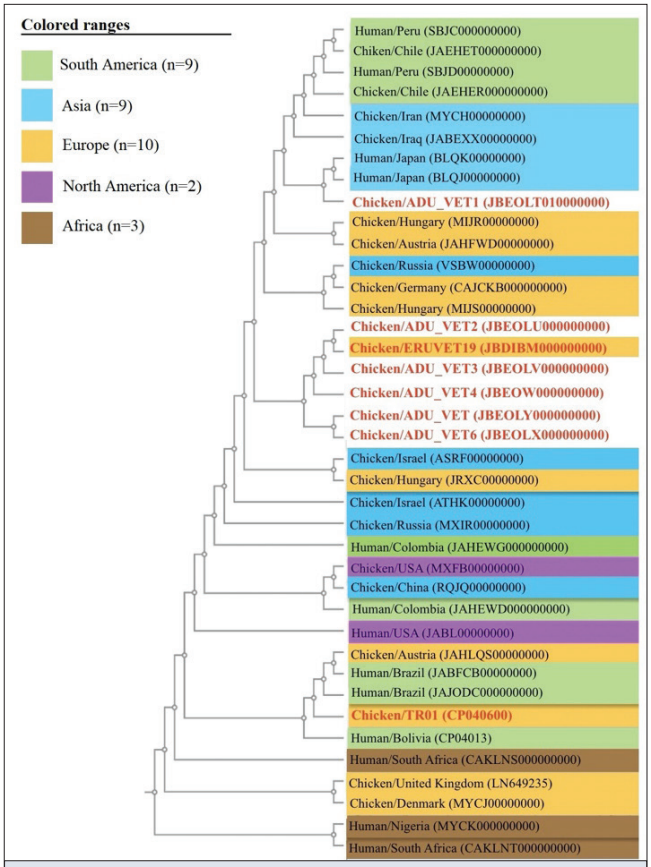

Fig 2. Maximum-likelihood phylogenetic tree based on core genome of 39 *S.* Infantis genomes from different regions of the World. ADU_VET and other Türkiye strains are depicted by bold red font. Geographic source of isolates is highlighted in different colors and GenBank accession numbers of each genome are given in parentheses

Data Availability

The genomic sequences for these isolates are available at NCBI under the BioProject number PRJNA1125001.

Discussion

In recent years, not only *S.* Infantis has become more prevalent serovar among broiler flocks, but also become one of the five serovars most frequently causing human salmonellosis in Europe, Israel, Japan, and United States, are widely related with the consumption of contaminated poultry meat [9]. In addition, among *S.* Infantis isolates from broiler flocks, both increasing rates of resistance and MDR have been reported. In this study, 96.4% (n=54) of *S.* Infantis isolates were found to be resistant to at least two or four antimicrobials, of which 46 (82.1%) were MDR with SXT-CIP-TE phenotype. The highest resistance rates were observed against ciprofloxacin (96.4%), sulfamethoxazole-trimethoprim (76.8%), and tetracycline (96.4%), respectively. In a recent study, Sarıçam İnce and Akan [25] examined a total of 133 *Salmonellae* belonging to four different serovars from chickens for their antimicrobial susceptibilities, and found higher resistance rates against resistance to nalidixic acid, sulphanamid, trimethoprim, sulfamethoxazole-trimethoprim, and tetracycline and high MDR (78.7%, 37/47) rate among *S.* Infantis isolates, compared to other serovars. In a similar study conducted by Kaya et al.[26], higher rate of MDR (89.3%) among 150 *S.* Infantis isolates were also reported, with high resistance rates against to nalidixic acid (94.6%), tetracycline (93.3%), sulphanamide (92.6%), sulphametoxazole-trimethoprim (81.3%), streptomicin (78%), but low resistance rates for chloramphenicol (7.3%), and ampicillin (6.6%). Higher tetracycline, ciprofloxacin and sulphametoxazole-trimethoprim resistance rates detected in this study could be attributed to ongoing the misuse and overuse of these antimicrobials in humans, animals and plants for years.

In abovementioned studies, ciprofloxacin resistance was not reported. However, increased rate of resistance to ciprofloxacin in the current study is important finding because ciproflaxacin is broadspectrum antimicrobial, active in a variety of infectious diseases, considered as one of critically important antimicrobials by WHO [27]. In *Salmonella* isolates, ciprofloxacin resistance is mainly attributed to mutations in quinolone resistance determining regions (QRDR) of *gyrA* and *parC* genes. Indeed, WGS analysis revealed mutation with Ser83Thr in *gyrA* and Thr57Ser in *parC* in ciprofloxaxin-resistant isolates. The co-existence of two single substitutions in these genes (*gyrA*: Ser83Tyr and *parC*: Thr57Ser) have been reported to be primary cause for ciprofloxacin resistance in *Salmonella* isolates by many researchers [28,29].

Among all tetracycline resistance mechanisms, *tetA* and *tetB* genes encoding efflux pump are recognized as the most common genes associated with resistance in *Salmonella* [29]. The WGS analysis revealed presence of *tetA* gene in tetracycline resistant isolates. Similar observation also reported by Sarıçam İnce and Akan [25], who found *tetA* as the most dominant gene in tetracycline resistant isolates.

The most common mechanism of resistance to sulfonamides is the acquisition of the dihydropteroate synthase enzyme encoded by the *sul1*, *sul2* and *sul3* genes, while main resistance mechanism for trimethoprim is dihydrofolate reductase enzyme encoded by the *dfr* genes [30]. WGS analysis revealed presence of *sul1*, *sul2*, *sul3*, *dfrA12* and *dfrA14* genes among trimethoprim-sulfamethoxazole resistant isolates. Of these genes, *sul1* is associated with class 1 integron, on the contrary, *sul2* genes has been detected on various plasmids but not associated with integrons [31]. Ahmed and Shimamoto [32] also reported *dfrA17*, *dfrA1* and *dfrA12* genes in class 1 integron in *Salmonella* isolated from diseased broilers. Lee et al.[33] reported that *dfr* genes confer to the highest levels of resistance to trimethoprim. The results suggest that presence of *sul* and *dfr* genes in *S.* Infantis isolates could

mainly attributed to the acquisition and dissemination of class 1 integron.

Several factors contribute the emergence and dissemination of antimicrobial resistance among *S.* Infantis strains. The bacteria develop antimicrobial resistance through acquisition of resistance genes via horizontal gene transfer (HGT) or target gene mutation. HGT usually occurs through mobile genetic elements such as plasmids, transposons and integrons, which allow resistance genes to spread rapidly among different species. The misuse and overuse of antibiotics pose selective pressure on bacteria, leading to the selection of resistant strains. This selective pressure also contributes to the maintenance and spread of resistance genes among S. Infantis strains, leading to a serious threat to public health. The combination of these factors increases the likelihood of resistant strains entering the food chain, thereby increasing the potential for transmission and treatment difficulties to humans [7,10].

Integrons are known to disseminate ARGs among bacteria via transmissible plasmids and ISs, posing a threat to public health [11]. In this study, class 1 integron was detected in all isolates, while class 2 integron was detected in 78.6% of the isolates, which is consistent with findings of Kaya et al.[26] who detected class 1 integron in all isolates. This show wide distribution of integrons among *S.* Infantis isolates. Without sequencing PCR products belonged to integrons is not possible to determine gen cassettes. However, when previous studies were evaluated, it has been seen that gene cassettes confer mostly resistance to aminoglycosides *(aadA1*, *aadA2*, *aadA5*, *aadA7*, *aadB*, *aac(3')-IIa*, *aph(4')-Ia*, *aac(4')-IV*, *aph(6')-Id),* beta-lactams *(bla*$_{\text{TEM-1}}$, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{PSE-1}}$*),* sulfonamides *(sul1, sul2),* amphenicoles *(floR)* and diaminopyrimidines *(dhfr17, dhfrA12, dhfrA1)* [34,35]. It could be speculated that some resistance genes detected in this study by WGS are of integron origin. Further studies are therefore needed to elucidate class 1 and 2 integron-associated gene cassettes from *Salmonella* isolates.

Regarding the virulence genes that were examined, all isolates carried *sopB*, *pipD*, *sifA*, *stn*, *spaN*, *slyA*, *hilA*, *invA* genes, of which *invA* was used PCR target gene for confirmation of *Salmonellae*, but *sopE* (n=10), *spvC* (n=5) and *spvR* (n=3) were detected in a limited number of the isolates. On the other hand, one isolate had all virulence genes examined. The *sopB* and *sopE* genes promote acute intestinal inflammation and fluid secretion by disrupting tight junctions between epithelial cells and challenging the inositol phosphate signaling pathways that prevent adequate chloride secretion [36]. In a study conducted in Türkiye, prevalence of *sopB* gene in the *S.* Infantis isolates was determined as 92.41% [26]. In other countries, higher prevalence rates of *sopB* above 94.1% have also been reported [37,38]. However lower prevalence of *sopE* (17.9%) was recorded in this study. In conrast, Karacan Sever and Akan [39] reported a higher rate for *sopE* (93.3%) among *S.* Infantis isolates. It was reported that *sopE* gene, which is carried by lysogenic bacteriophage, could contribute to the emergence of new epidemic strains and the epidemic success of strains carrying this gene [40].

The studies investigating the *hilA* gene, which is the transcriptional master regulator of the type III secretion system (T3SS), are very limited in *S.* Infantis is isolates [41]. The frequency of *hilA* gene among *Salmonella* isolates belonging different serovars were reported as 90% [42] and 94.4% [43].

While the *sifA* gene regulates the molecular mechanisms required for *Salmonellae* to enter and replicate in host cells, the *spaN* gene facilitates the entry of bacteria into non-phagocytosing cells and enables intracellular invasion through apoptosis in macrophages. Karacan Sever and Akan [39], in Türkiye, the rate of *sifA* in *S.* Infantis isolates was reported as 90.62%. In Spain, Lamas et al.[44] reported prevalence of *sifA* gene to be 67.16%. The *stn* and *slyA* are the genes responsible for enterotoxin and salmolysin production, respectively. The prevalence of *stn* gene has been reported to be 72.22% in Egypt [43] and 100% in India [45]. However, no study investigating *slyA* gene among *Salmonella* serovars from poultry has been present in Türkiye. However, in studies conducted abroad, the prevalence of this gene for *S.* Infantis *is* was reported as 100% [44,46].

In this study, *spvC* (8.9%) and *spvR* (5.4%) genes were detected in low rates. Chiu et al.[47] reported the *spv* genes are rarely seen in the *Salmonella* genome and are responsible for the systemic infection and multidrug resistance in humans and animals. The *spvC* gene has the ability to inhibit the activation of macrophages and initiate their apoptosis, and giving *Salmonellae* capacity to cause systemic infections [48]. Low prevalence rate of *spvC* gene was also reported by Karacan Sever and Akan [39], who reported a rate of 8.92% in *S.* Infantis isolates. On the other hand, Chaudhary et al.[48] reported the absence of this gene in all isolates. Deguenon et al.[49] therefore claimed that this gene is not systematically present in the *Salmonella* genome but is of paramount importance when present. The *spvR* gene encode a LysR-like transcriptional regulator that positively regulates the independent transcription of its own gene and that of the spvABCD operon [50]. There is no study investigating the *spvC* and *spvR* genes together in *S.* Infantis isolates of poultry origin in Türkiye and abroad. Therefore, it was not possible to compare the results obtained for *spv*R in our study. Lozano-Villegas et al.[38] state that presence of *spv* genes in different strains of *Salmonella* isolated from broilers and humans is associated with increased the possibility of *Salmonella* strains being clinical importance.

To conclude, the present study characterized 56 strains of *Salmonella* belonging to serovar Infantis. The findings of this study indicate higher rate of MDR and virulence genes among *S.* Infantis strains. This imply that these isolates might be able to evolve into a dominant clone with high zoonotic potential. Therefore, regardless of the serovars, continuous monitoring and surveillance of *Salmonella* strains for their antimicrobial resistance and virulence characteristics among the poultry industry is necessary to provides relevant risk assessment data and help to evaluate targeted interventions using advanced molecular technicques.

Declarations

Availability of Data and Materials: The authors declare that data supporting the study findings are also available from the corresponding author on reasonable request.

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Author Contributions: ÖA and ST planned, designed, and supervised the research procedure, ST performed all microbiological and molecular experiments, ÖA performed bioinformatic analyses, and ÖA wrote the manuscript. Both ÖA and ST have read and approved the manuscript.

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Research Article

Effect of Beetroot Extract *(Beta vulgaris)* Against Olanzapine on the Pituitary, Thyroid, and Fertility in Adult White Male Rats

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Abstract

Olanzapine (OLZ) is a second-generation atypical drug that is commonly used to treat schizophrenia. However, it is known to affect male sexual functions, reproductive processes, and spermatogenesis. This study aims to investigate the effectiveness of beetroot extract in reducing the side effects of Olanzapine. The study involved 46 male Wistar rats weighing 150-200 g, administered intraperitoneal doses of OLZ and beetroot extract at (10 mg/kg) daily for six weeks. Thyroid-stimulating hormone (TSH), triiodothyronine (T3), tetraiodothyronine (T4), (T3/T4), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone levels were measured. The study found that beetroot extract treatment significantly increased thyroid-stimulating hormone levels by 81% and TSH by 82% in the third and sixth weeks, while Triiodothyronine levels decreased by 42% and thyroxin levels by 115% in the third and sixth weeks, also, there was a significant decrease in testosterone levels in the group treated with Olanzapine by 108 % in the third week and by 116% in the sixth week when compared to a control group; additionally, the study found a 25% increase in absolute body weight in the beetroot extract group and 26% increase in a combined group treated with Olanzapine compared to Olanzapine. Histological sections of the thyroid gland and testis were taken at the end of the sixth week. OLZ caused atrophy of follicular epithelium in the thyroid gland and shrunken seminiferous tubules, with disorganized germinal epithelium in the testis; however, beetroot extract treatment resulted in normal testicular histology, characterized by regular tubules with stratified germinal epithelium and Leydig cell clusters. However, some sperm aggregation was observed in the tubular luminae. It concluded that administrating beetroot extract (10 mg/kg) daily for six weeks significantly improved the pituitary, thyroid, and fertility in adult white male rats.

Keywords: *Olanzapine, Beetroot, Beta vulgaris, Thyroid, Male fertility*

Introduction

Animal models have significantly advanced our understanding of mental disorders, including their underlying mechanisms, progression, symptoms, and potential treatment strategies [1]. Nevertheless, animal models cannot accurately reproduce the intricate conditions found in people. Hence, it is essential to meticulously choose animal models that exhibit similarities to human diseases and circuit-specific modifications that may result in pathology [2]. To be considered a valid model of a human mental disorder, an animal model should exhibit face validity, construct validity, and predictive validity. Face validity refers to the similarity between the behavioral and physiological symptoms observed in the model and those experienced by patients. Construct validity requires that the model replicates the underlying neurobiological mechanisms of the disorder. Predictive validity is demonstrated when the model accurately predicts the response to therapeutic interventions [3].

The Middle East exhibits a significantly higher prevalence of schizophrenia compared to developed countries, as evidenced by the age-standardized disability-adjusted life years associated with schizophrenia in 2004 [4]. The estimated rates per 100.000 inhabitants were approximately

273 in Egypt, 270 in Saudi Arabia, 269 in Kuwait, and 267 in the United Arab Emirates, whereas in Australia, the rate was 164 per 100,000 inhabitants [5]. However, a recent study conducted at Jazan Health, Saudi Arabia, specifically in an "adult psychiatry clinic," revealed that the most commonly prescribed initial antipsychotic drugs were Olanzapine (48.8%), haloperidol (13.9%), and aripiprazole (11.3%) [6]. Hence, we evaluated the protective effects of beetroot extract against the side effects of Olanz apine. Additionally, w e investigated the potential mechanisms of action by studying its impact on various gland functions [7].

Antipsychotic medications are utilized to treat a severe and chronic illness that affects 21 million individuals worldwide [8]. Olanzapine (OLZ) is a type of second-generation antipsychotic drug and is commonly prescribed for the immediate treatment of schizophrenia. It exhibits a strong affinity for various binding regions, including dopaminergic, serotonergic, muscarinic, adrenergic, and histaminergic regions [9]. Second-generation anti-psychotics, such as dopamine D2 receptor antagonists, can increase the levels of thyroid-stimulating hormone (TSH) in the bloodstream, thereby impacting other thyroid hormones like triiodothyronine and thyroxine [10]. In men, OLZ can lead to sexual dysfunction by inhibiting gonadotropin-releasing hormone, luteinizing hormone, and testosterone, resulting in hypogonadism and various adverse effects on sperm production, semen quality, sperm motility, and testicular tissue morphology [11].

Herbal remedies are generally considered safe and can significantly treat various diseases [12]. Beetroot extract, derived from the fleshy root of the *Beta vulgaris* plant, is known for its thin skin and a wide range of colors, including purple-pink, reddish-orange, and brownish tones. The deep crimson-red pulp of the beetroot has a pleasant, sweet taste [13].

Beetroot extract is recognized as one of the top ten potent vegetable sources of phytochemicals, exhibiting strong antioxidant and anticancer properties [14]. It is highly beneficial in improving hormonal levels related to fertility and can be advantageous for maintaining pregnancy and treating infertility [15]. There are no available studies on the effect of beetroot extract on Olanzapine toxicity; therefore, this study hypothesized that Beetroot supplementation would mitigate the adverse effects of Olanzapine on the pituitary and thyroid glands in rats. Also, its effect on reducing the increase in prolactin levels induced by Olanzapine, attenuating the decrease in thyroid hormones (T3 and T4) caused by Olanzapine, Simultaneously, improve the histological architecture of the pituitary and thyroid glands in olanzapine-treated rats and reduce oxidative stress markers in the pituitary and thyroid glands of olanzapine-treated rats.

Materials and Methods

Ethical Approval

The animal study has been approved by the Unit of Biomedical Ethics, Research Ethics Committee (REC HA-02-J-008, King Abdul Aziz's University). The accommodation and administration of the animals and the experimental protocols were conducted per the principles delineated in the Guide for the Care & Use of Lab Animals following the National Committee of Bioethics NCBE (2023). The Ethical Code number 511-89.

Beetroot Extract

Beetroot was purchased from the popular market in Jeddah, Beetroot material was ground to a uniform particle size of 0.5 mm. Subsequently, it was subjected to ultrasound-assisted extraction. A variety of solvents and co-solvents were used in these extractions. The resulting extracts were concentrated using a rotary evaporator (BÜCHI Rotavapor R-114 and BÜCHI Vacuum Controller B-721) and then dried under reduced pressure. The dried extracts were stored at -20°C for further analysis [16].

20 g of dried and ground material were added to an Erlenmeyer flask. 250 mL of water was added to the flask. The flask was then submerged in an ultrasonic bath (Iskra-Pio, Slovenia) and subjected to ultrasonic waves at a frequency of 40 kHz. The liquid level in the flask was maintained below the water level in the bath. The extraction process was carried out at a constant temperature of 40°C for 90 min [16].

Extraction yield, expressed as the ratio of the mass of the extract to the mass of the dry beetroot material, was used to assess the efficiency of the different extraction methods and conditions.

Experimental Animals' Layout

In this study, forty-eight adult male rats of Caucasian descent weighing between 150 and 250 g were utilized. The rats were housed in standard cages designed for rats and kept in a room with a 12:12 h light/dark cycle and a controlled temperature of 22±1ºC. To allow for acclimatization, the rats were kept in the laboratory for one week before the commencement of the study. The beetroot extract was filtered using filter paper, and intraperitoneal administration of a daily dose of 10 mg/kg was carried out for six weeks. Throughout the experiment, the rats were divided into four groups, each consisting of twelve rats.

Blood Biochemistry

After three weeks, serum samples were collected from each rat. Blood samples were collected using K3-EDTA tubes to measure various hormone levels, including thyroid-stimulating hormone (TSH), thyroid hormones

(triiodothyronine T3, tetraiodothyronine T4, and the percentage of T3 to T4) as follow, the blood samples (1 mL) were collected from control and treated rats, centrifuged (20 min at 1.500 x g). The serum was frozen at -70°C for later hormone analysis. T3 and T4 concentrations were measured using a competitive chemiluminescent enzyme immunoassay (Immulite 1000, Siemens). All samples were run in duplicate under standardized conditions with intra-assay CVs <5%.

Serum samples were analysed for LH, FSH, and testosterone levels using an Enzyme Immunoassay (EIA) kit (Cayman Chemical Company, Ann Arbor, MI, USA). The assay was performed following the manufacturer's protocol and the guidelines outlined in Tietz [17]. Results for LH, FSH, and progesterone were reported in ng/mL-1, while estradiol levels were expressed in pg/mL^{-1}.

Histological Studies

At the end of the sixth week, the rats were euthanized, and serum and tissue samples were obtained from their thyroid glands and testicles. The thyroid glands and testes were weighed immediately after dissection for further analysis. After the collection of blood samples all animals were sacrificed by cervical dislocation. Rats dissected one testis, and both the femora of each animal and the thyroid gland were dissected. The shape, color and location were recorded before the fixation, and the photographs of the thyroid were taken to depict the gross anatomy by using a digital camera Sony cyber-shot (14.2 megapixels) for histological and histochemical study samples of the thyroid gland was fixed in 10% neutral buffered formaldehyde and sectioned serially at 5 µm. Sections were stained with Hematoxylin and eosin, and for histochemical studies PAS and Masson Trichrome stain [18]. The histological sections of this study were examined by using a light microscope type (Olympus/Japan) with different magnifications (X20 and X40). The sections were photographed by using (Olympus/Japan) microscope and digital camera; an ocular micrometer calibrated with a stage micrometer was used for histological parameters, which include the thickness of the capsule, the diameter of different sizes of follicles and the height of lining epithelium [19].

Statistical Studies

The statistical analysis of the data was carried out using the software package STATISTICA 10.0 (StatSoft Inc., Tulsa, OK, USA). Each determination was performed three times, and the resulting data were averaged. The final results are presented as mean values. The means were compared with the LSD test at P<0.05.

Results

Thyroid-Stimulating Hormone (TSH)

The outcomes of this study indicate that, as compared to the control group, there was a statistically significant reduction in TSH levels in the olanzapine-treated groups during the third and sixth weeks, as well as in the combined group treated with olanzapine and beetroot extract during the third week. These data are displayed in *Fig. 1-A,B*. In contrast, the group that received beetroot extract treatment every week and the group that received Olanzapine plus beetroot extract treatment just for the sixth week showed no discernible changes in TSH levels when compared to the control group. Furthermore, compared to the olanzapine group, the beetroot extract group and the combination group treated with Olanzapine plus beetroot extract showed a statistically significant increase in TSH levels in both the third and sixth weeks.

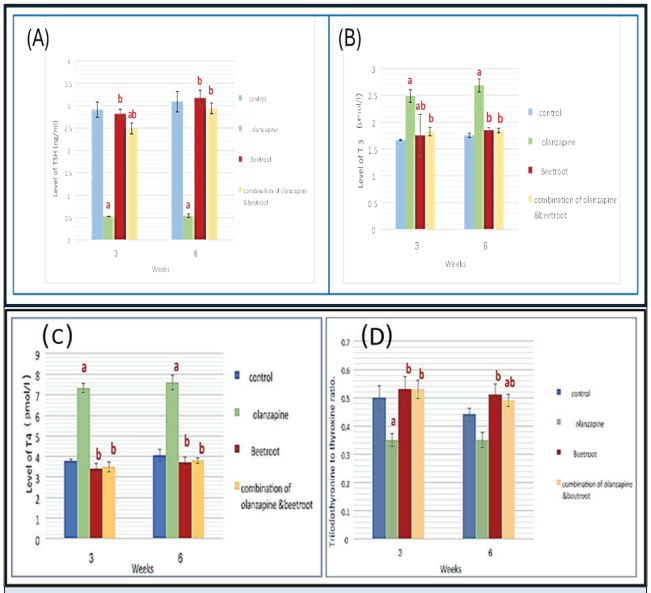

Fig 1. Effect of daily administration of Olanzapine (10 mg/kg), Beetroot extract (10 mg/kg), and Combination of Olanzapine & Beetroot on thyroid stimulating hormone (TSH) (mLU/mL) (A), and Triiodothyronine (T3) pmol/L (B), Thyroxine (T4) (mLU/mL) (**C**), and Triiodothyronine to thyroxine ratio (T3/T4) (D) of adult male rats for 6 weeks

Triiodothyronine (T3)

The data displayed in *Fig. 1-A* indicate that, in comparison to the control group, there was a discernible rise in the level of T3 in the groups treated with Olanzapine in the third and sixth weeks, as well as in the group treated with beetroot extract in the same week. In contrast to the control group, no discernible change in the level of T3 was seen in the group administered beetroot extract in the sixth week or in the combined group administered olanzapine and beetroot extract throughout all weeks. Additionally, it was shown that during both the beetroot extract treatment group and the combined group treated with olanzapine and beetroot extract, there was a substantial drop in the level of T3.

Thyroxine (T4)

As observed in *Fig. 1-C*, the group receiving Olanzapine experienced a considerable increase in T4 levels in the

third and sixth weeks when compared to the control group. Comparing the beetroot extract group and the combination group treated with olanzapine and beetroot extract to the control group, however, did not demonstrate a statistically significant difference in the amount of T4 in any of the weeks. In contrast, compared to the olanzapine group, there was a noteworthy drop in T4 levels in the beetroot extract group and the combined group treated with olanzapine and beetroot extract in both the third and sixth weeks.

Triiodothyronine to thyroxine ratio (T3/T4)

In *Fig. 1-D*, it is evident that the group receiving olanzapine treatment experienced a significant increase in the third week, while the combined group treated with olanzapine and beetroot extract showed a significant increase in the sixth week, both in comparison to the control group. On the other hand, no significant impact was observed on the triiodothyronine to thyroxine ratio (T3/T4) in the group treated with beetroot extract throughout all weeks, as well as in the combined group treated with olanzapine and beetroot extract during the third week, when compared to the control group. Furthermore, a significant increase in the group treated with beetroot extract and the combined group receiving Olanzapine and beetroot extract was evident during both the third and sixth weeks, in comparison to the olanzapine group.

Follicle-Stimulating Hormone (FSH)

Throughout all weeks, the group receiving olanzapine treatment exhibited a notable rise in FSH levels. Furthermore, during the third week, both the group treated with beetroot extract and the combined group receiving both olanzapine and beetroot extract demonstrated a significant increase in FSH levels in comparison to the control group, as depicted in *Fig. 2-A*. Conversely, there was a significant decrease in FSH levels observed during both the third and sixth weeks for the group treated with beetroot extract and the combined group receiving Olanzapine and beetroot extract. This finding contrasted with the group solely treated with Olanzapine.

Luteinizing Hormone (LH)

The level of LH increased significantly in the group treated with Olanzapine and the combined group treated with olanzapine and beetroot extract in both the third and sixth weeks. We also observed a significant increase in the level of LH in the group treated with beetroot extract only in the third week when compared to the control group *(Fig. 2-B)*. However, there was no significant change in the level of LH in the group treated with beetroot extract in the sixth week when compared to the control group. Furthermore, we noticed a significant decrease in the level of LH in the group treated with beetroot extract and the combined group treated with olanzapine and beetroot extract in both the third and sixth weeks when compared to the olanzapine group.

Testosterone

The group subjected to olanzapine treatment exhibited a significant reduction in testosterone levels during both the third and sixth weeks, as indicated in *Fig. 2-C*,

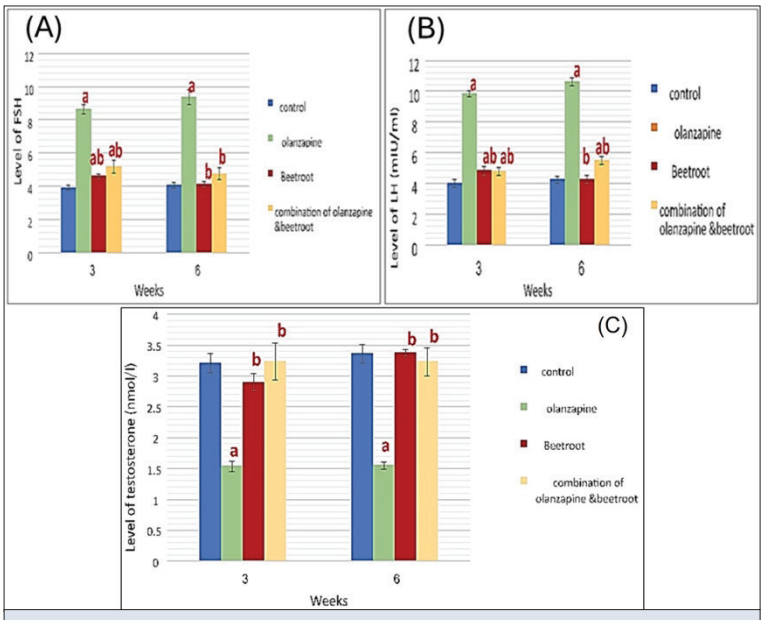

Fig 2. Effect of daily administration of Olanzapine (10mg/kg), Beetroot extract (10mg/kg), and Combination of Olanzapine & Beetroot on Follicle-stimulating hormone (FSH) (mlU/mL) (A), and Luteinizing hormone (LH) (mLU/mL) (B) testosterone hormone (nmol/l) (C) of adult male rats for 6 weeks

Table 1. *Effects of daily administration of Olanzapine (10mg/kg), Beetroot extract (10mg/kg), and Combination of Olanzapine & Beetroot on the absolute body weight in grams of adult male rate during 6 weeks*

Weeks	Control	Olanzapine	Beetroot	Combination
3	222.16±3.02	157.83±4.63^{a}	211.5±3.13^{ab}	214.7±4.75^{b}
6	192.5±8.55	205.8±3	199.3±5.47	204.6±2.57

Table 2. *Effects of daily administration of Olanzapine (10mg/kg), Beetroot extract (10mg/kg), and Combination of Olanzapine & Beetroot on absolute testes weight (g)of adult male rate during 6 weeks*

Weeks	Control	Olanzapine	Beetroot	Combination
3	1.86±0.08	1.01±0.02^{a}	2.04±0.09^{b}	1.77±0.11^{b}
6	1.94±0.08	1.02±0.02^{a}	2.05±0.07^{b}	1.81±0.06^{b}

Table 3. *Effects of daily administration of Olanzapine (10mg/kg), Beetroot extract (10 mg/kg), and Combination of Olanzapine & Beetroot on relative testes weight % of adult male rate during 6 weeks*

Weeks	Control	Olanzapine	Beetroot	Combination
3	0.83±0.02	0.64±0.02^{a}	0.96±0.01^{ab}	0.82±0.02^{b}
6	1.02±0.05	0.49±0.008^{a}	1.025±0.05^{b}	0.92±0.02^{b}

compared to the control group. However, there was no notable impact observed in the group treated with beetroot extract or the group receiving a combination of olanzapine and beetroot extract throughout all weeks, when compared to the control group. On the other hand, both the group treated with beetroot extract and the group receiving a combination of olanzapine and beetroot extract demonstrated a significant increase in testosterone levels in comparison to the olanzapine group.

Absolute Body Weight (BW)

During the third week, both the olanzapine-treated group and the beetroot extract group experienced a significant decrease in absolute body weight compared to the control group *(Table 1)*. However, we observed a significant increase in absolute body weight in the group treated with beetroot extract and the combined group treated with both olanzapine and beetroot extract in the third week when compared to the olanzapine group. There was no significant effect on absolute body weight in the group treated with Olanzapine, the group treated with beetroot extract in the sixth week, and the combined group treated with both olanzapine and beetroot extract in the third and sixth weeks when compared to the control group.

Absolute Testes Weight

The group receiving olanzapine treatment exhibited a significant decrease in absolute testis weight during both the third and sixth weeks, as depicted in *Table 2*, in contrast to the control group. However, there was no notable impact observed on the group treated with beetroot extract or the combined group receiving both olanzapine and beetroot extract during both the third and sixth weeks, when compared to the control group. On the other hand, a significant increase in absolute testis weight was observed in the group treated with beetroot extract and the combined group receiving both olanzapine and beetroot extract during both the third and sixth weeks, in comparison to the group treated with Olanzapine.

Relative Testes Weight

The study revealed significant findings regarding the effects of treatment interventions. Specifically, the group receiving olanzapine treatment demonstrated a notable decrease during both the third and sixth week in comparison to the control group, as illustrated in *Table 3*. Conversely, the group treated with beetroot extract exhibited a significant increase in the third week when compared to the control group. Moreover, the group treated with beetroot extract consistently displayed significant improvements throughout all weeks, while the combined group treated with both Olanzapine and beetroot extract showed a significant enhancement, specifically during the sixth week, relative to the group treated with Olanzapine alone.

Histological Studies

The light microscopic micrograph of the thyroid gland showed normal Follicles surrounding a homogeneous colloid and epithelium cell with regular capsule and fenestrated capillaries in the control group *(Fig. 3-A,B)*. while the histological structure of the thyroid gland in the group treated with OLA showed irregular follicles, cell disintegration, dark pigmentation heterogeneity of the colloidal substance, degenerated epithelium and connective tissue, and focal proliferation of C cells *(Fig. 3-C,D)*. On the other hand, the gland's structure in the beetroot extract is like the control group, with normal follicles, homogenous colloid, and epithelium cells with

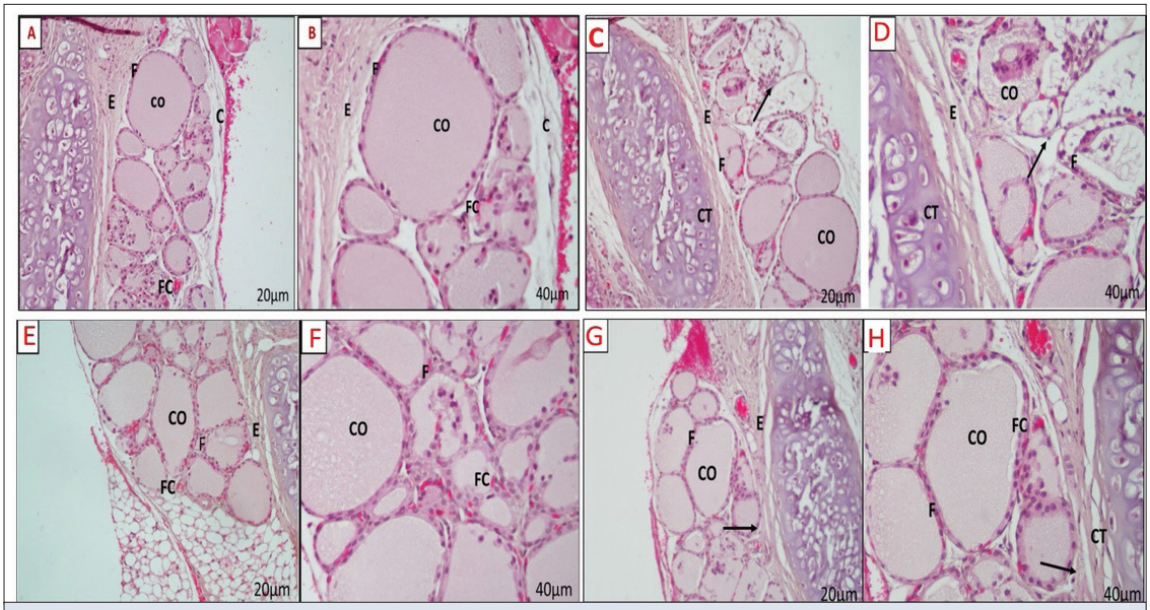

Fig 3. A, B: Light microscopic micrograph of the thyroid gland from adult rats in the control group showing normal Follicles (F), surrounding a homogenous colloid (CO) Follicular and epithelium cell (E) With normal capsule(C) and fenestrated capillaries (FC); **C, D:** Marked Irregular Follicles (F), heterogeneity of the colloidal substance (CO), degenerated Epithelium and Connective Tissue (CT) (E) Focal proliferation of C cells *(black arrows);* **E, F:** The structure of the gland is similar to the control group, normal Follicles (F), homogenous colloid (CO) Follicular, and epithelium cell (E) With normal capsule (C) and fenestrated capillaries (FC); **G, H:** Irregular Follicles and cell disintegration (F) and the colloidal substance are heterogeneous (CO), and less amount of epithelium and degenerated connective tissue (CT) (E) *(black arrows)* at a lower rate than the olanzapine group

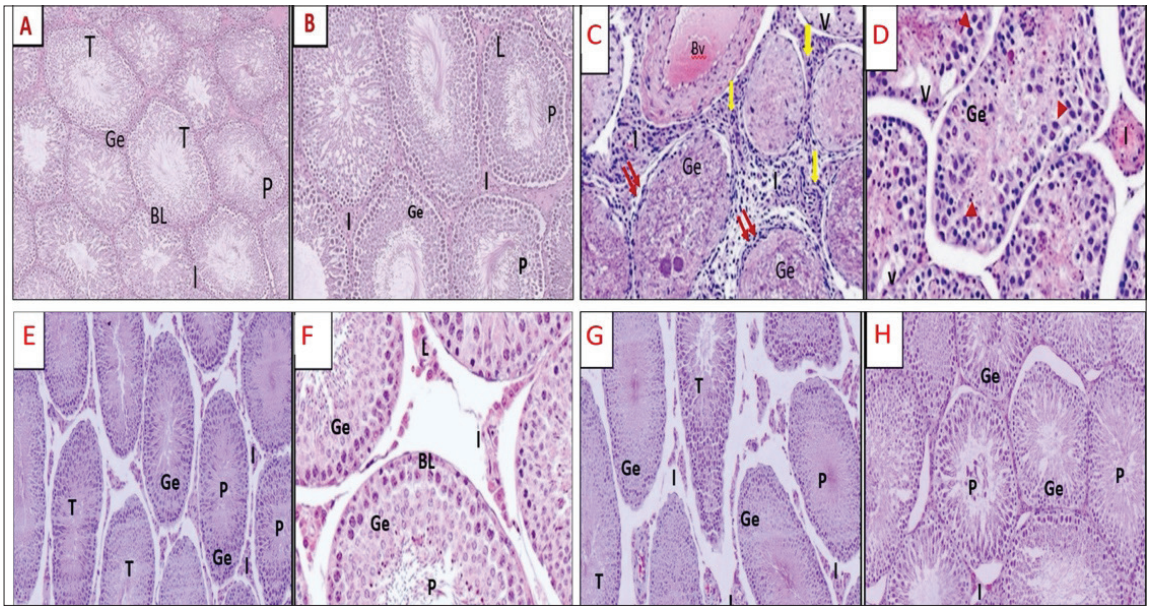

Fig 4. A, B: Light microscopic micrograph of testicular tissue from an adult rat in the control group showing normal seminiferous tubules (T) ensheathed with basal lamina (BL) and containing normal lined by stratified germinal epithelium (Ge). Aggregations of sperms (P) are seen in the lumina. Narrow interstitial spaces (I) show clusters of Leydig cells (L); **C, D;** Showing shrunken seminiferous tubules *(doubble arrow)* with disorganized germinal epithelium and marked vacations (V). Some other tubules are filled with degenerated germ cells (Ge), and darkly stained nuclei (arrowhead). Also, wide interstitial spaces (I) with scattered leucocyte infiltration *(arrow)* are noticed. Congested dilated blood vessels are also seen (Bv); **E, F:** Regular seminiferous tubules with stratified germinal epithelium cells (Ge) resting on regular basal lamina (BL). Tubules appear with aggregation of sperms (P) in the lumina. Normal interstitial spaces (I) show Leydig cells (L) clusters; **G, H:** Restoration testicular structure in most seminiferous tubules (T) and having nearly regular contour and are lined by stratified germinal epithelium (Ge). Their lumina contain aggregations of sperms (P). However, the interstitial spaces (I) are relatively wide compared with the control group

regular capsules and fenestrated capillaries *(Fig. 3-E,F)*. The combined group treated with olanzapine and beetroot extract showed shows no similarity with the control group. The Irregular Follicles and cell disintegration and the colloidal substance are heterogeneous, and less amount of degenerated epithelium and connective tissue at a lower

rate than the olanzapine group *(Fig. 3-G,H)*. The results showed normal testicular tissue structure in the control group *(Fig. 4-A,B)*. The seminiferous tubules appear normal and are ensheathed with basal lamina.

Additionally, they usually contain lined, stratified germinal epithelium. The lumina of the tubules shows aggregations of sperms, and narrow interstitial spaces display clusters of Leydig cells. While the histological structures of seminiferous tubules appear shrunken with disorganized germinal epithelium and marked vacuolations in the group treated with OLA *(Fig. 4-C,D)*. Some of the tubules are filled with degenerated germ cells and darkly stained nuclei. However, *(Fig. 4-E,F)* demonstrated regular testicular tissue in the treated group with beetroot extract. The cotreatment with beetroot extract and OLA restored testicular structure in most seminiferous tubules, with a nearly regular contour, lined by stratified germinal epithelium *(Fig. 4-G,H)*. However, the interstitial spaces are relatively wide compared with the control group.

Discussion

Schizophrenia often manifests around late adolescence or early adulthood and is associated with a range of symptoms that are traditionally categorized into three groups: positive, negative, and cognitive symptoms [20]. Manifestations of positive symptoms include hallucinations, delusions, and mental impairments. Negative symptoms encompass diminished emotional expression, apathy, lack of pleasure, retreat from social interactions, and avolition. Cognitive dysfunction includes impairments in working memory, attention, processing speed, and difficulties in maintaining focus, including genetic predisposition and exposure to severe socioenvironmental challenges [21], which influence The development of schizophrenia. To replicate the characteristics of schizophrenia, animal models in the study of the disease extensively employ genetic models, prenatal interventions, pharmacological models, and stress-based protocols during prepubertal periods.

The thyroid gland is a crucial player in metabolism thanks to its secretion of two vital hormones: thyroxine (T4) triiodothyronine (T3) and thyroid-stimulating hormone (TSH). KhoshvaghtiAbtahi [22], suggested that the unchanged levels might be due to flavonoid compounds with suppressive effects on the thyroid gland in the short term. The thyroid gland may also adapt to these compounds with long-term extract administration [23]. Another possible explanation is the inhibitory effect of flavonoid compounds on prostaglandin production via cyclooxygenase inhibition, which has been linked to the stimulatory effect of prostaglandins on the production and secretion of pituitary-thyroid hormones [23].

According to recent research of Śmierciak et al.[24], thyroid abnormalities such as hypothyroidism and hyperthyroidism have been identified as risk factors for various neuropsychiatric disorders, including schizophrenia. In our study, we observed a significant increase in Triiodothyronine (T3) and thyroxin (T4) levels in the group treated with Olanzapine when compared to a control group. These findings align with previously published studies [25], which have found that patients with schizophrenia tend to exhibit higher levels of T3 and T4.

Additionally, research by Jose et al. Jose et al.[26] suggests that higher levels of free T3 may be linked to suicide ideation in male schizophrenia. Zhu et al.[27] also discovered that free radical-induced hyperthyroidism can enhance thyroid hormone synthesis in schizophrenia. Notably, higher levels of total plasma peroxides and MDA have been observed to correlate with T3 in schizophrenics. Furthermore, long-term negative endocrine feedback resulting from the duration of the disease can reduce the effectiveness of negative feedback regulation on TSH secretion during the stage of neuroregulation. Finally, research by Li et al.[25] has suggested that thyroid hormones may serve as biomarkers of agitation in schizophrenia and could play a role in the disorder's pathogenesis.

Beetroot is a rich source of bioactive phytochemicals with potential health benefits, including antioxidant, antibacterial, antiviral, and analgesic properties. It has been explored for its therapeutic applications in various diseases, such as cancer and atherosclerosis. The food industry has utilized beetroot and its derivatives as natural colorants and preservatives due to their stability and non-toxicity. Beetroot is a rich source of flavonoids and phenolic compounds, such as 5-hydroxy-6,7-methylenedioxyflavone, 3,5-dihydroxy-6,7-methylenedioxyflavanone, 2,5-dihydroxy-6, and 7-methylenedioxyisoflavone [28].

Androgens, a class of steroid hormones, play a vital role in the reproductive system and overall homeostasis. Imbalances in androgen levels can contribute to a range of physiological disorders and diseases. Flavonoids, a diverse group of natural polyphenols widely found in plants and foods, have gained significant attention due to their potential health benefits and their ability to interact with hormone systems [29]. Emerging evidence suggests that flavonoids can influence androgen synthesis and metabolism, offering potential therapeutic benefits for androgen-related disorders. Flavonoids can influence androgen levels and actions by targeting multiple mechanisms, including the hypothalamic-pituitary-gonadal axis, androgen synthesis and metabolism, receptor binding, and antioxidant effects. However, the complex interplay between flavonoids, individual factors, and dietary matrices poses challenges in translating these findings into clinical applications [30].

The study found that beetroot extract treatment significantly increased thyroid-stimulating hormone levels by 81% and TSH by 82% in the third and sixth weeks, while Triiodothyronine levels decreased by 42% and thyroxin levels by 115% in the third and sixth weeks.This study agrees with Peepre et al.[31] who found increasing the level of thyroxin in rats after 15 days of Vitamin C and E administration. Also, Li et al.[32] reported that Elevated LH and FSH levels, combined with normal or decreased testosterone, can lead to testicular failure and germinal cell degeneration, significantly affecting spermatogenesis.

Schizophrenic psychoses often result in hyperprolactinemia and gonadal dysfunction, leading to estrogen deficiency in women and testosterone deficiency in men [33]. This can lead to infertility or sexual dysfunction, which are major problems for individuals with psychiatric disorders [34]. Antipsychotic drugs can also cause reproductive toxicity [34].

In our study, there was a significant decrease in testosterone levels in the group treated with Olanzapine by 108 % in the third week and by 116% in the sixth week when compared to a control group. This is consistent with Korkut et al.[35], who found that serum LH and testosterone levels decreased in 20 and 40 mg/kg quetiapine-administered rats.

Our study found a notable increase in LH levels in the group treated with beetroot extract, showing a 17% rise in the third week when compared to the control group. This aligns with previous research by Widhiantara et al.[36], which suggests that flavonoid compounds may positively stimulate the pituitary gland to produce LH and improve male fertility.

The study found a significant decrease in FSH and LH levels but an increase in testosterone levels in the beetroot extract and olanzapine-beetroot extract group compared to the olanzapine group. These findings are consistent with previous studies by Hussein [37].

Testosterone plays a crucial role in regulating spermatogenesis, initiating the process during puberty, and maintaining it in adulthood. It also helps with meiosis and spermatid differentiation, according to Elsheikh et al.[38]. Elsheikh et al.[38] found that Beetroot extract contains flavonoids and betalain pigments, which have anti-inflammatory and antioxidant properties, and areoflavones that inhibit enzymes like aromatase and 5-α reductase, preventing disease. Elsheikh et al.[38] also reported that beetroot extract significantly improves sperm count and motility. Furthermore, Hussien et al.[37] demonstrated that flavonoids promote sperm production and maintain the function of spermatogenic cells. Sarfaraz et al.[39] suggested that beetroot extract could improve fertility and maintain hormonal levels during fertility.

The study found a 25% increase in absolute body weight in the beetroot extract group and a 26% increase in a combined group treated with Olanzapine compared to Olanzapine. These results agree Abbas et al.[40] reported that this increase was due to beetroot's positive anabolic effect by improving lipids and glucose metabolism. Zhao et al.[41] found that Flavonoids enhance osteoblast differentiation and inhibit osteoclasts, affecting bone weight. Beetroot extract treatment significantly increased testes weight by 50% in the third and sixth weeks compared to the olanzapine group. This is consistent with the results of Almuoswi et al.[42]. They reported that increased testes weight increases sperm concentrations and improves male fertility.

The study found that the rat thyroid's histological structure in the control group exhibited normal follicles, a homogeneous epithelium cell, capsule, and fenestrated capillaries. Meanwhile, these findings are very similar to those reported in the literature BaqerAlaridhi [33].

The study reveals irregular follicles, heterogeneity of colloidal substance, degenerated epithelium, connective tissue, and focal C cell proliferation in an adult rat's thyroid gland, consistent with previous research. Samawi et al.[44]. However, Samawi et al.[44] showed that thyroid function parameters were altered due to olanzapine medication, which was related to Olanzapine's cytotoxic effect [45].

Dopamine-releasing substances can impede thyroid-releasing hormone (TSH) secretion, potentially leading to abnormal thyroid test results in patients taking conventional antipsychotics due to their pharmacologic profile and dopaminergic activity [46]. Nevertheless, the outcomes of this research are consistent with prior investigations that have identified certain irregularities, such as thyroid function tests and liver enzymes, subsequent to Olanzapine treatment [47].

Olanzapine induces cytotoxicity and degenerative changes in thyroid cells through excessive reactive oxygen species production, leading to mitochondria collapse, lysosomal membrane leakage, reduced lipid peroxidation, and glutathione depletion [48].

The research shows that rat thyroid's structure is similar to the control group, with normal follicles, cells, and capillaries. It also confirms that nitrate-rich beetroot juice does not significantly alter plasma T3 and T4 levels or promote thyroid gland dysfunction. These findings are crucial in terms of ensuring safety [49]. However, these findings are congruent with those of Krajka-Kuzniak et al.[50], who discovered that beetroot may stimulate the expression of phase II detoxifying enzymes via Nrf2 activation as a result of mitogen-activated protein kinase stimulation.

Additionally, beetroot may be advantageous to the thyroid gland. Iodine deficiency reduces thyroid hormone production. Because beetroot is high in iodine, it may aid with thyroid management [51].

The study found that rats treated with olanzapine and beetroot extract showed irregular follicles, cell disintegration, and a heterogeneous colloidal substance with higher drug effects, possibly due to dose or experiment duration.

Olanzapine detrimentally induces sexual dysfunction. However, a restricted body of research indicates that it additionally triggers structural alterations within the reproductive system [52]. Furthermore, scholarly research suggests that Olanzapine may contribute to male infertility by causing sexual dysfunction [15]. However, histopathological analysis is widely recognized as a highly sensitive biomarker in regulatory toxicology investigations for identifying the detrimental reproductive consequences of toxicants [53].

The research reveals that olanzapine-treated rats exhibit distinctive vacuations, diminished seminiferous tubules, disorganized germinal epithelium, degenerated germ cells, darkly stained nuclei, expansive interstitial spaces, blood vessel congestion, and dilation. Histopathological alterations include seminiferous tubule lumen openings, interstitial area losses, germinal epithelium disorders, and cell degeneration, potentially indicating testicular toxicity. However, the results in this study agree with those of de Siqueira Bringal et al.[54]. The study found that Olanzapine may cause dose-dependent toxicity in testicular tissue, with histopathological alterations observed in high-dose groups and similar features in low-dose groups [52]. The impact of Olanzapine on testicular histology has been established in a limited number of studies that have been published [15].

Similarly, seminiferous tubule structures in the control group were discovered to be normal, according to a recent study. The Leydig cells in the interstitial region exhibited a consistent morphology and arrangement [53].

Additionally, the control group exhibited regular spermatogenic series and Sertoli cells within the tubules and the detection of sperm within the lumen of seminiferous tubules [53]. Lipofuscin granule proliferation indicates differentiation in Leydig cells. Vacuolization, sperm count decreases, and basement membrane thickening indicate Sertoli cell degeneration. Germ cells typically undergo degeneration, exfoliation, disorganization, vacuolization, and edema. Sertoli cell functional deficiencies can lead to germ cell degeneration [53]. Histopathological analysis reveals vacuolization, swelling, and other pathologies as signs of germ cell injury or reproductive toxicity induced by Olanzapine, indicating degenerative processes in testicular tissue. Additionally, previous research for assessing the genotoxic and oxidative damage potential of Olanzapine revealed that high drug concentrations caused oxidative stress. It has been stated that oxidative stress may result in tissue injury [51]. Oxidative stress has additionally been demonstrated to be an effective factor in testicular degeneration, which has been linked to a variety of causes, according to numerous studies [7].

Moreover, a recent study has demonstrated that efficacy-based olanzapine-induced oxidative stress in testicular tissue may account for aberrant sperm morphology and degenerative histological findings in the structure of the testicular glands observed in groups administered high doses of the drug [52]. Numerous studies have confirmed that betalains derived from red beetroots possess formidable antioxidant properties [38].

In the present study, the histological structure of rat testis in the group treated with beetroot extract showed that the tubules were regular, with stratified germinal epithelium cells reclining on regular basal lamina. However, sperm aggregation occurs in the lumina of tubules. Normal interstitial spaces are lined with Leydig cell clusters. Thus, normal histological appearance was observed in the beetroot groups with respect to seminiferous tubule structures, interstitial area characteristics, and cells. However, these results are very similar to those reported in the literature of Elsheikh et al.[38]. It was found that the antioxidants found in beets protect against the potentially harmful effects of excessive oxidative stress and prevent potential pathological diseases. They also maintain the integrity of the structure and tissues.

Research shows that Olanzapine and beetroot extract treatment restore testicular structure in seminiferous tubules with stratified germinal epithelium, with sperm aggregations, and slower degenerative changes than the Olanzapine group, suggesting beetroot administration reverses degenerative impact. However, these results agree with Elsheikh et al.[38].

The protective properties of beetroot and its constituents against various xenobiotic toxins have been unequivocally acknowledged, including their potent antioxidant, anti-inflammatory, and vascular-protective effects [51]. Furthermore, prior research has demonstrated that beetroot juice serves as a safeguard against the toxicity of detrimental chemicals. For instance, it prevented oxidative stress in male rats exposed to carbon tetrachloride and diminished DNA and plasma protein carbonyl damage [54]. Additionally, the male reproductive system of albino rodents is protected from the toxicity of cadmium chloride by beetroot [13]. In the end, it can be concluded that beetroot (*Beta vulgaris*) is one of the most important medicinal plants, scientifically proven to contain natural antioxidants that have preventive and therapeutic effects

against oxidative stress. our results indicate that the antioxidants in beetroot extract have therapeutic effects against Olanzapine on the pituitary, thyroid, and fertility in adult white male rats.

Declaration

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author (H. Y. Alnahary) on reasonable request.

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Ethical Approval: The animal study has been approved by the Unit of Biomedical Ethics, Research Ethics Committee (REC HA-02-J-008, King Abdul Aziz's University). The accommodation and administration of the animals and the experimental protocols were conducted per the principles delineated in the Guide for the Care & Use of Lab Animals following the National Committee of Bioethics NCBE, (2023). The Ethical code number 511-89.

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Research Article

The Assessment of White Blood Corpuscles by Geometric-Morphometric Analysis After the Application of Calcium Aluminate and Calcium Silicate Dental Cements in Wistar Rats [1]

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Abstract

The aim of the research was to determine possible changes in the morphology of cells of the leukocyte order of peripheral blood, using geometric morphometric tests, after the application of calcium-aluminate and calcium-silicate cements to the dental pulp in rats. The study included 27 Wistar rats, divided into an experimental group (n=18) and a control group (n=9). Trepanation of the tip of the pulp cavity was performed, and placement of calcium-aluminate and calcium-silicate dental cements directly on the pulp. Peripheral blood samples were collected by vena caudalis puncture, with the aim of making blood smears. In the tpsUtil program, two-dimensional models of the examined leukocytes were created and they were converted into tps files, on which sixteen specific points were marked in the tpsDig program. We analyzed their shape in the MorphoJ program. The results of discriminant functional analysis determined that there was a statistically significant difference in the shape of the lymphocytes between the experimental animals, to which dental cements were applied, compared to the lymphocytes from the control group. Morphological differences were determined between the lymphocytes to which calcium aluminate and calcium silicate were applied. The results indicate that there were statistically significant morphological differences between these two groups of lymphocytes (P=0.02). The results obtained indicate the possibly unfavorable influence of the tested dental cements, especially calcium silicate, on leukocytic cells.

Keywords: Biomaterials, Leukocyte cells, Dentin, Rat

Introduction

More detailed research began to be carried out into the potential biomedical application of calcium aluminate and silicate cements at the beginning of the twenty-first century [1]. There are reports that these biomaterials would be a suitable alternative to the existing dental cements, and overcome their evident shortcomings. The failings of commonly used dental cements are generally known, namely their undesirable granular consistency of the fused material, and the prolonged period of attachment [2]. On the other hand, research by Janković et al.[1] produced results clearly illustrating the good tolerance of nanostructured calcium aluminate biomaterials, as well as comparable nanostructured calcium silicate ALBO-CSHA and commercial calcium-silicate cement (MTA). Definitely, calcium-aluminate and silicate cements have been identified as good materials for dentistry, especially for dental procedures that come into contact with the dental pulp. In addition, calcium-silicate and calcium-aluminate cements also cause biomineralization, i.e. deposition of hydroxyapatite (HA), and in this way additionally protect the dental tissue [3]. Moreover, according to Fu et al.[4] calcium aluminate cements have excellent resistance to high temperatures, better mechanical resistance to wear compared to other dental cements, and improved resistance to aggressive environments.

The most commonly used rodents for experimental studies in the application of dental cements are laboratory rats. Adult rats are the animal model of choice for these types of studies due to their extremely strong dentine that closely resembles human dentin [5,6].

Interpretation of white blood cell (WBC) changes provides valuable information to guide human and veterinary doctors in establishing diagnoses for a wide range of diseases. Changes in WBC, in a quantitative sense, as well as, for example, the presence of inclusions and/or degenerative changes within the WBC cytoplasm, determined by microscopic examination of blood corpuscles, are additional indicators when establishing a more precise diagnosis [7,8]. However, successful control and therapeutic success in the treatment of the patient largely depend on identification of the primary, or qualitative state of the observed WBCs.

Geometric analysis greatly helps in this, since the same type of morphometric analysis is performed on a 2- or 3-dimensional plane, and the process of geometric morphometric analysis excludes factors such as position and size, where only shape-related differences are detected [9,10].

In research by Katica et al.[2] calcium-silicate and calcium-aluminate cements were used, and no occurrence of inflammatory processes was determined after analysis of the leukogram and the total WBC count. In that study, all the corpuscles of the leukocytic order were within the reference limits in all experimental groups, without degenerative changes, with the exception of lymphocytes, which had slightly lower values than the reference values.

According to the literature available, that Ajanović et al.[11] analyzed the effects of hyperthermia on WBC in rats using geometric morphometric methods on leukocyte cells.

The aim of the research was to determine possible changes in the morphology of cells of the leukocyte order of peripheral blood, using geometric morphometric tests, after the application of calcium-aluminate and calcium-silicate cements to dental pulp in adult rats.

Material and Methods

Ethics Committee Approval

This study was approved by the Ethics Committee of the University of Sarajevo-Veterinary Faculty under registration number 07-03-535-3/22, Bosnia and Herzegovina.

Animals

In the experiment, 27 adult albino Wistar rats of both sexes were used, aged 70-77 days, with a body weight of 265-280 g. All animals were kept in the same laboratory conditions (accommodated in standard plastic cages with a sawdust mat) and lived according to the standards for adequate environmental air temperature (20 and 23°C), with 60%±10% air humidity, and a 12-h light/darkness schedule. The rats were regularly visited, and had free access to food and water *(ad libitum)* [2].

Testing Materials

The materials used for testing are calcium aluminate systems: a $CaO•Al_2O_3$ + $CaCO_3$ + Bi_2O_3, a mixture called ALBOMCCA obtained by mixing $CaCO_3$ and Bi_2O_3, and BaS04 with calcium aluminate phase in a ratio of 2:2:1. Finally, water was added to the mixture in a 1:2 ratio, to create cement paste. The second material used was calcium silicate (CS): 60% of the total quantity was β-C2S and C3S phase, with added components: 20% calcium carbonate ($CaCO_3$) and 20% $BaSO_4$ (Merck, Germany) [1,12].

General Experimental Procedures and Experimental Groups

In the experiment, 27 adult rats were used, divided into two groups. The first group (control) underwent surgical (dental) procedures, but without the application of dental cements. This group consisted of nine (9) animals.

The second experimental group, to which dental cements, were applied consisted of 18 animals. Ca aluminate

was applied to the dental pulp of nine (9) rats, and Ca silicate was applied to the remaining nine (9) rats of the experimental group.

Surgical (Dental) Procedures

General anesthesia was administered to the rats in the experimental groups (Ketamine HCl, Injection USP) Rotexmedica-German, i.m., 90 mg/kg body weight, for one h. Dental procedures followed, with the use of sterile instruments, including the plates on which dental cement was prepared. The application site on the teeth was relatively dry following use of absorbent cotton and a saliva pump. Cavity preparations on the occlusal surface of the non-carious first and second maxillary molars were made with a technical micromotor and a sterile round diamond ISO 008 drill, with continuous cooling with water. Blood from the pulp was removed with sterile cotton balls, and the cavity was flushed with saline to remove possible blood debris, as well as dentin dust. Trepanation of the apex of the pulp cavum and placement of cement was performed directly on the pulp. Rats from the control group were subjected to surgery in an identical manner, but without any application of dental cements.

Hematological-Histological Procedures

Peripheral blood samples were collected by *v. caudalis* puncture into 3 mL tubes containing ethylene-diaminetetraacetic acid (EDTA) and gel. The injection site was previously disinfected with a standard disinfectant (0.2% chlorhexidine spray).

A drop of blood from each rat from the control and experimental groups was transferred to the slides, and smears were made at an angle of 45 degrees. After air-drying, according to standard laboratory practice, the smears were stained by the Giemsa method [13].

Geometric Morphometric Tests

The research was performed on two-dimensional models of leukocytes from the peripheral blood smear of the experimental rats. The tested leukocytes were divided into two groups: the group of leukocytes from the experimental animals to which the investigated dental cements were applied (Group 2), and the group of leukocytes from the experimental animals to which no dental cement was applied (Group 1).

After detailed microscopic observation of the blood smears of peripheral blood, a binocular microscope (Boeko, Germany) was used, at 1000 times magnification. Two-dimensional models of the visual fields with the most representative leukocytes of the examined samples were then made using Motic Images Plus 2.0 software. In the tested samples, lymphocytes dominated, followed by neutrophils and eosinophils, while the number of basophils and monocytes was not sufficient to meet the statistical criteria for inclusion in the study. Furthermore, an analysis of the shape of the lymphocytes, neutrophils and eosinophils from the peripheral blood smear was performed on two-dimensional models using geometric morphometry.

The two-dimensional models of the examined leukocytes were converted in the tpsUtil program into tps files on which sixteen (16) specific points (landmarks) were marked in the tpsDig program. Eight points were marked on the outside of the tested cell and eight points were marked on the core of the tested cell. Specific points were marked in the same order on all the cells of the tested sample, which was necessary for the correct implementation of geometric morphometry.

After marking the specific points, data on their position in the coordinate system (based on x and y axis values) were used to analyze the morphological differences in the leukocytes between the two examined groups. For the analysis of morphological differences, the MorphoJ program was used, in which the x and y axis values of specific points for each examined cell were entered. After centering, scaling and rotation, generalized Procrustes analysis and principal component analysis were performed.

Statistical Analysis

All statistical analyses were performed using SPSS version 13.0 for Windows (Chicago, IL, USA). The distribution of quantitative variables was tested using the Kolmogorov-Smirnov test. The descriptive statistics results for continuous variables are expressed as mean and standard deviation (SD) for normally distributed variables, or as the median and interquartile range for variables with skewed distributions. Categorical variables are expressed as frequencies and percentages. An independent two-sample Student's t-test assessed the significance of the mean differences between two groups. The Mann-Whitney U-test assessed the differences in parameter values that showed a non-normal distribution. Differences between categorical variables were assessed using the chi-square test. P-values less than 0.05 were considered statistically significant [11].

Results

After centering, scaling and rotation, generalized Procrustes analysis and principal component analysis were performed. *Table 1* shows the results of morphological variability described by principal components (PCs).

Fig. 1 shows the percentage of variability according to the principal components.

The examined leukocyte cells were divided according to their specificities (lymphocytes, neutrophils and

Table 1. *Eigenvalues and percent variability defined by principal components (PCs)*

Principal Components	Eigenvalues	% Variance	Cumulative %
PC1.	0.00617628	39.681	39.681
PC2.	0.00192800	12.387	52.068
PC3.	0.00146929	9.440	61.507
PC4.	0.00139279	8.948	70.456
PC5.	0.00115537	7.423	77.879
PC6.	0.00070528	4.531	82.410
PC7.	0.00068653	4.411	86.821
PC8.	0.00053314	3.425	90.246
PC9.	0.00037407	2.403	92.649
PC10.	0.00035343	2.271	94.920
PC11.	0.00029818	1.916	96.836
PC12.	0.00022685	1.457	98.293
PC13.	0.00015913	1.022	99.315
PC14.	0.00010656	0.685	100.000

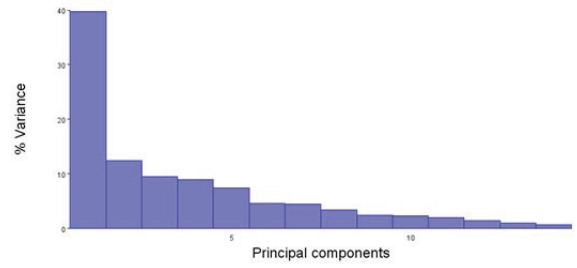

Fig 1. Presentation of the eigenvalues of the principal components and the percentage of variability that they describe (PC1 always describes the highest variability, then PC2 and so on)

eosinophils), and within these groups they were divided into two other groups depending on whether dental cements were applied or not. A discriminant functional analysis was performed comparing the two groups by morphological characteristics.

Fig. 2 shows the position of the leukocytes in the examined groups in the morphological space, defined by the first two principal components (Principal Component 1 and Principal Component 2). The graph clearly shows that there is separation based on morphological characteristics between the control group and the experimental group with dental cements applied.

The results of the discriminant functional analysis show that there was a statistically significant difference in the shape of the lymphocytes between the two experimental groups, i.e. the shape of the lymphocytes of the experimental animals that received dental cements was statistically significantly different from the lymphocytes

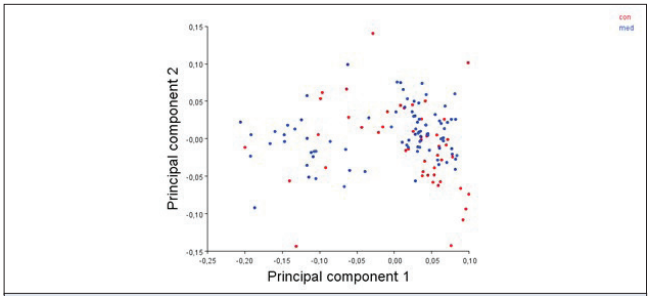

Fig 2. The position of the investigated peripheral blood smear leukocytes in the morphological space defined by the first two principal components (Principal Component 1 and Principal Component 2)

of the control group. The Procrustes distance was 0.036. The Mahalanobis distance value was 1.3179, while the P value (with 1000 permutations) was 0.0135. The T-test value is 0.0110.

Table 2 shows the results of the classification, where it can be seen that, on the basis of the shape, as many as 83.33% of lymphocytes from the group with applied dental cements were correctly classified (Group 2).

Fig. 3 shows the results of the discriminant functional analysis.

Table 2. *Results of the correct classification test based on the morphological characteristics of lymphocytes*

Group	Group 1	Group 2	Total
Group 1	19	16	35
Group 2	9	45	54

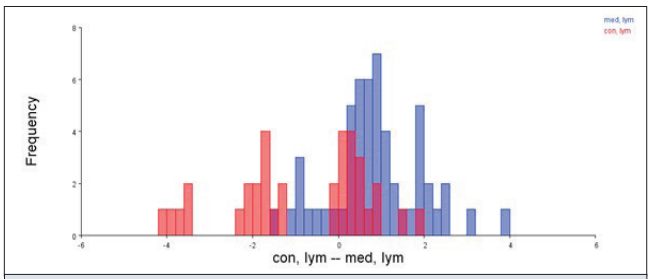

Fig 3. Histogram representation of the results of the discriminant functional analysis of the morphological differences of the lymphocytes of the two examined groups (Blue histograms represent lymphocytes of animals with applied dental cements (med, lym), red histograms represent lymphocytes of animals from the control group (con, lym)

Taking into account that statistically significant differences were found in the shape of lymphocytes between the control group and the group where dental cements were applied, further research was conducted in the direction of examining the morphological changes in the lymphocytes between the control group and the two groups with dental cements, i.e. calcium aluminate and calcium silicate. The results of the discriminant functional analysis of the

morphological differences in lymphocytes between these groups showed that there were statistically significant differences in the shape of the lymphocytes between all these groups.

When comparing the morphological characteristics between the control group of lymphocytes and the lymphocytes where calcium aluminate was applied, the discriminant functional analysis showed statistically significant differences (P=0.03). The results are shown in *Fig. 4.*

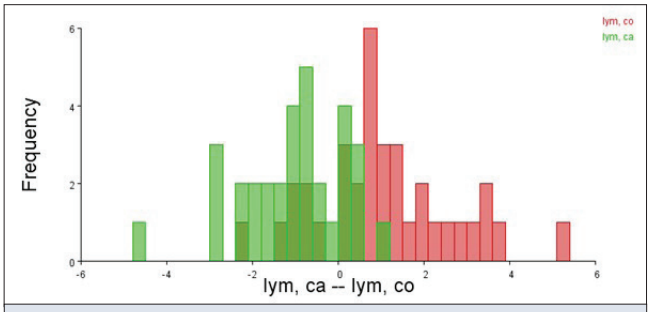

Fig 4. Histogram presentation of the results of the discriminant functional analysis of the morphological differences in the lymphocytes of the two examined groups, the control group of lymphocytes (lym, co) is shown in red, lymphocytes where calcium aluminate was applied (lym, ca) are shown in green

When comparing the morphological characteristics between the control group of lymphocytes and lymphocytes where calcium silicate was applied, the discriminant functional analysis showed statistically significant differences (P=0.030). The results are shown in *Fig. 5*.

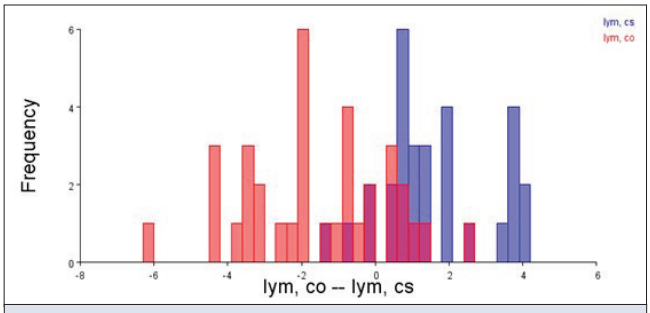

Fig 5. Histogram of the results of the discriminant functional analysis of the morphological differences of the lymphocytes of the two examined groups, the control group of lymphocytes (lym, co) is shown in red, the lymphocytes where calcium silicate was applied (lym, cs) are shown in blue

The discriminant functional analysis examined the morphological differences between lymphocytes when two different types of dental cements, calcium aluminate and calcium silicate, were applied, and the results showed that there are statistically significant morphological differences between these two groups of lymphocytes (P=0.02). The results are shown in *Fig. 6.* and *Fig. 7*.

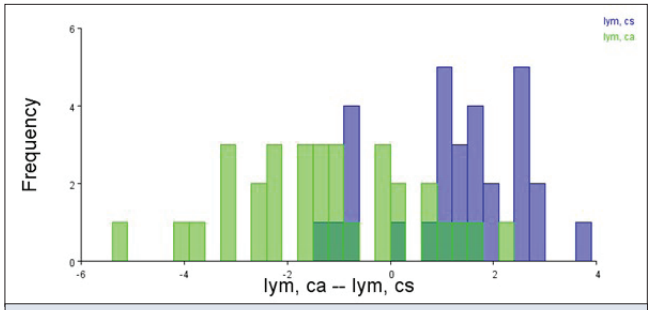

Fig 6. Histogram of the results of the discriminant functional analysis of the morphological differences in the lymphocytes of the two investigated groups, the lymphocyte group where calcium aluminate was applied (lym,ca) is shown in green, the lymphocyte group where calcium silicate (lym, cs) was applied is shown in blue

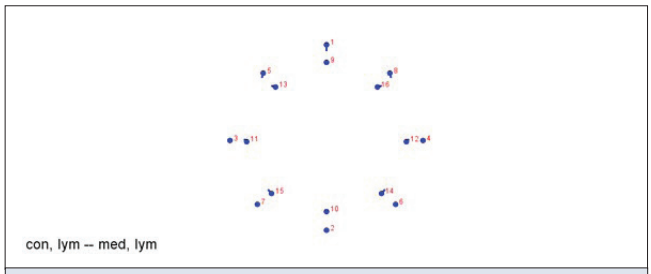

Fig 7. Location of specific points on the tested lymphocytes. The blue dot represents the mean value of the position, the blue line represents the intensity and the direction of change between the control group of lymphocytes (con, lym) and the group where dental cement was applied (med, lym)

No statistically significant morphological differences in neutrophils and eosinophils were found on the two-dimensional models of peripheral blood smears using geometric morphometry between the two investigated groups (the P-value in the analysis of the morphological differences in neutrophils was 0.1635, while the P-value in the analysis of the morphological differences in eosinophils between the two investigated groups was 0.9036.

Discussion

In general, the bioactive materials applied achieve a specific response with the surface of the examined tissue [2].

In addition, according to research by Paraš et al.[14] and Janković et al.[1] the biocompatibility was determined of dental cements based on calcium aluminate and calcium silicate, when observing the potential adverse effects on the liver and subcutaneous tissue of the tested animals. Immunohistochemical studies revealed that nanostructured biomaterials induced hepatocyte proliferation to a certain extent, but this was not clinically relevant and represented a normal and reversible response [14]. Furthermore, the *in vitro* studies by Čolović et al.[15] and Janković et al.[16], determined that calcium aluminate did not show any adverse cytotoxic and genotoxic effects on the MRC-5 diploid cell line of lung fibroblasts.

The research by Janković et al.[17] also confirmed that the newly synthesized Ca aluminate tested did not cause inflammation of the pulp tissue, with a lower number of neutrophils observed, and their number was not statistically significant compared to the control group.

Thus, all these studies indicate that these dental cements did not have unfavorable local effects on different types of tested tissues in *in vivo* and *in vitro* conditions. However, the influence of the tested dental cements on the blood corpuscles of the peripheral blood shows possible adverse effects, especially on the erythrocytes, in the form of mild hypochromic anemia of the normocytic type. A more unfavorable effect was determined in the case of calcium silicate, where pronounced annulocytosis was determined, and moderate stomatocytosis was determined in the tested rodents that had calcium aluminate applied to the dental pulp [2]. The same study Katica et al.[2] determined that, after the application of calcium aluminate and calcium silicate to the dental pulp of the tested rats, the total number of leukocytes remained within the reference intervals of Car et al.[18], but leukocyte values gravitated towards the lower physiological limit. Mild lymphopenia was found on the leukogram [18], which was more pronounced in rats treated with calcium silicate. Neutrophils and other leukocyte cells were within the physiological limits. The exception was monocytes, where a significant difference was observed between the mean values of these cells treated with calcium silicate compared to monocytes from the control group [2].

In our research, using geometric morphometric tests, we obtained representative results in relation to the lymphocytes in the tested rats. These animals, in conditions of physiological balance, had the highest percentage of lymphocytes in their leukograms [19]. The results of our study indicate statistically significant differences in lymphocyte morphology between the control group and the group where dental cements were applied. Accordingly, it may be understood that dental cements have an adverse effect on lymphocytes to a certain extent. In support of this claim, the results from a similar study by Katica et al.[2] indicate the occurrence of lymphopenia in the experimental groups to which calcium aluminate and calcium silicate dental cements were applied. The main risk factors for the development of lymphopenia in both human and veterinary medicine are inadequate nutrition, and in particular, various infections, diseases, and the use of certain medications that contribute to lymphopenia, which above all increases the risk of developing lymphopenia [20].

A less favorable effect in terms of lymphopenia was caused by calcium silicate compared to calcium aluminate [2]. Calcium silicate cement is a well-known material used in dental practice, and is valued for its exceptional physical properties. However, this dental cement needs a long time for binding and washing from the application site, which is not the case with calcium aluminate [16]. Furthermore, according to the same author, Janković et al.[16], nanostructured biomaterial based on calcium aluminate did not show any genotoxic potential on human lung fibroblasts. The percentage of DNA damage at all the applied concentrations was at the level of untreated control fibroblast cells. However, calcium silicate showed a certain amount of genotoxic potential.

In the light of this, the results from our study are interesting, where morphological differences between the lymphocytes were determined through geometrical and morphometric tests where two different types of dental cements, calcium aluminate and calcium silicate, were applied. The results showed that there were statistically significant morphological differences between these two groups of lymphocytes (P=0.02), which indirectly points to the possible unfavorable diversity of the influence of these dental cements after application to the tooth bud, and indicates that our results correspond with similar studies by Katica et al.[2], Janković et al.[16] and Janković et al.[17].

In addition to analyzed lymphocytes, the subject of geometric-morphometric tests in our study were neutrophils, as well as acidophilus. However, no statistically significant morphological differences in neutrophils and eosinophils were found on the two-dimensional models of the peripheral blood smear of the tested rats. As already stated, the number of basophils and monocytes was not sufficient to meet the statistical criteria for inclusion in the study. The reason for this is the usual low percentage values of acidophils, basophils and monocytes in the peripheral blood of rats under physiological conditions [6,11].

Our research is partly limited since we did not classify lymphocytes into B and T lymphocytes. More detailed knowledge within this classification, and in the context of our research goals, would significantly help in a better understanding of the possible adverse impact of dental cements on lymphocytes.

The results obtained from our study unequivocally indicate that the tested dental cements have a somewhat unfavorable effect on leukocyte cells. It is to be assumed that human and veterinary dentists should be careful when applying them to vulnerable populations, especially geriatric and puerperal populations, as well as other immunocompromised patients.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding authors (M.K.) on reasonable request.

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Ethics Committee Approval

This study was approved by the Ethics Committee of the University of Sarajevo-Veterinary Faculty under registration number 07–03-535–3/22, Bosnia and Herzegovina.

Competing Interests: The authors declare that they have no conflicts of interest.

Declaration of Generative Artificial Intelligence (AI): Authors declare that the article and/or tables and figures were not written/created by AI and AI-assisted technologies.

Author Contributions: MK, ZA, AB, OJ, ED and VJ supervised the study. MK, NH, TF, SP and AL collected the data. ZA, AL and AB made the statistics. The first draft of the manuscript was written by MK, ZA, AB, ED, OJ, SP, TF and NH and all authors contributed to the critical revision of the manuscript and have read and approved the final version.

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Research Article

CXCR1 Gene SNP Variability that Affects Mastitis Resistance in Holstein Cows in Türkiye

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Abstract

Genotyping 16 SNPs of C-X-C motif chemokine receptor 1 (CXCR1) gene region which affects host resistance against mastitis disease was carried out in Holstein cows raised in Türkiye. In this study, the frequency of the undesirable C allele in the CXCR1 c.771C>G polymorphic region, associated with an incomplete response, was found to be high. Additionally, the genotypes c.1016AA and c.1016GG, which contribute to mastitis resistance, were observed at low frequencies. Several SNP loci in the CXCR1 gene, including c.606G>A, c.678G>A, c.1104G>A, c.1119+6C, c.1119+7A, and c.1119+10, significantly deviated from Hardy-Weinberg equilibrium (HWE) (P<0.0001), indicating violations of HWE assumptions such as random mating and absence of selection. The deviations at c.606G>A, c.678G>A, and c.1104G>A suggest strong selection pressures, likely due to artificial selection in Holstein cattle. These variants are synonymous mutations that do not alter the amino acid sequence but may influence protein synthesis through effects on mRNA stability, splicing, or translation efficiency. Furthermore, the absence of heterozygotes at loci c.1119+6C, c.1119+7A, and c.1119+10, which are located on untranslated regions (UTRs), potentially affecting gene expression by regulating mRNA stability, localization, or translation initiation, points to genetic drift or population substructure. These findings are important for understanding genetic variability and can inform marker-assisted selection programs to enhance breeding strategies while preserving genetic diversity for traits like disease resistance and milk production.

Keywords: Cattle, PCR, SNP, Mastitis, Resistance

Introduction

In recent years, to increase profitability and meet the growing demand for milk, enhancing daily milk yield has become essential, alongside intensive genetic selection and improved nutrition and management conditions. Achieving this high yield requires the cows to possess a healthy and well-developed mammary structure. However, mammary health is consistently threatened by pathogens attempting to enter the mammary gland through the teat canal. Intramammary infections cause inflammation known as mastitis. The disease can manifest with or without symptoms. The terms clinical mastitis and subclinical mastitis are used for observable symptoms and unobservable symptoms, respectively. Clinical mastitis is characterized by clotting in the milk and swelling, redness, or an overall increase in body temperature in the affected udders [1]. Subclinical mastitis can be identified by the increased somatic cell concentration in the milk obtained from the affected quarter. Both clinical and subclinical mastitis lead to a reduction in milk yield and quality [2]. Additionally, clinical mastitis affects animal welfare due to the pain it causes [3]. Research related to bovine mastitis has been conducted for more than 100 years. Mastitis is a multifaceted disease influenced by pathogens, host, and management factors that affect its prevalence and incidence. Over hundred different pathogens have been isolated from the milk of mastitic cattle. Intramammary infections are primarily caused by bacteria, but algae, yeasts, and fungi can also lead to bovine mastitis. Under similar infection pressure, some cows are affected by mastitis, while others remain healthy. For pathogens to establish an intramammary infection, they

need to penetrate the teat canal and multiply within the mammary gland. In cows with high resistance to mastitis, pathogens are less likely to penetrate the teat canal and are more effectively eliminated by mammary immunity. Management factors affect both the infection pressure and host resistance, leading to low or high mastitis prevalence and incidence [4].

Neutrophils play a crucial role in mammary gland immunity. Genes related to neutrophil function are potential genetic markers for mastitis, as the migration of neutrophils to infection sites via blood is essential for combating mastitis. Neutrophils interact via specific C-X-C motif chemokine receptors (CXCR1 and CXCR2) on their surfaces. The recognition of pathogens by CXCR1 leads to neutrophil activation and ultimately the elimination of the pathogen [5]. CXCR1 is becoming more well known for its role as a cancer stem cell identifier and therapeutic target [6].

Inflammatory cytokines and the genes that control them have been given great importance in expanded research to combat mastitis. The chemokine receptor Interleukin-8 receptor α (IL-8RA), which is found on neutrophil cells and binds with high affinity to pro-inflammatory IL-8, is encoded by the CXCR1 gene. IL8RA plays an important role in the control of mastitis by strengthening immunity and counteracting the immunosuppressive effect of *S. aureus* mastitis [7]. However mRNA transcription levels of five genes, including CXCR1, were significantly higher in the *E. coli*-induced mastitis group than in the *S. aureus*-induced group, therefor *E. coli* often causes acute mastitis, while *S. aureus* causes chronic mastitis [8]. Bacteria activate CXCR1, which regulates NF-κB signaling after interaction with TLR4. NF-κB binds to DNA and induces expression of the CXCR1 gene [9]. In addition, the interaction of IL-8 with CXCR1 or CXCR2 genes causes some changes that lead to chemotaxis of neutrophils against infection in the mammary gland and increase cell survival, migration and phagocytosis activity [10]. During predicting mastitis resistance in buffalo CXCR2 was identified as a potential gene [11].

The importance of the CXCR1 gene in this context has been highlighted in several studies suggesting that single nucleotide polymorphisms (SNPs) within this gene can affect an animal's susceptibility to mastitis. The ability to monitor and understand these SNPs may provide critical information for improving mastitis resistance in Holstein cattle [5]. The relationships of CXCR1 gene polymorphisms with clinical mastitis, reproductive disorders and performance characteristics in Hardhenu cattle were investigated, and it was reported CXCR1 gene could be associated with both increased mastitis susceptibility and high milk production [12]. Beyond CXCR mechanism other genes are also effect host resistnacy, Aksel et al.[13] reported that two SNP loci in mannose-binding lectin (MBL1) gene may lead a role in subclinic mastitis in Holstein cattle. However CXCR1 could be the highest priority gene for host resistance in mastitis among all candidate genes [14].

Holstein cattle are the most widely used breed in dairy production due to their high milk yield; however, this very characteristic also makes them more prone to diseases such as mastitis. The identification of genetic markers, such as SNPs within immune-related genes like CXCR1, has become a valuable tool in the field of animal breeding for disease resistance [15]. In particular, certain SNPs in the CXCR1 gene have been associated with variations in neutrophil function and immune response, which may influence the severity of mastitis infections. Some studies have reported a strong association between milk SCC and CXCR1 mutations in dairy cattle. Exploring the relationship between these SNPs and mastitis resistance could offer potential for selective breeding programs aimed at enhancing disease resistance without compromising milk production [15-17].

This study aims to investigate the key SNPs in the CXCR1 gene in Holstein cattle, with a focus on their association with mastitis resistance. By following the presence and frequency of these SNPs in the population, this study seeks to contribute to the growing body of knowledge on genetic markers for mastitis resistance, providing a foundation for more effective breeding strategies that combine high milk yield with enhanced disease resilience.

Material and Methods

Ethical Statement

All experimental procedures were applied in this study received approval from the the Istanbul University-Cerrahpaşa, Animal Experiments Local Ethics Committee: 2018/118480. Additionally, informed consent forms were obtained from the owners

Sampling and DNA isolation

In this study, a total of 240 blood samples from 24 different farms located within the boundaries of Kırklareli province were collected from Holstein cows using a sterile double-ended cannula from the caudal vena of the animals into vacuum sterile EDTA blood tubes. The ear tag numbers of the animals were recorded on the blood tubes. The collected blood samples were transported in insulated bags with ice packs and were kept at 4°C until they reached the laboratory within 2-3 days. Each blood sample in the EDTA vacuum tubes was aliquoted into two 1.5 mL sterile Eppendorf tubes, approximately 600 µL each, with one being a backup. The blood samples were stored at -20°C in sealed storage racks. DNA isolation from the blood samples was performed using Bioneer Exiprep 16 Plus

Genomic DNA innovation robot (Bioneer Corporation, Korea). To visualize the genomic DNA samples obtained from whole blood 0.8% agarose gel was used. For this purpose, 100 mL of TBE Buffer was measured, and 0.8 mg of agarose was weighed, dissolved with the help of a microwave and then 3 µL of Red Safe dye (iNtRON) was added. The isolated DNA samples were mixed with 6x loading dye and loaded into the gel wells. The agarose gel electrophoresis system was run at 100 volts for 35 min, allowing the genomic DNA samples to migrate within the gel.

Amplification of the CXCR1 Gene

The PCR process was conducted with using AccuPower PCR PreMix (Catalog No.pcr K-2012, Bioneer, Korea) which is a lyophilized (Taq DNA polymerase 1U, each: dNTP -dATP, dCTP, dGTP, dTTP- 250 µM Tris-HCl pH 9.0 10 mM, KCl 30 mM, $MgCl_2$ 1.5 mM, stabilizer and tracking dye) mixture ready-to-use in 0.2 mL attached 8-tubes. The PCR mixture content (20 µL) was an adequate volume of sterile bidistilled water (15 µL), 50 ng of DNA (3 µL), and 0.25 µM of each primer pair (0.5 µL each) were added. Amplification of CXCR1 gene was performed with TCCTTGATGAGAGTGATTTGGA and TTGACATGGGACTGTGAACG primer pairs under the conditions of: denaturing at 95°C for 5 min, and 35 cycles of 95°C for 30 sec, 62°C for 30 sec, 72°C for 75 sec, and final extension at 72°C for 4 min [5]. Following the amplification of the CXCR1 gene region, 1% agarose gel was used to visualize the PCR products with 1 µg of 1 kb ladder (NEB) under UV imaging system. The agarose gel electrophoresis system run at 120 volts for 30 min. PCR products bands associated with the products to be made visible using UV tools of E-Box imaging system (Vilber Lourmat).

Detection of SNPs by Sequence Analyses

In order to analyse SNP regions within the amplified PCR products were sent to Genoks Company for sequencing (ABI 3130xl DNA Analyser; Thermofisher; USA). The sequences were analyzed and aligned using CLC Genomics Workbench 10.1. 1 (QIAGEN, Hilden, Germany). Sequencing data were manually inspected and edited, and consensus sequences were generated from both forward and reverse reads. The aligned sequences were screened for SNPs by comparison with the reference genome.

Once the variants were identified, they were further analyzed using the Ensembl genome browser *(https://www.ensembl.org)*. This tool was used to annotate the SNPs and predict their potential impact on gene function.

Statistical Analyses

Genetic variation in SNP regions terms of the minor allele frequency, observed (Ho) and expected (He) heterozygosity and the probability of a deviation from Hardy-Weinberg equilibrium (HWE) were calculated using PopGene ver. 1.32 [18] program.

Results

CXCR1 gene was obtained as 1083 bp with PCR process and visualised with 1% agarose gel electrophoresis *(Fig. 1)*.

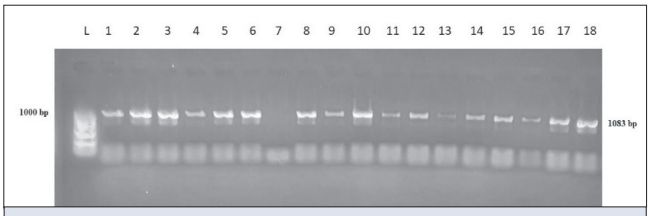

Fig 1. Visualization of PCR products of CXCR1 gene on 1% agarose gel electrophoresis in lines 1-6 and 8-18 (1083bp), line 7 is negative control. L: 1kb ladder

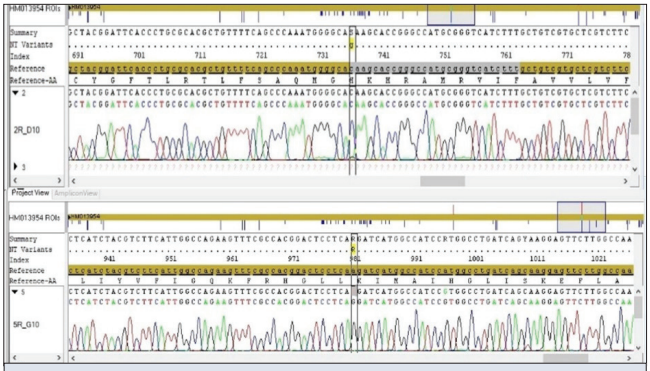

Fig 2. Two SNP loci data obtained with Sanger sequence and alligned in order to find variations of CXCR1 gene in Holstein cattle of Türkiye

Through bioinformatic mining, 16 SNPs identified in CXCR1 gene *(Fig. 2)*. The genotypic and allelic distributions of 16 SNP loci in the CXCR1 gene were evaluated in the Holstein cow population raised in Türkiye *(Table 1)*.

The frequency of the undesirable C allele in c.771C>G, associated with an incomplete immune response, was moderate (C = 0.4630), while the G allele was more prevalent (G = 0.5370). For c.1016A>G, the A allele, which contributes to mastitis resistance, was at low frequency (A = 0.1761), whereas the G allele was predominant (G = 0.8239). The loci c.606G>A (P<0.05), c.678G>A (P<0.05), c.1104G>A (P<0.05), c.1119+6C>A, c.1119+7A>C, c.1119+10A>C, and c.1119+10C>T exhibited significant deviations from HWE, indicating selection or non-random mating. In contrast, other loci, such as c.318C>T, c.369T>C, c.771C>G, c.852C>A, and c.855G>A, were in HWE (P>0.05).

Loci c.1119+6C>A, c.1119+7A>C, and c.1119+10A>C showed complete absence of heterozygotes. For c.606G>A, c.678G>A, and c.1104G>A, the observed heterozygosity was lower than expected.

Table 1. *The genotypic and allelic distributions of 16 SNP loci in the CXCR1 gene in the Holstein cow population raised in Türkiye*

SNP Locus	Variant Type	Variant Code	Alleles	Allele Frequency	Genotypes	Obs_ Gen	Obs_ Hom	Obs_Het	Exp_ Hom^{+}	Exp_ Het^{+}	Nei^{++}	Ave_ Het	HWE
c.318C>T	Missense	rs208795699	C	0.9978	CC	229	0.9957	0.0043	0.9957	0.0043	0.0043	0.0043	NS
			T	0.0022	CT	1							
					TT	0							
c.369T>C	Synonymous	rs209401160	T	0.9978	TT	229	0.9957	0.0043	0.9957	0.0043	0.0043	0.0043	NS
			C	0.0022	TC	1							
					CC	0							
c.606G>A	Synonymous	rs137262611	G	0.8478	GG	180	0.8696	0.1304	0.7414	0.2586	0.2580	0.2580	*
			A	0.1522	GA	30							
					AA	20							
c.678G>A	Synonymous	rs109587131	G	0.8652	GG	183	0.8609	0.1391	0.7663	0.2337	0.2332	0.2332	*
			A	0.1348	GA	32							
					AA	15							
c.771C>G	Missense	rs43323012	C	0.4630	CC	51	0.5174	0.4826	0.5016	0.4984	0.4973	0.4973	NS
			G	0.5370	CG	111							
					GG	68							
c.852C>A	Synonymous	rs210501501	C	0.7935	CC	147	0.6913	0.3087	0.6715	0.3285	0.3277	0.3277	NS
			A	0.2065	CA	71							
					AA	12							
c.855G>A	Synonymous	rs134348371	G	0.4717	GG	56	0.5435	0.4565	0.5005	0.4995	0.4984	0.4984	NS
			A	0.5283	GA	105							
					AA	69							
c.1016A>G	Synonymous	rs134348371	A	0.1761	AA	3	0.6739	0.3261	0.7092	0.2908	0.2902	0.2902	NS
			G	0.8239	AG	75							
					GG	52							
c.1031A>G	Missense	rs43323013	A	0.2391	AA	18	0.6783	0.3217	0.6353	0.3647	0.3639	0.3639	NS
			G	0.7609	AG	74							
					GG	138							
1044C>T	Missense / Synonymous	rs43323014	C	0.7761	CC	143	0.6913	0.3087	0.6517	0.3483	0.3476	0.3476	NS
			T	0.2239	CT	71							
					TT	16							
c.1054T>C	Synonymous	rs722933381	T	0.9978	TT	229	0.9957	0.0043	0.9957	0.0043	0.0043	0.0043	NS
			C	0,0022	TC	1							
					CC	0							
c.1104G>A	Synonymous	rs132989373	G	0.6826	GG	129	0.7565	0.2435	0.5657	0.4343	0.4333	0.4333	*
			A	0.3174	GA	56							
					AA	45							
c.1119+6C>A	-	rs446949386	C	0.8522	CC	196	1.0000	0.0000	0.7475	0.2525	0.2519	0.2519	*
			A	0.1478	CA	0							
					AA	34							
c.1119+7A>C	-	rs446949386	A	0.8565	AA	197	1.0000	0.0000	0.7537	0.2463	0.2458	0.2458	*
			C	0.1435	AC	0							
					CC	33							

Table 1. *The genotypic and allelic distributions of 16 SNP loci in the CXCR1 gene in the Holstein cow population raised in Türkiye (continued)*

SNP Locus	Variant Type	Variant Code	Alleles	Allele Frequency	Genotypes	Obs_ Gen	Obs_ Hom	Obs_Het	Exp_ Hom⁺	Exp_ Het⁺	Nei⁺⁺	Ave_ Het	HWE
c.1119+10A>C	-	rs452865404	A	0.9957	AA	229	1.0000	0.0000	0.9913	0.0087	0.0087	0.0087	*
			C	0.0043	AC	0							
					CC	1							
c.1119+10C>T	-	rs452865404	C	0.8674	CC	199	0.9957	0.0043	0.7695	0.2305	0.2300	0.2300	*
			T	0.1326	CT	1							
					TT	30							
						Mean	0.8291	0.1709	0.7495	0.2505	0.2499	0.2499	
						St.Dev.	0.1770	0.1770	0.1690	0.1690	0.1687	0.1687	

⁺ *Expected homozygosty and heterozygosity were computed using Levene (1949),* ⁺⁺ *Nei's (1973) expected heterozygosity, NS: non-significant, *P<0.001*

Discussion

This study analyzed genetic variation in the CXCR1 gene in Holstein cattle raised in Türkiye, focusing on the association of 16 SNP loci with mastitis resistance. The findings obtained from this study were found to be consistent with previous studies. Loci c.606G>A, c.678G>A and c.1104G>A ($p < 0.05$), which had significant deviations from the Hardy-Weinberg equilibrium (HWE), indicate that they probably experienced strong selection pressures. The absence of heterozygosity at c.1119+6C>A, c.1119+7A>C and c.1119+10C>T loci may suggest genetic drift or the presence of population substructure. A higher prevalence of the G allele (0.5370) at c.771C>G locus, may indicate a selection advantage for this allele in the population, as it is potentially associated with improved immune response efficiency [16]. Besides that, the frequency of the C allele (0.4630) which has been associated with a deficiency in neutrophil response and is therefore less desirable [5], is concerning. On the contrary, it is also thought-provoking that the A allele in the c.1016A>G locus which is defined to have a protective effect against mastitis [16,19], is found at low frequency (0.1761) in the relevant population.

Previous studies have emphasized the need to increase the frequency of resistance alleles through breeding programs to increase host resistance to combat mastitis in dairy cattle [20]. Nei's gene diversity (0.2499) and mean heterozygosity across all loci (0.2505) indicate moderate genetic variability. In order to increase mastitis resistance in Holstein cattle, it is important to adopt marker-assisted selection (MAS) programs to reduce the frequency of the C allele in c.771C>G and increase the prevalence of advantageous genotypes such as c.1016AA and c.1016GG. These findings, together with previous studies, highlight the impact of CXCR1 SNP variations on disease resistance and the essential role of genetic diversity in improving herd health and productivity.

In this study, it was found that c.606G>A, c.678G>A, c.1104G>A, c.1119+6C, c.1119+7A and c.1119+10 loci in the Holstein cattle population raised in Türkiye deviated significantly from Hardy-Weinberg equilibrium (HWE) ($P<0.0001$). HWE based on the principles of random mating, the absence of mutation, migration or selection, and a sufficient population size, helps maintain constant allele and genotype frequencies across generations. Significant deviations from HWE of c.606G>A, c.678G>A ve c.1104G>A ($P<0,0001$) loci and the complete absence of heterozygosity at the c.1119+6C, c.1119+7A and c.1119+10 ($P<0.0001$) loci indicate significant population substructure or genetic drift. Genetic diversity may be reduced due to small effective population sizes or controlled matings [21]. Understanding the genetic diversity and evolutionary forces that influence traits such as disease resistance and milk production heavily relies on deviations from HWE. For marker-assisted selection (MAS) programs we gain valuable insights by identifying loci that are under selection. MAS can improve breeding strategies for economically important traits and also maintain the genetic diversity essential for the long-term health of the population.

There are synonymous mutations in c.606G>A, c.678G>A and c.1104G>A that do not affect the amino acid sequence but cause specific changes in the coding region. These variations do not cause any changes in protein structure, but may affect protein synthesis through changes in mRNA stability, splicing, translation efficiency, protein folding and quantity. On the other hand, c.1119+6C, c.1119+7A and c.1119+10 mutations are located in the untranslated regions and can regulate gene expression by affecting mRNA stability, localization or translation initiation, thus not changing the protein structure but may affect the amount of protein produced. Therefore, while protein structure is unaffected by synonymous mutations, its expression and regulation may be influenced.

This study examined 16 SNP loci in the CXCR1 gene of Holstein cattle in Türkiye, considering their relationship with mastitis resistance. The moderate occurrence of the undesirable C allele at c.771C>G and the low presence of the protective A allele at c.1016A>G, as well as the absence of heterozygotes at certain loci, highlight the effects of population substructure and genetic drift in this managed breeding program. These results provide important information about the genetic structure of the CXCR1 gene in Holstein cattle raised in Türkiye and the potential role of selective breeding strategies in increasing disease resistance.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author (K. Avanus) on reasonable request.

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Author Contributions: KA, AY, AA, HG: design of the study, AY, BE, HY, DK, NÖ: determine the farms, BE, HY, DA, NÖ: funding acquisition, KA, AY: field work and sample collection, KA: laboratory analyze, KA, HG, AA: statistical analyze, KA, AY, HG, AA: writing the manuscript. All authors have read and agreed to the published version of the manuscript.

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Research Article

Antimicrobial Susceptibility Patterns of *Bacillus anthracis* Isolates Obtained from Different Origins

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Abstract

In this retrospective study, it was evaluated the antimicrobial susceptibility profiles of *Bacillus anthracis* isolates obtained from human, various samples (blood, spleen, lung, liver, meat) of dead animals (cattle, sheep, dog, horse) that died from anthrax and soil samples from the animal burial areas between 2012 and 2023 from Kars province of Türkiye. In this context, a total of 87 *B. anthracis* isolates obtained from 1 human, two horses, 51 cattle, 7 sheep, 1 dog, and 25 soil were investigated. The isolates were confirmed as *B. anthracis* using protective antigen *(PA)* and capsule *(Cap)* gen specific PCRs. The Kirby-Bauer disk diffusion method was used for deternination of antimicrobial susceptibility. Ten antimicrobials including penicillin, amoxicillin, trimethoprim-sulfometoxazole, erythromycin, meropenem, streptomycin, ofloxacin, ciprofloxacin, chloramphenicol, and clindamycin were tested. As a result of PCR, all isolates were confirmed as fully virulent field strains of *B. anthracis*. All isolates were found as susceptible to penicillin, amoxicillin, ofloxacin and ciprofloxacin. Since the last studies in the region, a change in the antimicrobial profile of *B. anthracis* strains was observed only for trimethoprim-sulfomethoxazole among the antimicrobials tested, a transition from susceptibility to resistance. In conclusion, penicillin and amoxicillin, are still the antibiotic of first choice for the prophylaxis and treatment of anthrax. Ofloxacin and ciprofloxacin are also effective enough to be prescribed for treatment.

Keywords: Animal, Antimicrobial susceptibility, *Bacillus anthracis,* Human, PCR, Soil

Introduction

Anthrax is a sporadic infection of many warm-blooded animals (camels, horses, cats and dogs, etc.) in particular of herbivores (cattle and sheep) [1]. Although this disease, which can occasionally be transmitted from infected animals to humans by direct or indirect routes, is becoming less common in the world and our country, it continues its zoonotic existence and continues to be a global threat associated with bioterrorism as a biological weapon in both developed and developing countries [2].

The agent of anthrax is *Bacillus anthracis*. The agent is a pathogen that is Gram-positive, immobile, encapsulated, spore forming, and can grow as aerobic or facultative anaerobic [3,4]. Spores of *B. anthracis* are highly resistant to unfavourable environmental conditions. Spores remain viable for many years in contaminated environments and constitute an important source of infection in grazing animals for long term [5]. The expression of the pathogenic activity of *B. anthracis* in animals is mediated by the capsule localised on the pXO2 plasmid, which confers antiphagocytic properties, and a complex of three toxic proteins: protective antigen (PA), edema factor (EF), and lethal factor (LF) localised on the pXO1 plasmid [6,7].

In regions where anthrax is endemic, contact with infected

livestock or animal products during domestic slaughter, disposal of butchery waste, processing and disposal of cattle carcasses are the main sources of human infections; these are the primary factors that put livestock keepers and farmers at high risk of infection [8,9]. The severity of disease in humans depends on the patient's natural and adaptive immunity, the virulence of the agent and the number of times it enters the body [10]. Anthrax occurs in humans in different forms such as cutaneous, injection, gastrointestinal or inhalation, and cutaneous anthrax accounts for approximately 95% of infections [3]. Sepsis and meningoencephalitis are rare complications resulting from the spread of primary lesions [3,11]. The incidence of other infection types has been reported for inhalation anthrax, gastrointestinal anthrax and primary meningitis as 12% 5% 4%, respectively [12,13].

In the treatment of anthrax, the use of antibiotics is vital in line with the diagnosis [11,14,15]. The range of antibiotics to which *B. anthracis* is susceptible *in vitro* is quite wide and clinical isolates are sensitive to various antibiotics such as penicillin, carbapenems, aminoglycosides, macrolides, quinolones, vancomycin, rifampicin, tetracyclines, clindamycin, cefazolin, and linezolid [9,11]. The region and the severity of the disease influence the application of different antibiotic treatment strategies in anthrax [9,16]. Therefore, the first-line drugs for naturally occurring cases of anthrax are penicillin G and amoxicillin. Especially in the treatment scheme for uncomplicated and mild cutaneous and complicated cutaneous and systemic cases of anthrax, ciprofloxacin and doxycycline are alternative agents [17]. In severe cases, the initial choice of antibiotic must be combined with one or two of the antibiotics such as penicillin, imipenem, ampicillin, meropenem, ciprofloxacin, rifampicin, clindamycin, aminoglycoside, linezolid or vancomycin [18].

Bacillus anthracis is resistant to late-stage cephalosporins such as cefoxime, cefotaxime, ceftazidime, aztreonam and trimethoprim-sulfomethoxazole [14]. In general, genes encoding acquired antibiotic resistance are found on mobile genetic elements such as transposons or plasmids. Through horizontal gene transfer, these elements can lead to the emergence of antibiotic resistance between Bacillus and other clinical pathogens [19]. It is a critical process to start treatment before *B. anthracis* begins to release toxins into the bloodstream. The use of a beta-lactam antibiotic such as penicillin is recommended by the Centers for Disease Control and Prevention (CDC) and the World Health Organisation (WHO) [20]. In developing countries where anthrax is endemic, penicillin drug is recognised as first choice for treatment due to its efficacy, widespread availability and low cost [21].

In this retrospective study, it was aimed to evaluate the antibiotic susceptibility, of *B. anthracis* isolates obtained from one human, various samples (blood, spleen, lung, liver, meat) of animals (cattle, sheep, dog, horse) that died from anthrax and soil samples taken from the animal burial areas between 2012 and 2023 from Kars province of Türkiye.

Material and Methods

Ethical Statement

This study vas approved by the Kafkas University Animal Experiments Local Ethics Committee (Approval no: KAÜ-HADYEK/2023/15).

B. anthracis Isolates

A total of 87 *B. anthracis* isolates obtained from 1 human, various samples (blood, spleen, lung, liver, meat) of 61 animals (51 cattle, 7 sheep, 1 dog, and 2 horses) that died from anthrax and soil samples (25) taken from the animal burial areas brought to the Department of Microbiology, Faculty of Veterinary Medicine, Kafkas University between 2012 and 2023 from Kars province, Türkiye, were used.

Isolation of *B. anthracis*

For isolation of *B. anthracis* from animal and human samples, 5% sheep blood agar was used. Medusa head-like and non haemolytic colonies grown after 24 h of incubation in aerobic coditions at 37ºC were evaluated. PLET Agar (Polymyxin B - Lysozyme - EDTA - Thallous acetate Agar) (Milipore, 55678) with Anthracis-Selective-Supplement (Milipore, 72659) was used for isolation of the agent from environmental samples. Petri dishes were incubated at 37°C for 36-40 h. The colonies that were circular, creamy-white with a ground glass texture were evaluated. Subcultured from this medium to blood agar was performed to confirm the isolates.

Phenotypical Identification

Bacillus anthracis suspected isolates were identified using the classical microbiological methods such as microscopic and macroscopic morphology, motility, and penicillin (10 U, Oxoid, UK) and gamma phage susceptibility [22].

Molecular Identification

DNA Extraction: A commercial extraction kit (56304, Qiagen, Germany) was used for the genomic DNA extraction of the *B. anthracis* isolates according to the manufacturer's instructions.

PCR Analysis: PCR targeting the amplification of capsule *(Cap)* and protective antigen *(PA)* genes was used for molecular identification of the *B. anthracis* isolates [22]. Amplification was performed using primers Cap6-5'-TACTGACGAGCAACCGA-3' and Cap103-5'-GGCTCAGTGTAACTCCTAAT-3', PA5-5'-GAGGTAG AAGGATACGGT-3' and PA8-5'-TCCTAACACTAACGA

AGTCG-3'. The PCR mixture was adjusted as 50 µL reaction volume including 25 µL Taq PCR Master Mix Kit (Qiagen, UK), 15 µL distilled water, 5 µL primer mix, and 5 µL template DNA. Thermal cycle conditions were as 30 cycles of initial denaturation at 94°C for 5 min, followed by denaturation at 94°C for 40 s, primer binding at 58°C for 40 s, elongation at 72°C for 40 s and final elongation at 72°C for 5 min. Amplified products were analysed by 1.5% gel electrophoresis (1640300, Bio-Rad, USA). Products of 1035 bp and 596 bp were considered positive for *Cap* and *PA* genes, respectively. *B. anthracis* Sterne strain lack of capsule was used as reference strain.

Antimicrobial Susceptibility Test

For determination the *in vitro* antimicrobial susceptibility of the *B. anthracis* isolates, the Kirby-Bauer disk diffusion method was applied [23]. Ten antimicrobials from 8 different groups including beta-lactams (penicillin [Oxoid, 10 U], amoxicillin [Oxoid, 25 µg]), sulfonamide (trimethoprim-sulfomethoxazole [Bioanalyse, 25 µg]), macrolide (erythromycin [Oxoid, 15 µg]), carbapenem (meropenem [Bioanalyse, 10 µg]), aminoglycoside (streptomycin [Oxoid, 10 µg]), fluoroquinolone (ofloxacin [Oxoid, 5 µg], ciprofloxacin [Oxoid, 5 µg]), fenicol (chloramphenicol [Oxoid, 30 µg]), lincosamide (clindamycin [Oxoid, 2 µg]) were used.

Bacterial inoculum was prepared from colonies of fresh *B. anthracis* cultures on 5% sheep blood agar in 0.9% physiological saline. Turbidity of the inoculum was adjusted to the 0.5 McFarland standard (approximately 10^8 cfu.mL^{-1}). 0.1 ml of bacterial inoculum was spread on Mueller Hinton agar and let to dry for 10 min. Then, antimicrobial discs were placed on the inoculated agar plates. The inhibition zone diameters formed after 24 h of incubation at 37ºC in air were evaluated. Inhibition zone was measured in milimeter using a ruler. Evaluations were made according to the standards of the Clinical and Laboratory Standards Institute (CLSI) [24], and the European Antibiotic Susceptibility Testing Committee (EUCAST) [25]. Since the inbition zone of *B. anthracis* in disk diffusion method has no been determined by CLSI, diameter of the Staphyloccal inhibition zone was used to interpretation. Since the breakpoints in EUCAST are given excluding *B. anthracis*, the evaluations were made using the breakpoints given for *Staphylococcus* spp. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as control strains for the purpose of test reliability.

Statistical Analysis

The Pearson Chi Square test, one of the nonparametric tests, was used to evaluate the changes in the antibiotic susceptibility of *B. anthracis* strains according to sample origin and years.

Results

Phenotypic Identification Findings

All isolates were seen as encapsulated, large, square ended rods in tissue preparations, medusa head-like and non haemolytic colonies in 5% sheep blood agar, Typical circular, creamy-white with a ground glass texture colonies grown on Plet agar and Gram-positive bacterial cells with hair thread morphology in culture preparations. All of them were non-motile, susceptible to penicillin and gamma phage and showed mucoid and encapsulated colonies on bicarbonate agar. All of the isolates in this study were classified as *B. anthracis* according to the mentioned clasical phenotypic tests.

Molecular Identification Findings

As a result of *Cap* and *PA* specific PCR performed for the confirmation of the *B. anthracis* isolates, it was determined that all of them were virulent *B. anthracis* with the presence of fragments of 1035 and 596 bp for *Cap* and *PA* genes, respectively. *B. anthracis* Sterne showed fragment of 596 bp because of has not capsule gene *(Cap -, PA +) (Fig. 1)*.

Antimicrobial Susceptibility Findings

As a result of the disk diffusion method, all (100%) *B. anthracis* isolates were sensitive to penicillin, amoxicillin, ciprofloxacin and ofloxacin, whereas the sensitivity rate of the isolates to chloramphenicol, erythromycin, clindamycin, streptomycin, meropenem was found 95.4%,

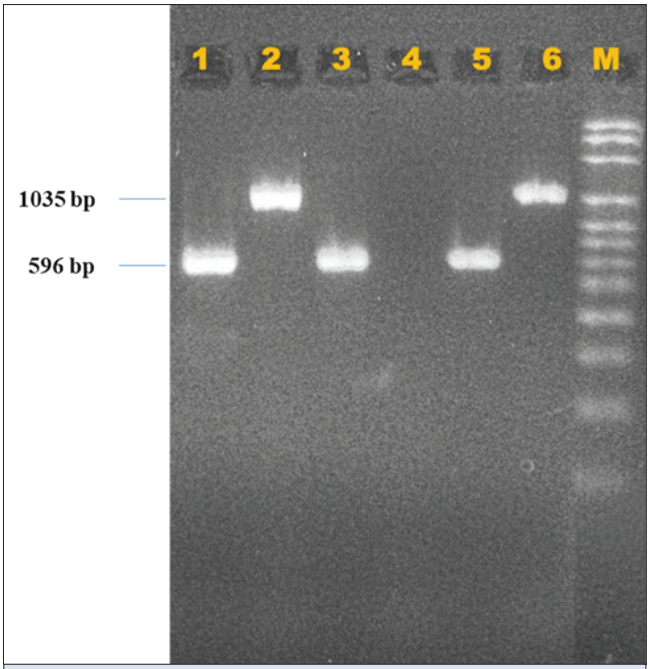

Fig 1. *PA* 5/8 (596 bp) and *Cap* 6/103 (1035 bp) PCR for *B. anthracis* confirmation. M: HyperLadder 100 bp Plus (Bioline); 1, 2, 5, and 6: *B. anthracis* field isolates; 3 and 4: *B. anthracis* Sterne strain

Table 1. *Antibiotic susceptibilities of 87 Bacillus anthracis strains isolated between 2012-2023*

Year	Origin	Antimicrobial Agents																														P Value
		P			AML			SXT			E			MEM			S			OFX			CIP			C			DA			
		S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	
2012	Cattle	12	-	-	12	-	-	-	2	10	12	-	-	5	7	-	11	1	-	12	-	-	12	-	-	11	-	1	9	3	-	0.000
	Sheep	1	-	-	1	-	-	-	-	1	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	0.000
	Soil	8	-	-	8	-	-	-	-	8	8	-	-	3	5	-	8	-	-	8	-	-	8	-	-	7	1	-	5	3	-	0.000
2013	Cattle	12	-	-	12	-	-	-	2	10	10	1	1	4	8	-	8	4	-	12	-	-	12	-	-	12	-	-	11	1	-	0.000
	Sheep	1	-	-	1	-	-	-	-	1	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	0.000
	Dog	1	-	-	1	-	-	-	-	1	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	0.000
	Human	1	-	-	1	-	-	-	-	1	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	0.000
	Soil	9	-	-	9	-	-	-	-	9	9	-	-	4	5	-	8	1	-	9	-	-	9	-	-	8	1	-	7	2	-	0.000
2014	Cattle	9	-	-	9	-	-	-	1	8	8	1	-	5	4	-	9	-	-	9	-	-	9	-	-	8	1	-	8	1	-	0.000
	Soil	8	-	-	8	-	-	-	1	7	6	2	-	2	6	-	6	2	-	8	-	-	8	-	-	8	-	-	6	2	-	0.000
2015	Cattle	4	-	-	4	-	-	-	-	4	4	-	-	1	3	-	3	1	-	4	-	-	4	-	-	4	-	-	4	-	-	0.000
	Sheep	1	-	-	1	-	-	-	-	1	1	-	-	-	1	-	-	1	-	1	-	-	1	-	-	1	-	-	1	-	-	0.000
2016	Cattle	2	-	-	2	-	-	-	-	2	2	-	-	-	2	-	1	1	-	2	-	-	2	-	-	2	-	-	2	-	-	0.000
2018	Cattle	3	-	-	3	-	-	-	-	3	3	-	-	-	3	-	3	-	-	3	-	-	3	-	-	3	-	-	3	-	-	0.000
	Sheep	2	-	-	2	-	-	-	-	2	2	-	-	-	2	-	1	1	-	2	-	-	2	-	-	2	-	-	2	-	-	0.000
	Horse	2	-	-	2	-	-	-	-	2	1	-	1	2	-	-	2	-	-	2	-	-	2	-	-	2	-	-	2	-	-	0.000
2019	Cattle	4	-	-	4	-	-	-	-	4	3	1	-	-	4	-	3	1	-	4	-	-	4	-	-	4	-	-	4	-	-	0.000
	Sheep	1	-	-	1	-	-	-	-	1	1	-	-	-	1	-	-	1	-	1	-	-	1	-	-	1	-	-	1	-	-	0.000
2020	Cattle	2	-	-	2	-	-	-	-	2	2	-	-	-	2	-	1	1	-	2	-	-	2	-	-	2	-	-	2	-	-	0.000
2021	Cattle	1	-	-	1	-	-	-	-	1	1	-	-	-	-	1	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	0.000
2022	Cattle	2	-	-	2	-	-	-	-	2	1	-	1	2	-	-	1	1	-	2	-	-	2	-	-	2	-	-	2	-	-	0.000
2023	Sheep	1	-	-	1	-	-	-	-	1	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	1	-	-	0.000
Total (%)		**87 (100)**	-	-	**87 (100)**	-	-	-	**6 (6.9)**	**81 (93.1)**	**79 (91)**	**5 (6)**	**3 (3)**	**33 (38)**	**53 (61)**	**1 (1)**	**71 (82)**	**16 (18)**	-	**87 (100)**	-	-	**87 (100)**	-	-	**83 (95.4)**	**3 (3.4)**	**1 (1.1)**	**75 (86)**	**12 (13.8)**	-	

P: Penicillin; AML: Amoxicillin, SXT: Trimethoprim-sulfometoxazole, E: Erythromycin; MEM: Meropenem; S: Streptomycin; OFX: Ofloxacin; CIP: Ciprofloxacin; C: Chloramphenicol; DA: Clindamycin; S: Susceptible; I: Intermediate; R: Resistant

Table 2. *Antibiogram test results as percent and origins of B. anthracis strains*

Antimicrobial Agents	Zone Diameter Breakpoints (mm)			Origin and Percent of Isolates																	
	S (≥)	I	R (≤)	Cattle (n: 51)			Sheep (n: 7)			Dog (n: 1)			Horse (n: 2)			Human (n: 1)			Soil (n: 25)		
				S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
P[a]	29	-	28	100	-	-	100	-	-	100	-	-	100	-	-	100	-	-	100	-	-
AML[b]	26	-	26	100	-		100	-	-	100	-	-	100	-	-	100	-	-	100	-	-
SXT[a]	16	11-15	10	-	9.8	90.2	-	-	100	-	-	100	-	-	100	-	-	100	-	4	96
E[a]	23	14-22	13	90	6	4	100	-	-	100	-	-	50	-	50	100	-	-	92	8	-
MEM[a]	22	-	22	33	65	2	43	57	-	100	-	-	100	-	-	100	-	-	36	64	-
S[a]	22	-	14	80.4	19.6	-	57	43	-	100	-	-	100	-	-	100	-	-	88	12	-
OFX[a]	18	15-17	14	100	-	-	100	-	-	100	-	-	100	-	-	100	-	-	100	-	-
CIP[a]	21	16-20	15	100	-	-	100	-	-	100	-	-	100	-	-	100	-	-	100	-	-
C[a]	18	13-17	12	96	2	2	100	-	-	100	-	-	100	-	-	100	-	-	92	8	-
DA[a]	21	15-20	14	90.2	9.8	-	100	-	-	100	-	-	100	-	-	100	-	-	72	28	-

[a]*According to S. aureus inhibition zone from CLSI M100-30th ed.*[24],
[b]*According to S. aureus inhibition zone from EUCAST- Version 13.1* [25],
P: Penicillin; AML: Amoxicillin, SXT: Trimethoprim-sulfometoxazole, E: Erythromycin; MEM: Meropenem; S: Streptomycin; OFX: Ofloxacin; CIP: Ciprofloxacin; C: Chloramphenicol; DA: Clindamycin; S:Susceptible; I: Intermediate; R: Resistant

91%, 86%, 82%, and 38%, respectively. Out of the isolates 61% were moderately susceptible to meropenem, 18% to streptomycin, 13.8% to clindamycin, 6.9% to trimethoprim-sulfomethoxazole, 6% to erythromycin and and 3.4% to chloramphenicol. Among the isolates, 93.1% of them were found to be resistant to trimethoprim-sulfometoxazole, 3% to erythromycin, 1.1% to chloramphenicol and 1% to meropenem. *Table 1* displays antibiotic susceptibilities of 87 *B. anthracis* strains isolated between 2012-2023.

When the antimicroabial susceptibilities of the *B. anthracis* isolates were evaluated according to their origin, it was observed that while dog and human origin isolates were susceptible to all antibiotics (except trimethoprim-sulfomethoxazole), all of bovine and soil origin isolates were susceptible to penicillin, amoxicillin, ofloxacin and ciprofloxacin antibiotics, all sheep origin isolates were susceptible to penicillin, amoxicillin, ofloxacin, erythromycin, ciprofloxacin, clindamycin, and chloramphenicol antibiotics, all horse origin isolates were susceptible to penicillin, amoxicillin, meropenem, streptomycin, ofloxacin, ciprofloxacin, chloramphenicol and clindamycin. Erythromycin susceptibility rates were 90.2%, 90% and 50% in soil, cattle and horse isolates, respectively; streptomycin susceptibility rates were 88%, 80.4% and 57% in soil, cattle and sheep isolates, respectively; chloramphenicol susceptibility rates were 96% and 92% in cattle and soil isolates; chloramphenicol susceptibility rates were 96% and 72% in cattle and horse isolates, respectively. In addition, 65% of the bovine origin isolates were moderately susceptible to meropenem and 9.8% to trimethoprim-sulfomethoxazole, and 64% of the soil origin isolates were moderately susceptible to meropenem and 4% to trimethoprim-sulfomethoxazole. All sheep, horse, dog and human isolates, 90.2% of bovine isolates and 96% of soil isolates were resistant to trimethoprim-sulfomethoxazole. Antibiogram test results as percent and origins of *B. anthracis* strains were given in *Table 2*.

When antimicrobial susceptibilities are analysed according to years, it was seen that cattle, sheep, and soil isolates were susceptible to penicillin, amoxicillin, ofloxacin and ciprofloxacin in all years tested (2012-2023). All sheep origin isolates were also susceptible to chloramphenicol, erythromycin, and clindamycin and resistant to trimethoprim-sulfomethoxazole in all years. Bovine isolates were also susceptible to cholaramphenicol and erythromycin in 2015 and later years. While in 2012, 2013 and 2014 years, 83.3%, 83.3% and 88.9% of bovine isolates were resistant to trimethoprim-sulfomethoxazole, 16.7%, 16.7% and 11.1% were moderately susceptible, respectively. All isolates identified in 2015 and subsequent years were resistant to trimethoprim-sulfomethoxazole. Dog (1 isolate from 2013), human (1 isolate from 2013)

and horse (2 isolates from 2018) isolates were susceptible to all antimicrobial tested. Just 1 horse isolate was resistant to erytromycin. Antibiotic susceptibilities of dog, human, and horse isolates were presented in *Table 1*.

Statistical Analysis Findings

At a result of the Chi-Square analysis ($\chi 2$ = 141.897; P=0.000), a statistically significant difference in the antibiotic susceptibility of *B. anthracis* strains according to sample origin and years was found.

Discussion

Bacillus anthracis is sensitive to many antibiotics such as penicillin, oxytetracycline, amoxicillin, chloramphenicol, ciprofloxacin, doxycycline, erythromycin, gentamicin and sulphonamides [3]. Among these, penicillin is used both in the diagnosis of the agent and in the treatment of the disease [26]. Penicillin is the first preferred antibiotic in anthrax treatment [11,27]. All 87 *B. anthracis* strains used in the present study were found to be susceptible to penicillin. This result is consistent with the studies of Aydın et al.[26], Doğanay and Aydın [27], Eşel et al.[28], Otlu et al.[29], Chun et al.[30], Habrun et al.[31], and Perçin et al.[32].

The drugs preferred for post-exposure prophylaxis of anthrax include amoxicillin, ciprofloxacin or ofloxacin as well as penicillin G [33]. Also oral amoxicillin may be used in mild, uncomplicated cutaneous anthrax [2]. In addition ciprofloxacin is one of the first choices in bio-terrorism or biological weapon related anthrax. Amoxicillin, ofloxacin and ciprofloxacin evaluated in the present study showed very good activity on all *B. anthracis* strains. This result is consistent with the studies of Doğanay and Aydın [27], Eşel et al.[28], Chun et al.[30], Habrun et al.[31] and Cavallo et al.[34]. As can be seen, *B. anthracis* remains susceptible to amoxicillin, ofloxacin and ciprofloxacin. According to these results obtained, it is thought that it may be significant to continue to include these antibiotics in the treatment prescription in clinical cases.

In patients with penicillin allergy, erythromycin, streptomycin, chloramphenicol and clindamycin are among the alternative drugs that can be used in the treatment of anthrax [5,35]. Also, in systemic anthrax, penicillin must be combined with one or two antibiotics to which the bacteria are susceptible. In cases of pulmonary anthrax, penicillin G must be combined with clindamycin or ciprofloxacin, and in gastrointestinal anthrax, it must be combined with aminoglycoside (preferably streptomycin) [2]. The penicillin drug combined with streptomycin is also curative [3]. In the present study, 95.4% of the strains were susceptible to chloramphenicol, 91% to erythromycin, 86% to clindamycin and 82% to streptomycin. Effectivity of these antibiotics on all *B. anthracis* strains tested suggest that there may be several suitable alternative antimicrobial agents for the prophylaxis and/or treatment of *B. anthracis*.

In anthrax meningitis, which is a life-threatening clinical picture, a combination of quinolone (such as ciprofloxacin) + carbapenem (such as meropenem) + a protein synthesis inhibitor antibiotic (such as clindamycin/chloramphenicol) can be used [36]. Another antibiotic used in the present study was meropenem. As a result of the study, 33% of the strains were susceptible to meropenem, 66% were moderately susceptible and 1% were resistant. According to this result of the present study, it is useful to pay attention to results of antibiogram to be performed in case of the use of meropenem and to organise the treatment accordingly.

B. anthracis has been found to develop high rates of resistance to trimethoprim-sulfomethoxazole [2,5]. Although not associated with sulfoamethoxazole, trimethoprim resistance has been reported in some studies [7,31,37,38]. In the present study, resistance to trimethoprim-sulfomethoxazole was determined in 93.1% of the isolates and this result is in parallel with the other studies [7,39]. This result supports studies reporting that *B. anthracis* has an intrinsic resistance to this antibiotic [40], and the claim that this antibiotic should not be used in anthrax prophylaxis or treatment in humans [28]. Therefore, bacterial antimicrobial resistance, which reduces the efficacy of drugs used to treat bacterial infections, is therefore a major public health problem and studies on the mechanisms and spread of antibiotic resistance are very important [41].

Before deciding on treatment, it should be remembered that *B. anthracis* has developed resistance to trimethoprim-sulfomethoxazole as well as third generation cephalosporins. Aydın et al [26], in a study on the number of anthrax cases in humans and animals in Kars region between 1995 and 2000, determined that all of the 61 *B. anthracis* strains (45 cattle, 6 sheep and 10 human origin) were susceptible to trimethoprim-sulfomethoxazole. Unlike from this, Otlu et al.[29], reported that all 61 cattle and 13 sheep *B. anthracis* isolates were resistant to trimethoprim in their study on the antibimicrobial susceptibility/resistance of sheep and cattle origin *B. anthracis* strains raised in Kars and Ardahan regions. In the present study, while 16.7%, 16.7% and 11.1% of the bovine *B. anthracis* isolates identified in 2012, 2013, and 2014 were moderately susceptible to trimethoprim-sulfomethoxazole, respectively, all bovine isolates identified in 2015 and later years were resistant to trimethoprim-sulfomethoxazole. This result can be considered as an indication that *B. anthracis* strains develop intrinsic resistance to SXT as mentioned above.

Anthrax is one of the serious diseases in animals and humans throughout history and remains a major zoonotic

concern. Timely recognition of *B. anthracis* infection is essential for determination of appropriate treatment, identification of outbreaks and veterinary and public health interventions. Therefore, in determining preventive and therapeutic strategies including the using of antibiotics in anthrax, which carries a high risk for public health, it will be useful further monitoring *B. anthracis* with the consideration of its resistance and susceptibility profile and to decide on the best therapeutic strategy (current or alternative treatment options) considering the results of antibiotic susceptibility test. The results showed no change in the susceptibility profile of *B. anthracis* isolates, especially penicillin and amoxicillin susceptibility profiles which are the first antimicrobial agents preferred in naturally occurring anthrax cases of *B. anthracis* strains in Kars region in the current study.

Anthrax is important as a potential weapon of bioterrorism. Therefore, if the antimicrobial resistance of the causative agent *B. anthracis* increases, the treatment of this pathogen in bioterrorism events may become more difficult. In particular, the development of resistance to potent and broad-spectrum antibiotics could pose a major threat to public health. In cases of increasing antibiotic resistance, the need for more complex treatment strategies, the use of antitoxins, combination therapies and rapid diagnostic methods may also increase. Ciprofloxacin, one of the first choice antibiotics in anthrax associated with biological weapons or bioterrorism, was found to be fully effective against *B. anthracis* in the present study. However, as mentioned above, the possibility that the resistance to trimethoprim-sulfomethoxazole antibiotic may similarly develop against ciprofloxacin and penicillin antibiotic, which is the first choice in prophylaxis and treatment protocols, should not be ignored. Therefore, in order to be prepared for resistance in possible bioterrorism attacks, there is an intense need for practices for monitoring antibiotic susceptibility/resistance, comprehensive treatment strategies and strong integration of effective interventions for public health. Conclusion, changes in the antimicrobial susceptibility of *B. anthracis* significantly affect treatment options in bioterrorism events, which can make rapidly spreading infections more difficult to control. Therefore, it is important to develop more comprehensive treatment strategies, faster and more accurate diagnostic methods, and effective intervention protocols against bioterrorism events. But as of right now, athough *B. anthracis* strains are sensitive to certain antibiotics, it is important that drugs should not be used in anthrax prophylaxis or medical and veterinary management without prior susceptibility testing.

Considering the results obtained from the current study, it is thought that further studies involving standardized testing methodologies for the antibiotic resistance profile of *B. anthracis* and the continuation of these studies will elucidate the resistance mechanisms that may develop in such a bioterrorism agent.

Declerations

Availability of Data and Materials: The data and materials of this study are available from the corresponding author (E. Çelik).

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Research Article

Effect of Peppermint Oil (*Mentha piperita*) Supplementation in Quail Diets on Egg Production and Some Egg Quality Characteristics, Oxidative Stress Parameters and Lipid Profile

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Abstract

This study investigated the effects of peppermint oil supplementation in the diet of Japanese quail on egg production, quality characteristics, oxidative stress parameters, and lipid profile. A total of 90 female quail were divided into three groups: a control group, a low-dose group receiving 150 mg/kg peppermint oil, and a high-dose group receiving 300 mg/kg supplementation. The study lasted for 8 weeks during which external and internal egg quality characteristics, biochemical and lipid profile analyses were performed. The results indicated that low doses of peppermint oil had beneficial effects on eggshell weight and quality. In contrast, high doses of peppermint oil had negative effects on egg internal quality unit (IQU) and increased egg pH. A significant increase in omega-3 fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), was observed in the high-dose peppermint oil group, indicating an improvement in the lipid profile. While the low dose of peppermint oil improved certain egg quality parameters, the high dose had negative effects, highlighting the importance of determining the correct dosage of supplementation. Overall, the study shows that peppermint oil, when used at the appropriate dose, can improve both egg quality and lipid profiles, potentially offering health benefits by increasing beneficial fatty acids such as omega-3 in quail eggs.

Keywords: Egg quality, Lipid profile, Oxidative stress, Peppermint, Quail

Introduction

The growing global demand for poultry products has led to a significant increase in production, particularly of eggs, which are now one of the most widely consumed sources of animal protein. This surge in consumption can be attributed to factors such as easy accessibility, affordability and broad cultural acceptance. In addition to chicken eggs, quail eggs have become a popular alternative due to their nutritional benefits and ease of production. As poultry production intensifies, there is growing interest in using natural feed additives and plant-based products to improve animal health and productivity in response to dietary regulations, consumer preferences and animal welfare concerns [1-5].

With increasing restrictions on the use of antibiotics in animal agriculture, there has been a growing shift towards the use of botanical extracts as natural alternatives. These plant-derived compounds are used not only to prevent disease, but also to promote growth, enhance development and improve overall performance in poultry and livestock [6,7]. Among these, polyphenols-micronutrients found in abundance in plant extracts-have attracted attention for their diverse biological activities. They are known for their cardiovascular, cognitive, antioxidant, anti-inflammatory and immunomodulatory properties, among others [1]. However, despite these benefits, excessive levels of polyphenols may have uncertain or even negative effects on gastrointestinal health, nutrient digestion, enzyme

activity, vitamin and mineral absorption, and ultimately on laying performance and egg quality [6-8].

Peppermint is an aromatic plant that is widespread throughout the world and includes 25 different species. It can grow in all climatic conditions. Peppermint is widely used in medicine as an antiseptic, antibacterial, antispasmodic, emetic and diuretic due to its essential oils (menthol, menthyl acetate, menthone, mentofuran, carvone) [2,9,10]. Herbal extracts have also been reported to have a beneficial effect on biochemical parameters, particularly by lowering blood cholesterol levels [3,4,10-12]. The use of caraway seeds and black cumin seeds in the diets of chickens also reduced the levels of total cholesterol (TCHOL) and low-density lipoprotein cholesterol (LDL-CHOL) in the blood serum of broiler chickens [13,14]. It has been reported that sage and thyme extracts from aromatic plants have positive effects on some performance and quality parameters, can be used as lipid oxidation inhibitors instead of vitamin E, which is an antioxidant, and can be added to laying hen diets as cholesterol lowering agents [15]. Studies with peppermint oil and peppermint extract show the potential of peppermint as an additive [16]. Among the herbal extracts, the addition of chamomile, wild mint and thyme extracts had no significant effect on egg production, egg weight, feed intake, feed conversion ratio, egg quality characteristics and hatching performance [17]. In another study, peppermint oil was found to significantly decrease total oxidative status (TOS) and increase total antioxidant status (TAS) in blood [18]. They showed that the addition of lavender essential oil to the diet resulted in an increase in live weight, egg production, egg mass, egg weight, egg white height, Haugh unit (HU), omega-3 fatty acids and a decrease in malondialdehyde (MDA) concentration [19]. In the studies, the use of herbal extracts in different forms and ratios did not show significant effects on egg quality, but did show significant effects on egg biochemical profiles.

Considering the effects of different herbal extracts on different poultry species and products, the aim of this study was to investigate the effects of peppermint on egg quality parameters, biochemical properties and lipid profile. The main objective of the study was to investigate the effects of different levels (low and high doses) of peppermint oil in quail diets on eggs.

Material and Methods

Ethical Approval

This study was conducted with the permission of Kırıkkale University Animal Experiments Local Ethics Committee dated 19.07.2023 and numbered 2023/07/28.

Animal Material

The study utilized a total of 90 female Japanese quail *(Coturnix coturnix Japonica)* with an age of 16 days. The animals were weighed to ensure that there was no difference in live weight among all groups and then divided into three experimental groups with three replications, each with 10 quails. Each subgroup was housed in cages measuring 100x45x20 cm. The study continued for 8 weeks after the first eggs were observed at 16th day and were terminated at the conclusion of the period. Feed and water were provided *ad libitum*, and a comfortable temperature (24°C) was maintained for all animals throughout the study. Additionally, a 16-h light and 8-h dark cycle was applied during the laying period.

Diet Content

The animals utilized in the experiment were provided with the dietary regimen recommended by the National Research Council (NRC) [20], tailored to the specific age groups from hatching until the conclusion of the experiment, with consideration given to the basal diet. From the time of hatching until the 16th day, when the first egg was seen, the broiler chick feed was used and then the experiment was started by switching to the layer feed. The nutrient contents of the diets were determined according to the methods specified in AOAC [21]. Metabolizable energy of the diets was calculated according to TSE [22]. The nutritional values of the basal diet used during the pre-laying and laying periods have been determined *(Table 1)*. The experimental groups were structured as follows: Group 1, basal diet (control), Group 2, basal diet + 150 mg/kg peppermint essential oil (LM), and Group 3, basal diet + 300 mg/kg peppermint essential oil acid (HM).

Determining Egg Production and Egg Quality

In the study, the number of eggs collected daily was determined, and the number of eggs per week was calculated by including those with broken, cracked, and shell anomalies. For the 8-week experimental period, the groups were evaluated in terms of egg numbers. Fifteen eggs were randomly collected from each group at the beginning, middle and end of the experiment and egg weight (EW), egg shape index (ESI) and shell surface area (ESSA) were calculated. The formula of Yannakopoulos and Tserveni-Gousi [23] was used to calculate the egg shape index.

Table 1. *Basal diets nutritient values*

Nutrient (%)	Broiler Chicken	Laying Hen
Dry matter	88.61	90.41
Crude Protein	22.19	16.88
Crude fat	5.05	4.17
Crude Cellulose	4.69	5.54
Crude Ash	5.48	6.33
ME, Kcal/Kg	3093	2701

Egg shape index (%) = (Egg width/Egg length) x 100

The formula of Olawumi and Chiristiana [24] was used to calculate the eggshell surface area.

Shell surface area (cm^2) = 3.9782 x (Egg weight) $^{0.7056}$

After the measurements and calculations of external quality characteristics were completed, internal quality characteristics were evaluated. The examined characteristics were determined as membrane shell weight (ESW), eggshell ratio (ESR), albumen height (AH), albumen width (AW), albumen length (AL), yolk height (YH), yolk width (YW), yolk length (YL), albumen index (AI), yolk index (YI), albumen pH value, Haugh Unit (HU) and Internal Quality Unit (IQU). The pH of the egg yolk was determined using a digital pH meter (HANNA, HI 2221). The formulas given below were used in the calculations.

Shell ratio (%) = (Shell weight (g)/Egg weight (g)) x 100 [25]

Albumen index (%) = (Albumen height (mm)/[(Albumen length + Albumen width)/2] x 100 [24].

Yolk index (%) = (Yolk height (mm)/Yolk diameter (mm)) x 100 [23].

Haugh Unit = 100 x log (Egg height (mm) + 7.52 – 1.7 x Egg weight (g) $^{0.37}$) [26].

IQU (%) = 100 x log [Egg height (mm) + 4.18 – (0.8989 x Egg weight (g) $^{0.6674}$)] [25].

Determining the Amount of Cholesterol in Egg Yolk

One day before the end of the experiment and on the day the experiment was to be terminated, three eggs from each quail group were collected and the cholesterol and triglyceride content of the egg yolk was determined by Uyanık et al.[27] with slight modification. The egg yolk was separated from the egg white, and then the yolk was collected from the vitelline membrane with a syringe. Weigh out 0.1 g of egg yolk and place it in a homogenizing tube. Add 1 mL of 99.7% isopropyl alcohol. The raw egg yolk was vortexed until dissolved and homogenized. The homogenate in the tube was incubated at 37°C for 10 min and centrifuged at 3000 rpm for 5 min at 4°C. Egg yolk cholesterol (CHO) concentration was determined in supernatant samples using a commercial kit (Otto Scientific, Türkiye) in a autoanalyzer (Mindray BS-400, China).

Determination of TOS and TAS Levels in Egg Yolk

One day before the end of the experiment and on the day the experiment was to be terminated, three eggs from each quail group were collected and the TAS and TOS levels of the egg yolk were determined according to the method reported by Kornbrust et al.[28] with modification. 1 mL of egg yolk was taken by piercing the vitelline membrane with an automatic pipette tip, and Raw egg yolk was diluted 1:3 with distilled water. The mixture in the tube was vortexed until a completely homogeneous solution was obtained. The tubes were centrifuged at 15000 rpm for 5 min at +4°C. After centrifugation, TAS and TOS values in supernatant were determined using commercial test kits (Rel Assay, Türkiye) on an autoanalyzer device (Mindray BS-400, China). To calculate the OSI, which is known as the percentage of the ratio of total oxidative capacity to total antioxidative capacity, the units of TAS and TOS levels were equalized. The results were expressed in arbitrary units (AU) [29]. Oxidative stress index (OSI) was calculated using the following equation:

$$OSI = \frac{TOS\ (\mu mol\ H2O2\ Equiv/L)}{TAS(\mu mol\ \ Trolox\ Equiv/L)}$$

Determination of Egg Lipid Profile

Egg yolk samples were obtained from freshly collected eggs at the conclusion of the study (eight eggs from each group, for a total of 24 eggs). The fatty acids (free and bound) in the egg yolk samples were subjected to a modified three-step methylation procedure, as described by Wang et al.[30]. The supernatants, comprising methylated fatty acids in n-hexane, were transferred to a 1.5-mL screw-neck ND-9 amber vial with 9-mm screw caps (silicone white/PTFE) and analyzed using a gas chromatograph (TRACE 1300, Thermo Scientific, USA) with automatic sampling (Thermo AI 1310, Thermo Scientific, USA). The FAME mix (37°C) standard solution in dichloromethane (Chem-Lab, CL.40.13093.0001, Zedelgem, Belgium) was employed for the identification of the peaks. Heptadecanoic acid (C17:0) was employed as the internal standard. The processing method employed was that of a fatty acid methyl esters column (length 60 m, internal diameter 0.25 mm, film thickness 0.25 µm, maximum temperature 250-260°C), with an injection split temperature of 255°C, a column temperature of 140°C, and a flow rate of 30 mL/min. This was conducted for a period of 42 min. The identification of the fatty acids was conducted by comparing the peaks in the chromatogram with the standard retention times [31].

Statistical Analysis

In this study, the full factorial model was employed with the use of IBM SPSS 27 [32] in order to analyze a number of egg quality parameters across three experimental groups: the control group, the low-dose peppermint oil group (with a dose of 150 mg/kg), and the high-dose peppermint oil group (with a dose of 300 mg/kg). The inclusion of interaction terms was not considered appropriate given the simplicity of the experimental design. A generalized linear model (GLM) was selected as the analytical method

for the comparison of continuous dependent variables (egg quality characteristics, biochemical parameters and lipid profiles) across categorical independent variables (treatment groups). This approach was deemed appropriate due to the experimental design, thereby facilitating a robust comparison of means between groups. Polynomial contrasts (linear, quadratic, cubic) were not applied in this analysis, as the primary objective was to compare the main effects of the treatment groups on the dependent variables without exploring trend analyses. The parameters subjected to analysis included: The external egg quality characteristics included in the analysis were shell weight and shell thickness. The internal egg quality characteristics were yolk weight, albumen weight, egg internal quality unit (IQU) and egg pH. The biochemical and lipid profile parameters were omega-3 fatty acids (EPA and DHA). A full factorial GLM model was employed to conduct statistical comparisons between the three experimental groups. After the GLM procedure, Tukey's multiple comparison test was used to separate the statistically different groups (P<0.001).

Results

In the study, the effects of adding different amounts of peppermint oil to the diet on egg production, and external egg quality parameters were investigated *(Table 2)*.

Upon analysis of the egg numbers, it was observed that the control group exhibited the highest average egg number, while the HM group demonstrated the lowest. Statistically significant differences were identified between the groups in regard to egg numbers (P<0.001). In terms of EW values, a significantly higher value was observed in the LM group compared to the control group, while this value was significantly lower in the HM group (P=0.014). No statistically significant difference was observed between the groups in terms of ESI (P=0.431). The ESW was found to be significantly higher in the HM group compared to the control and LM groups (P=0.003). Although no statistically significant difference was observed between the groups in terms of ESR, a marginally higher value was noted in the HM group (P=0.128). The ESSA values were found to be similar in the LM and control groups, but significantly lower in the HM group (P=0.005).

Table 2. *Egg external quality parameters in groups containing different amounts of peppermint oil (LSM±S_E)*

Parameters	Control	LM	HM	P
EN	136.87±2.33^{c}	125.87±1.27^{b}	114.12±1.27^{a}	<0.001
EW	9.89±0.10^{ab}	10.22±0.09^{b}	9.35±0.31^{a}	0.014
ESI	80.35±0.30	79.53±0.37	79.55±0.74	0.431
ESW	1.32±0.01^{a}	1.31±0.02^{a}	1.44±0.03^{b}	0.003
ESR	13.50±0.22	12.30±0.17	15.55±1.95	0.128
ESSA	19.78±0.13^{b}	19.72±0.11^{b}	18.40±0.54^{a}	0.005

EN: Egg number; EW: Egg weight, g; ESI: Egg shape index, %; ESW: Egg shell weight, g; ESR: Egg shell ratio, %; ESSA: Egg shell surface area. LM: Low mint group; HM: High Mint group.
^{a,b,c} *different letters in the same line represent statistical difference (P<0.01)*

Table 3. *Internal egg quality parameters in groups containing different amounts of peppermint oil (LSM±S_E)*

Parameters	Control	LM	HM	P
AH	4.57±0.11	4.61±0.08	4.54±0.12	0.907
YH	10.64±0.29	10.89±0.16	9.89±0.47	0.095
AW	33.36±0.54	33.83±0.35	32.91±0.38	0.327
AL	43.11±0.53^{ab}	44.71±0.39^{b}	40.67±1.24^{a}	0.003
YW	24.77±0.52^{b}	23.60±0.16^{a}	23.62±0.23^{ab}	0.026
pH	8.78±0.04^{a}	8.68±0.05^{a}	9.28±0.03^{b}	<0.001
YI	44.31±1.90	47.17±0.62	44.14±1.80	0.306
AI	3.02±0.11^{a}	2.89±0.05^{a}	6.34±0.83^{b}	<0.001
HU	94.47±0.99^{b}	90.92±0.29^{a}	88.23±1.03^{a}	<0.001
IQU	70.25±0.69^{c}	66.06±0.71^{b}	60.05±1.82^{a}	<0.001

AH: Albumen height, mm; YH: Yolk height, mm; AW: Albumen width, mm; AL: Albumen length, mm; YW: Yolk width, mm; YI: Yolk index, %; AI: Albumen index, %; HU: Haugh Unit; IQU: Internal quality unit
^{a,b,c} *different letters in the same line represent statistical difference (P<0.01)*

Table 4. *Effects of dietary peppermint oil supplementation on oxidative stress parameters (LSM±S_E)*				
Parameters	**Control**	**LM**	**HM**	**P**
TAS	0.210±0.02	0.350±0.07	0.199±0.06	0.159
TOS	6.30±1.45	7.97±4.17	8.18±1.39	0.856
OSI	3.78±1.37	6.28±4.45	6.03±1.32	0.777
TG	457.90±9.53	459.36±4.34	442.71±7.59	0.237
CHOL	29.29±1.81	34.66±3.04	31.07±2.39	0.336

*TAS: Total Antioxidant Status, TOS: Total Oxidant Status, OSI: Oxidative Stress Index, TG: Triglyceride, CHOL: Cholesterole. LM: Low mint group; HM: High mint gro*up

Table 5. *Egg lipid profile in groups containing different amounts of peppermint oil (LSM±SE)*				
Fatty Acids	**Control**	**LM**	**HM**	**P**
Lauric Acid Methyl Ester (C12:0) 4% (dodecanoate)	0.010±0.001	0.021±0.001	0.018±0.001	0.324
Tridecanoic Acid Methyl Ester (C13;0) 2%	0.005±0.001	0.006±0.002	0.002±0.001	0.450
Myristic Acid Methyl Ester (C14:0) 4%	0.246±0.025	0.260±0.021	0.240±0.020	0.817
Myristoleic Acid Methyl Ester (C14:1) 2%	0.015±0.008	0.010±0.000	0.008±0.001	0.531
Pentadecanoic Acid Methyl Ester (C15:0) 2%	0.046±0.007	0.057±0.005	0.046±0.004	0.356
cis-10-Pentadecenoic Acid Methyl Ester (C15:1) 2%	0.023±0.001	0.029±0.001	0.024±0.002	0.075
Palmitic Acid Methyl Ester (C16:0) 6%	20.029±1.233	23.658±1.568	20.614±1.436	0.177
Palmitoleic Acid Methyl Ester (C16:1) 2%	2.504±0.199	2.770±0.218	2.415±0.262	0.530
Heptadecanoic Acid Methyl Ester (C17:0) 2%	0.073±0.007$^{a.b}$	0.093±0.009^{b}	0.046±0.012^{a}	0.011
cis-10-Heptadecenoic Acid Methyl Ester (C17:1) 2%	0.036±0.001	0.040±0.003	0.040±0.004	0.702
Stearic Acid Methyl Ester (C18:0) 4%	6.190±0.365^{a}	8.445±0.656^{b}	6.552±0.724$^{a.b}$	0.033
Elaidic Acid Methyl Ester (C18:1n9t) 2%	0.027±0.011	0.003±0.002	0.008±0.004	0.062
Oleic Acid Methyl Ester (C18:1n9c) 4%)	30.969±1.063	37.018±2.172	36.641±2.462	0.078
Linolelaidic Acid Methyl Ester (C18:2n6t) 2%	26.165±3.638	11.490±5.223	19.388±5.436	0.124
Linoleic Acid Methyl Ester (C18:2n6c)2%	10.150±1.048	11.535±0.719	10.041±1.270	0.535
γ-Linolenic Acid Methyl Ester (C18:3n6) 2% (GLA)	1.775±0.081^{a}	2.245±0.104^{b}	2.031±0.147^{ab}	0.029
Arachidic Acid Methyl Ester (C20:0) 4% (Eicosanoic acid)	0.060±0.003	0.081±0.006	0.079±0.007	0.043
cis-11-Eicoenioic Acid Methyl Ester (C20:1) 2% (gondoic acid)	0.128±0.014	0.136±0.016	0.070±0.023	0.045
α-Linolenic Acid Methyl Ester (C18:3n3) 2% (ALA)	0.090±0.012	0.085±0.002	0.093±0.007	0.827
Heneicosanoic Acid Methyl Ester (C21:0) 4%	0.010±0.000^{a}	0.020±0.002^{b}	0.024±0.003^{b}	0.002
cis-11,14,17-Eicosadienoic Acid Methyl Ester (C20:2) 2%r	0.039±0.002	0.052±0.004	0.044±0.004	0.078
Behenic Acid Methyl Ester (C22:0) 4%	0.046±0.001	0.073±0.017	0.033±0.010	0.080
cis-8,11,14-Eicosatrienoic Acid Methyl Ester (C20:3n6) 2% (DGLA)	0.012±0.001	0.017±0.001	0.013±0.001	0.066
Erucic Acid Methyl Ester (C22;1n9) 2%	0.000±0.000	0.000±0.000	0.000±0.000	-
cis-11,14,17-Eicosatrienoic Acid Methyl Ester (C20:3n3) 2% (ETE)	0.100±0.011	0.145±0.017	0.124±0.007	0.071

Table 5. *Egg lipid profile in groups containing different amounts of peppermint oil (LSM±SE) (continued)*

Fatty Acids	Control	LM	HM	P
Arachidonic Acid Methyl Ester (C20:4n6) 2% (AA)	0.315±0.024	0.431±0.030	0.373±0.044	0.075
Tricosanoic Acid Methyl Ester (C23:0) 2%	0.055±0.002	0.074±0.005	0.066±0.008	0.120
cis-13,16-Docosadienoic Acid Methyl Ester (C22:2) 2%	0.290±0.020	0.306±0.037	0.171±0.071	0.122
Lignoceric Acid Methyl Ester (c24;0) 4% (Tetracosanoate)	0.241±0.026	0.255±0.033	0.274±0.031	0.756
cis-5,8, 11,14,17-Eicosapentaenoic Acid Methyl Ester (c20:5n3) 2% (EPA)	0.114±0.016[a]	0.200±0.013[b]	0.235±0.030[b]	0.002
Nervonic Acid Methyl Ester (C24:1) 2% (cis-15-tetracosenoate)	0.048±0.005	0.065±0.007	0.051±0.009	0.270
cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester (C22:6n3) (DHA)	0.178±0.033	0.331±0.016	0.241±0.074	0.099
Saturated Fatty acids, %	28.726±1.575	35.206±2.265	29.946±1.927	0.064
Unsaturated Fatty acids	71.261±1.579	64.745±2.245	70.056±1.926	0.061
MUFA	33.749±1.225	40.070±2.318	39.256±2.652	0.102
PUFA	37.513±2.783	24.675±4.543	30.800±4.368	0.098
ω-3	0.481±0.042[a]	0.761±0.039[b]	0.693±0.069[b]	0.003
ω -6	37.031±2.761	29.914±4.578	30.107±4.411	0.091
ω -9	31.139±1.087	37.166±2.185	36.726±2.462	0.082
ω -3/ω -6	0.013±0.001[a]	0.042±0.007[b]	0.031±0.008[a.b]	0.017

MUFA: Mono-unsaturated fatty acids; PUFA: Polyunsaturated fatty acids; ω-3, 6, 9: Omega 3, 6, 9. LM: Low mint group; HM: High mint group
[a,b] different letters in the same line represent statistical difference (P<0.01)

In this study, the effects of adding different amounts of peppermint oil to the diet on internal egg quality parameters in quails were investigated *(Table 3)*.

No statistically significant difference was observed between the groups in terms of albumen height (P=0.907). The egg yolk height was observed to be higher in the LM group; however, no statistically significant difference was identified (P=0.095). No statistically significant differences were observed between the groups in terms of albumen width and yolk width (P=0.327 and P=0.026). However, the yolk width was found to be numerically lower in the LM group. The lowest albumen length was observed in the HM group while the highest albumen length was observed in the LM group. This difference was statistically significant (P=0.003). With regard to yolk width, a significantly higher value was observed in the control group (P=0.026). The egg pH value was found to be significantly higher in the HM group, with a highly statistically significant difference (P<0.001). Upon examination of the albumen index, a significant increase was observed in the high peppermint oil group, which was found to be statistically highly significant (P<0.001). The Haugh Unit and the Internal Quality Unit were observed to be diminished in the HM group. Both parameters demonstrated statistically significant discrepancies (P<0.001).

In the study, the effects of adding different amounts of peppermint oil to the diet on egg yolk cholesterol and oxidative stress levels were investigated *(Table 4)*.

The present study examined specific biochemical and oxidative stress parameters in the control group, LM, and HM groups. The TAS values were observed to be higher in the LM group in comparison to the control group. However, TAS values in the HM group was found to be comparable to that of the control group. Nevertheless, no statistically significant difference was identified between the groups (P=0.159). With regard to TOS, the HM and LM groups exhibited higher values than the control group. However, these differences were not statistically significant (P=0.856). The oxidative stress index (OSI) was observed to be higher in the LM and HM groups in comparison to the control group. Nevertheless, this discrepancy was not statistically significant (P=0.777).

With regard to lipid parameters, TG levels were found to be in close proximity to those observed in the control group in the LM group and exhibited a slight decline in the HM group. However, these differences were not statistically

significant (P=0.237). The CHOL values were observed to be higher in the LM group than in the control group, and similar in the HM group. However, these differences were not statistically significant (P=0.336).

In the study, the effect of adding different amounts of peppermint oil to the diet on egg lipid profile was investigated *(Table 5)*.

As a result of the analysis, some differences were detected between the groups in terms of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). In particular, a significant increase was observed in the HM group compared to the control and LM groups in terms of omega-3 fatty acids (ω-3) (P<0.05). EPA and DHA levels were found to be significantly higher in the HM group. The HM group also exhibited a higher value compared to the other groups in terms of omega-3/omega-6 ratio (P<0.05). In addition, SFA concentrations were relatively higher in the control group, no statistically significant differences were detected between the groups (P>0.05). Some saturated fatty acids such as Stearic Acid (C18:0) and Palmitic Acid (C16:0) were observed to be at lower levels in the HM group. On the other hand, MUFA were observed at the highest levels in the HM group, but this difference was not found to be statistically significant (P>0.05). Although there were no statistically significant differences between the groups in terms of PUFA. PUFA levels were found to be higher in the HM group than in the LM group.

Discussion

In recent years, there has been a notable increase in research investigating the effects of peppermint oil on egg production, egg weight, shell quality, and other quality parameters. A substantial body of research has demonstrated the pronounced impact of peppermint oil and other essential oils on the productivity and egg quality of hens. It has been demonstrated that the addition of EOM to the diet of laying hens has a beneficial impact on egg-laying performance [33]. Upon examination of the results pertaining to egg number, the findings diverged from those of previous studies. These previous studies had indicated that the incorporation of herbal extracts into poultry diets did not result in a notable alteration in egg number [34]. The existing literature on the effect of aromatic plants and essential oils on egg quality presents a range of findings. A study by Çabuk et al. [35] reported that a 24 mg/kg EO mixture supplementation increased egg production, feed efficiency and decreased the rate of broken eggs. The same study reported that essential oil mixtures demonstrated favorable effects on egg production rate and feed conversion ratio (FCR) in quails. In a study conducted by Büyükkılıç Beyzi et al.[36], no significant difference was observed between the EOS and control groups with respect to egg quality parameters. However, a notable increase in egg specific gravity was evident in the group that received the essential oil and vitamin mixture during the final four weeks of the study period. It was observed that LM group resulted in an increase in egg weight. The significantly higher egg weight (10.22 g) observed in the LM group compared to the control group lends further support to this finding. It is also noteworthy that this value exhibited a marked decline in the high peppermint oil group (9.35 g). This suggests that while the use of essential oils at appropriate doses may have beneficial effects, excessive doses may result in adverse outcomes. This finding is consistent with previous studies indicating that optimal doses of essential oils can enhance egg quality [33,34]. Additionally, other findings related to the use of essential oils indicate differences in eggshell characteristics. The eggshell weight was found to be significantly higher in the HM group than in the control and LM groups, thereby confirming the effects of essential oils on shell quality. Similarly, although no statistically significant difference was observed in shell ratio, a slightly higher value was noted in the high peppermint oil group. However, this increase did not reach statistical significance, which requires further investigation. However, it should be noted that high doses may have negative effects on egg internal quality and shell surface area. In order to confirm these effects of peppermint oil, longer term studies covering different dose levels are required.

The results on the effects of diets supplemented with peppermint oil on internal quality parameters of quail eggs are consistent with the literature on the effects of essential oils on egg quality. Most studies indicate that essential oils do not always have a direct effect on albumin quality [17,19,36]. Deniz et al.[37], reported that different essential oils can cause some changes in yolk volume and color, but these effects usually depend on the type and dose of oil. Florou-Paneri et al.[33], reported that some essential oils, such as peppermint oil, may affect egg pH and thus egg storage time. In this study, Eisen and Bohren [25] identified albumin level and Haugh unit as important indicators of egg freshness and stated that essential oils may cause differences in these indicators. Studies such as Frankič-Korošec et al.[38], also emphasize that essential oils should be used with caution and report that high doses may have negative effects on egg quality. In this study, no significant difference was observed between the groups with respect to albumin level (P=0.907). The increase in yolk height observed in the LM group is noteworthy but not statistically significant (P=0.095). In terms of albumin length, the lowest value was observed in the HM group and the highest value was observed in the LM group (P=0.003), supporting the dose-dependent effect of

peppermint oil. However, a higher value for yolk width was observed in the control group (P=0.026), which is consistent with the findings that yolk components may differ. In this study, a significant increase in egg pH was observed in the HM group (P<0.001), suggesting that essential oils may affect pH and thus egg internal quality. The increase in albumin index and decrease in Haugh unit (P<0.001) indicate the importance of a balanced use of essential oils that affect different aspects of egg quality. These results are consistent with the literature showing that peppermint oil may contribute positively to quail egg quality at appropriate doses but may be detrimental at high doses.

Menthol and menthone, the major components of peppermint oil, have the ability to enhance antioxidant defenses by neutralizing free radicals and increasing cellular antioxidant activity [2]. Several previous studies have shown that moderate doses of essential oils, especially peppermint oil, potentiate endogenous antioxidant systems by increasing TAS in animals [9]. Previous studies have reported that high doses of peppermint oil may lead to lipid peroxidation, which may increase TOS levels [10]. Orzuna-Orzuna et al.[39], reported that essential oils may have pro-oxidant effects at high concentrations in poultry diets, which may lead to increased OSI levels. The non-significant differences in TAS, TOS and OSI levels between groups demonstrate the complex dose-dependent nature of the effects of peppermint oil on oxidative stress. The results of this study are consistent with broader research highlighting the importance of determining the optimal dose to maximize the antioxidant benefits of essential oils while avoiding adverse pro-oxidant effects. In the present study, oxidative stress parameters including TAS, TOS and OSI were found to be higher in the LM group than in the control group, but the difference did not reach statistical significance (P=0.159). This finding is consistent with the literature on the antioxidant properties of peppermint oil [10]. On the other hand, TOS levels were higher in both LM and HM groups compared to the control group, but these differences were not statistically significant (P=0.856). This suggests that while peppermint oil activates antioxidant pathways at low doses, it may trigger oxidative reactions at high doses. OSI levels followed a similar pattern and were higher in the LM and HM groups compared to the control group (P=0.777). Although not statistically significant, this trend indicates a balance between increased antioxidant capacity and oxidative stress.

The effects on the lipid parameters obtained in this study are in agreement with the existing literature and the research supports the possible effects of essential oil on lipid metabolism. Mehri et al.[40] and Barbalho et al.[41], reported that the effects of peppermint oil on serum lipid profile and cholesterol synthesis usually occur at low and moderate doses, but this effect depends on the type and dosage. Similarly, Arjun et al.[42], investigated the effects of peppermint oil on performance and lipid metabolism in poultry and reported positive results. The fact that TG levels in the LM group were close to the control group and slightly decreased in the HM group, but not statistically significant (P=0.237), is consistent with previous studies showing that peppermint oil may have a limited effect on lipid metabolism. The fact that CHOL levels were higher in the LM group compared to the control group and similar levels were observed in the HM group (P=0.336) is consistent with the findings that peppermint oil may affect cholesterol metabolism in different ways. These findings support the potential effects of essential oils in regulating lipid metabolism.

Studies suggest that peppermint oil may affect the biosynthesis and metabolism of polyunsaturated fatty acids (PUFAs), particularly omega-3 fatty acids (ω-3), due to its bioactive compounds [19]. Some studies have also highlighted the potential impact of peppermint oil on the synthesis and degradation of saturated fatty acids (SFAs) like stearic acid (C18:0) and palmitic acid (C16:0), as well as its role in modulating monounsaturated fatty acids (MUFAs) such as oleic acid [36]. However, the results regarding the effect of peppermint oil on lipid metabolism remain inconsistent in the literature [10]. In this study, the effects of adding different amounts of peppermint oil to the diet on the lipid profile of eggs were investigated. The most notable finding was the significant increase in ω-3 fatty acid levels in the HM group compared to the control and LM groups (P<0.05). This is consistent with previous studies suggesting that essential oils may enhance ω-3 fatty acid metabolism [43]. Additionally, the ω-3/ω-6 ratio was higher in the HM group, supporting the hypothesis that peppermint oil may contribute to a more favorable lipid profile by modulating PUFA synthesis and storage processes. This finding is important as a high ω-3/ω-6 ratio is associated with health benefits, including anti-inflammatory effects and reduced cardiovascular risk [44]. While no significant differences in SFA concentrations were observed among the groups (P>0.05), the lower levels of specific SFAs (C18:0 and C16:0) in the HM group are noteworthy. These findings are in line with studies indicating that peppermint oil may influence the synthesis or degradation of SFAs [43]. The MUFA levels, although highest in the HM group, did not differ significantly among the groups (P>0.05). Given the health benefits of MUFAs like oleic acid, the observed increase in the HM group may still hold biological significance, potentially attributable to the stimulating effect of menthol on lipid metabolism [45]. For PUFA levels, while the differences between groups were not statistically significant, higher values were recorded in the HM group compared to the

LM group. This observation suggests a potential positive effect of peppermint oil on PUFA synthesis or storage, aligning with previous literature on the role of essential oils in modulating lipid metabolism [46].

In conclusion, the results of the study show that peppermint oil increases egg weight and shell quality at the correct dose, but this effect is reversed at high doses. Biochemically, high doses of peppermint oil (HM group) increased the levels of omega-3 fatty acids (EPA and DHA) and caused a significant increase in the omega-3/omega-6 ratio. When the lipid profile was analyzed, a decrease in saturated fatty acids, especially stearic acid and palmitic acid, was observed along with lower levels of saturated fatty acids (SFA). In addition, changes in MUFA and PUFA levels were observed, with higher PUFA levels in the HM group. In biochemical parameters related to the antioxidant defense system (TAS, TOS, and OSI), peppermint oil produced positive effects at low doses, while oxidative stress increased at high doses. These results suggest that peppermint oil may modulate lipid metabolism and contribute positively to egg quality; however, it is emphasized that the optimal dose of use should be carefully determined due to the negative effects of high doses.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author (Ş. Evci) on reasonable request.

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Ethical Approval

This study was conducted with the permission of Kırıkkale University Animal Experiments Local Ethics Committee dated 19.07.2023 and numbered 2023/07/28.

Declaration of Generative Artificial Intelligence: The authors declare that Generative Artificial Intelligence applications or softwares were not used in the study.

Conflict of Interest: The authors declared that there is no conflict of interest.

Author Contributions: ŞE: Study design, data collection, supervision, writing and editing; EE: Study design, data collection, statistical analysis, writing and editing; KK: Data evaluation, laboratory analysis; AŞ: Study design, data evaluation, laboratory analysis; MÇ: Study design, data evaluation, laboratory analysis.

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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.

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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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Reviews are original manuscripts that gather the literature on the current and significant subject along with the commentary and findings of the author on a particular subject (It is essential that the author/s have international scientific publications on this subject). The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should be followed by introduction, text (with appropriate titles), conclusion, and references.

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Narrative reviews describe current published information on a scientific topic. However, it does not include a specific methodological process.

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7- The necessary descriptive information (thesis, projects, financial supports, etc.) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of the title.

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References should be listed with numerical order as they appear in the text and the reference number should be indicated inside the parentheses at the cited text place. References should have the order of surnames and initial letters of the authors, title of the article, title of the journal (original abbreviated title), volume and issue numbers, page numbers and the year of publication and the text formatting should be performed as shown in the example below.

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DOI number should be added to the end of the reference.

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