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EDITORIAL

Dear Readers,

We are filled with delight and pride as we present to you the third issue of our magazine for the year 2023. This edition encompasses a total of fifteen articles, delving into various realms of veterinary medicine, including twelve research papers, one short communication, and two case reports.

The selection of publications for this issue was based on the chronological order of acceptance dates. As the Editorial team, we strive to adhere to this sequence to the best of our ability. Being fair in presenting your valuable works to the scientific community is of utmost importance to us. On this occasion, I would like to extend my heartfelt gratitude and appreciation to you for choosing our journal as the platform to share your scholarly endeavors.

With the sincere hope that our latest issue will contribute to the world of science, I extend my warmest regards to every one of you.

Dr. Levent ALTINTAŞ
Editor in Chief
Ankara Üniversitesi Veteriner Fakültesi Dergisi

Teaching feedback skills to veterinary students by peer-assisted learning

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ABSTRACT

Feedback is considered an essential element of effective learning. Students who receive feedback from peers can improve their clinical and communication skills. This paper aims to testify for whether peer-assisted learning (PAL) is successful in teaching students with the ability to give feedback. The study was design as tutors (n=20), tutees (n=20) and control group (n=20). Tutors were educated to provide constructive feedback, and this group trained tutees in PAL to increase their skills in providing feedback. After the training, tutors and tutees used role play about veterinarian-client consultation. At the end of the consultations, each tutee provided feedback to the tutor about his/her communication skills. As findings, there was a statistically significant change between the mean scores of both "tutor and control groups" ($P<0.004$) and "tutors and tutees" ($P<0.001$). However, there was no statistically significant difference in terms of the academic year and gender between groups. Tutees benefited from being trained by peers, and tutors also improved their feedback skills by training peers. Conspicuously, PAL was found to be effective for not only tutors but also tutees. With this argument, it is predicted that other veterinary fields can also benefit from PAL throughout veterinary training.

Introduction

International organizations such as American Veterinary Medical Association (AVMA) and European Coordination Committee on Veterinary Training (ECCVT) have recognized the value of communication skills training in veterinary medicine (5, 15), and many veterinary schools have worked on training programs related to these skills (21, 40). According to the AVMA, communication skills are among those that new graduates should have, while the European Association of Establishments for Veterinary Education (EAEVE) states that these skills should be included in the undergraduate curriculum (5, 15). The development of communication in veterinarians has a positive effect on the animals that are their patients and on the humans who are their colleagues and clients (2).

Communication is a basic skill for healthcare providers, and effective communication is a core competency for patient or client satisfaction. Observing, listening, reinforcing and encouraging, questioning, responding, and giving information are just some of the steps in effective communication (27), and special training methods such as problem-based, skills-based or scenario-based approaches and simulation-based practices with simulated or real clients are needed for improving communication skills (2). One of the core elements of teaching and learning communication skills is descriptive feedback. Feedback is considered an essential part of effective learning (36) and evaluation (25, 33), and providing feedback to people who need it is a strong contribution to one's own learning (8, 22, 33, 38). Providing well-intentioned, detailed, and descriptive feedback is one of the basic skills to be learned in clinical communication training (1), because veterinarians need to provide feedback to their colleagues and peers while practicing their profession; they also need to properly evaluate the feedback provided to them (14).

The purpose of effective feedback is to reveal the strengths and weaknesses in practices. Improving one's

ability to provide effective feedback depends on clinical skills, communication skills, and a predetermined task (25). Feedback is effective if it emphasizes the crucial and specific steps of performance in real time (33). It should be clear, focused on specific behaviors and tasks, given just in time, motivating, unbiased, objective, descriptive, supportive, appropriate in amount, goal-oriented, focused on the process, and not critical (20, 25, 30, 37). Verification and detailed explanation are among the most important features of effective feedback (41). Moreover, it should be understandable, meaningful, well-defined, accurate, encouraging, non-judgmental, respectful, appropriate in amount, proposing, and guiding on how one can improve oneself (33). To develop feedback skills, experience-based learning methods should be applied to learners (2). In providing feedback training, role play and veterinarian-client simulations have drawn scholarly attention (4). These practices provided to the student by simulated clients (SCs), peers, or trainers (4, 14). The opportunity for students to obtain feedback from teachers, coaches, or peers can improve their clinical and communication skills (46).

Feedback is generally a teacher-oriented process (22). It may be preferable to give student/peer-oriented feedback instead of teacher-oriented feedback to enable students to receive effective and accurate feedback regarding their practices during their vocational education. Many students are known to be satisfied when they receive feedback as a general approach (8). The message that is given in the effective feedback plays an active role in the learning process of the student (8, 22, 33, 38). Students who provide feedback improve their clinical skills, perform better at clinical practices and make fewer mistakes (46). When students do not receive any feedback about their performance, they do not have the chance to evaluate themselves (20).

In the literature, there are studies involving peer-assisted learning (PAL) in the process of providing feedback (17, 31, 39). Peer feedback is usually formative, aids in learning, and enhances motivation (14). PAL is a method in which people obtain help from their peers to develop knowledge or skills (32). The access of a student to information with the help or guidance of another student is based on experiencing that information. This training method has become widespread in many health sciences, including medicine (18, 23), dentistry (10), nursing (11), pharmacy (13), and veterinary medicine (12). In a more specific framework, PAL has been used for training in areas such as anatomy (3), surgery (12), clinical skills (6, 26), laboratory studies (45), and communication skills (42).

According to the Day One Competences of ECCVT pertaining to the skills that a newly graduated veterinarian should have, people are expected to interact with their peer groups to increase their professional performances (15). PAL, which facilitates interaction between students, seems to contribute to professional teamwork that encourages successful cooperation within the same work environments (6). Furthermore, by using PAL, the learning activity can take place in a calmer environment, and students can ask questions more easily about topics they do not understand (23). The existence of programs such as communication education (University of Tennessee Peer-Assisted Communication Training (UT-PACT)) (42), role play applications (Peer Role Play (PRP)) (18), and scenario-based training (35) which focus solely on PAL, further highlights the importance of peer training.

Studies on feedback skills have been conducted within the scope of communication skills courses in medical education, of which Hacettepe University Faculty of Medicine is a pioneer in Türkiye. Within the scope of these studies, a guide (the *Providing Feedback Guide, in Turkish: Geribildirim Verme Rehberi*) that was prepared by Elçin et al. (16) has been used in training medical students, standardized patient programs, and the Training of Trainers courses organized at various times for academics at that medical school. But feedback training and PAL in the field of veterinary medicine in Türkiye is a nascent area of research. There is no assessment tool for feedback in veterinary medicine that has been adapted into Turkish and for which validity and reliability studies have been conducted. As of the beginning of 2021, no published study about PAL conducted with veterinary students in Türkiye could be found.

To draw attention to the deficiencies in this area, this study aims to improve the feedback skills of veterinary students through the PAL method. This study focuses on measuring the effectiveness of learning assisted by peers in developing veterinary students' feedback skills. Another goal is to investigate whether the students' academic year or gender have any impact on the acquisition of feedback skills via PAL. This study, which identifies the gap regarding feedback skills and PAL in the field of veterinary education, has been carried out to be a pioneer in this field and to form the basis for future studies.

Materials and Method

Study design: This study was carried out at a single university with a small group of students as a preliminary study on training veterinary students to provide feedback by peer-assisted learning. The population of the overall study was drawn from students at Ankara University Faculty of Veterinary Medicine in the 2019–2020 academic year, with the sample comprising 60 volunteer students (19 male and 41 female). In the study, tutor (n = 20), control (n = 20) and tutee (n = 20) groups were formed. Each group had four students from each year from

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first to fifth. Distribution of these students in the groups was based on the order in which volunteers applied.

The study was designed with a pre-test/post-test control group design. The working schedule of the tutors consisted of a pre-test, training, and post-test-1, while that of the control group consisted of a pre-test and post-test. Tutees' schedule consisted of a pre-test, post-test-1, training, and post-test-2.

First step of the study was designed to evaluate whether tutor students could gain the ability to give feedback from the client's perspective. For this purpose, a pre-test was applied to tutors. After the pre-tests, tutors received training on the feedback education of other students (tutees) using the PAL method. The training, which lasted four hours, included role play in veterinary medicine, examination of the scenarios used in the pre-test and post-test, the basis of the veterinarian-client-patient relationship, communication in veterinary consultation, basic communication skills for veterinarians, characteristics of effective feedback skills, and a roadmap on how to carry out peer-to-peer learning. As for educational material, basic theoretical knowledge on the above subjects was provided, and videos about communication skills in veterinary consultations and their feedback sessions were shown. Discussions about and evaluations of the videos were carried out, giving and receiving feedback exercises were practiced with the students, and the students gained experienced training one another. The students were given the opportunity to practice as much as they wanted in the communication room. In the training schedule, the above subjects, other than providing feedback, were practiced to avoid any problems during role play, but these subjects were not evaluated in the assessment of the students. During this phase of the effort, the control group did not receive any training, and after this step, post-tests were applied to all the students.

Second step of the study examined whether PAL could provide students with the ability to give effective feedback. All tutees took the pre-test and post-test-1. Before any intervention was undertaken with the tutees, the results of these two tests were examined to see if there were any differences. This analysis aimed to show whether there was a time-dependent difference between the pre-test and post-test-1 of the tutees. Immediately after post-test-1, the tutors trained the tutees using the PAL method. The training lasted four hours and included examinations of the scenarios used in the pre-test and post-test, the basis of the veterinarian-client-patient relationship, communication in veterinary consultation, and the characteristics of effective feedback skills. The participants were given the opportunity to practice with peers as much as they wanted. In the role plays, the tutors demonstrated their clinical communication skills as veterinarians, while tutees acted as clients. The mission of the tutees was to give feedback to the tutors about their clinical communication from the client's perspective. After the training, all tutors and tutees completed post-test-2.

All pre-tests and post-tests were conducted between two students as role play. Two similar scenarios were prepared to be used in pre-test and post-test for all students. The scenarios were based on the clinical examination of a pet brought to a veterinary clinic. The scenarios did not include specific topics such as a difficult client, breaking bad news, end-of-life conversation, and so on. In the pre-test and post-test, depending on the scenario, when a tutor acted as a client, a tutee acted as a veterinarian. Role plays about clinical consultation took about 7–8 minutes. After the role play in each feedback session, the student who acted as a client gave 3–4 minutes of feedback to the student playing the veterinarian about the latter student's communication skills.

The role plays were held in a room with a two-way mirror on one wall. The researcher observed all role plays from the control room behind the mirrored wall for as long as those conversations continued. After each role play, participants were checked according to the Providing Feedback Guide (Table 1) (16) whose content validity was provided by expert opinion. This guide had to be used in this study since no other scale or measurement tool has been adapted to Turkish for use in veterinary medicine. Students successfully completing the skills from the guide received one point for each item.

Table 1. Providing Feedback Guide.

Items	Yes	No
1. Stated the behavior rather than focusing on individual characteristics.		
2. Focused on observations rather than deducing.		
3. Described the problem rather than judging.		
4. Preferred terms in the identification of the behavior such as frequent/seldom rather than using adjectives such as good/bad.		
5. Stated behavior related to a special condition.		
6. Shared information and facts with the other person rather than making suggestions.		
7. Explained the options to the student rather than answering/providing solutions.		
8. Answered the needs of the recipient.		
9. Gave an appropriate amount of feedback.		
10. Gave feedback at the appropriate time.		
11. Focused on what to say rather than why.		

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As last step of the study, open-ended qualitative questions were posed to the students to learn their opinions on feedback and PAL. These questions covered whether the study met their expectations, how successful the student was in providing feedback, the advantages and disadvantages of the PAL method, and whether the student wanted to provide peer training later. In order to create a qualitative data set, answers were transcribed, and a thematic analysis was undertaken. The transcripts were coded by the author of this paper, and the coding was reviewed by an independent expert.

Statistical analysis: For the study, the descriptive statistics were presented as mean±standard deviation. Repeated measures ANOVA was used to determine the effect of pre-test and post-test (within factor), groups (between factor) and the interaction term of these factors. In case of the detection of any statistically significant effect in the interaction terms, simple effect analysis was performed with Bonferroni correction as a post-hoc test. SPSS 14.01 (SPSS Inc., Chicago, IL) was used to perform all the statistical analyses. $P<0.05$ was considered statistically significant.

Results

In the study, regardless of the groups (tutors, control group, and tutees), academic years, and gender of the participants, the researcher observed a statistically significant change between the mean pre-test and post-test scores ($P<0.001$, $P<0.001$, and $P<0.001$) (Table 2 and Table 3). When the researcher separately evaluated the groups, the tutors and the tutees showed a statistically significant difference and there was no difference in the control group (Table 2 and Table 3).

Table 2. Results of the tutors and control group.

		Pre-test Score	Post-test Score	Estimated Marginal Means	Score	Group	Score-Group
Group	Tutors	2.35±0.43[b]	7.95±0.41[a]	5.15±0.37			
	Control	2.90±0.53[a]	4.00±0.48[a]	3.45±0.37	<0.001	<0.001	<0.001
Estimated Marginal Means		2.63±0.34	5.98±0.32				
Academic Year	First	3.50±0.80	5.75±0.65	4.63±0.68			
	Second	2.50±0.96	5.50±1.16	4.00±0.68			
	Third	1.75±0.70	6.25±0.98	4.00±0.68	<0.001	0.914	0.764
	Fourth	2.50±0.78	5.88±1.02	4.19±0.68			
	Fifth	2.88±0.55	6.50±1.27	4.69±0.68			
Estimated Marginal Means		2.63±0.35	5.98±0.47				
Gender	Male	1.92±0.48	6.17±0.71	4.04±0.54			
	Female	2.93±0.43	5.89±0.57	4.11±0.36	<0.001	0.572	0.271
Estimated Marginal Means		2.42±0.37	6.03±0.49				

[a,b] Different lowercase letters indicate statistically significant difference among columns.

Table 3. Results of the tutors and tutees.

		Pre-test	Post-test-1	Post-test-2	Estimated Marginal Means	Score	Group	Score-Group
Group	Tutors	4.35±0.43[b]	9.75±0.46[a]	10.20±0.29[a]	8.10±0.33			
	Tutees	2.60±0.50[b]	3.65±0.51[b]	7.05±0.48[a]	4.43±0.33	<0.001	<0.001	<0.001
Estimated Marginal Means		3.48±0.33[c]	6.70±3.42[b]	8.63±0.281[a]				
Academic Year	First	3.63±0.71	6.13±1.27	9.00±0.68	6.25±0.88			
	Second	2.88±0.58	6.38±1.51	9.25±0.75	6.17±0.88			
	Third	4.00±0.91	7.63±1.07	8.13±0.90	6.58±0.88	<0.001	0.983	0.308
	Fourth	4.00±1.09	5.88±1.52	7.75±1.18	5.88±0.88			
	Fifth	2.88±0.69	7.50±1.45	9.00±0.68	6.46±0.88			
Estimated Marginal Means		3.48±0.36[c]	6.70±0.62[b]	8.63±0.38[c]				
Gender	Male	2.71±0.49	6.50±1.03	8.50±0.70	5.91±0.64			
	Female	3.88±0.47	6.81±0.74	8.69±0.45	6.46±0.47	<0.001	0.486	0.512
Estimated Marginal Means		3.30±0.36[c]	6.66±0.63[b]	8.60±0.40[a]				

[a,b,c] Different lowercase letters indicate statistically significant difference among columns.

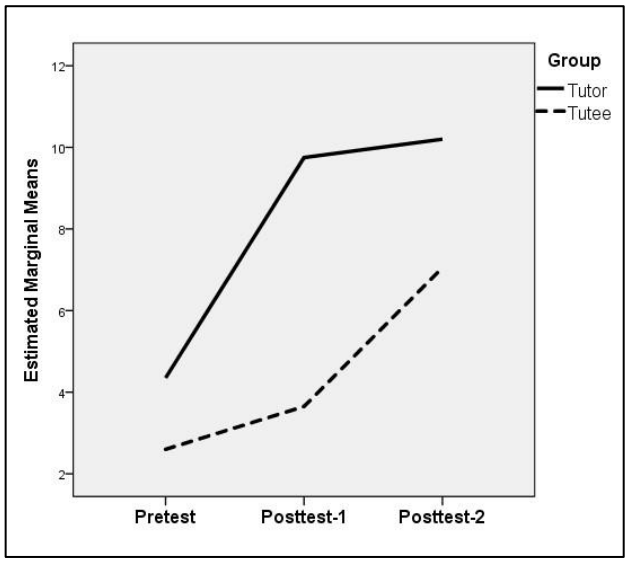

Figure 1. The contribution of PAL to the tutors and the tutees.

Regardless of the effect of repetition, there was a statistically significant change between the mean scores of both "tutor and control groups" (P<0.004) and "tutors and tutees" (P<0.001) (Figure 1). There was no statistically significant difference in terms of the academic year and gender between both "tutors and control group" (P=0.914 and P=0.572) and "tutors and tutees" (P=0.983 and P=0.486) (Table 2 and 3).

The frequency of the responses of the students to each skill in the Providing Feedback Guide was analyzed. In the pre-tests, the skills on which most students did not show were the fourth (n = 60) and seventh (n = 58) of the guide, while the skills on which the most students did show were the second (n = 40) and first (n = 35). In the post-tests, the skills on which most students did not perform were again the fourth (n = 45) and seventh (n = 38), while the skills on which most students performed were the second (n = 58) and first (n = 57) skills of the guide.

All tutors who gained the ability to give feedback volunteered to provide PAL by stating that they wanted to continue working. Some of the qualitative data collected from the students at the end of the study are reported below.

One student who acted as a client stated difficulty of feedback that, "It was difficult to give feedback to others, but I felt it was useful for someone." Some students' opinions on how successful they were in providing feedback follow.

"I had never given feedback before, so I do not know whether I was successful or not."

"I think I gave him effective feedback. I focused on the highlights I learned in the training."

"While I was giving feedback, I constantly envisioned that veterinary-client consultation. Thus, I focused on giving accurate feedback."

"Trying to give feedback was tiring me."

One tutee noted one of the strengths of the PAL that "A senior student trained me. We became friends after training."

Another stated the following: "I also want to teach other students. Can I join any further studies?"

A tutor student mentioned that "The study was exactly as I expected. It was enjoyable to interact with friends, to teach them something."

One tutee stated that "It was comfortable to have a role play with a person I met before." and "It relieved my tension that the person who showed my mistakes was not a teacher."

Discussion and Conclusion

The statistically positive change between the pre-test and post-test scores of the tutors over time (Table 2) can be evaluated as the result of the training this group received. Although the students in the control group did not receive training, an increase, which was not statistically significant, was also observed between their pre-test and post-test scores. This increase may be an effect of repetition because of their exposure to the same skills a second time to facilitate their ability to give feedback. The increase in the scores of tutors was greater than that of the control group. This result confirms the hypothesis that the efficiency of the training is responsible for the positive change in the scores of the tutors.

The researcher evaluated the enthusiasm of all students with regard to whether they viewed providing tutoring as one of the advantages of the study. The findings indicate that tutors will be capable of participating voluntarily in future studies. According to similar results of the one study (6), many students wanted to provide peer training voluntarily.

In the second step of the study, there was an overall positive change over time between the pre-test and post-test scores of both the tutors and tutees (Table 3, Figure 1). However, as Figure 1 shows, in spite of the increases in both groups after training, the changes in the scores of the tutees were more remarkable than those of the tutors. This is an indication that PAL can help students to enhance their skills in providing feedback. Furthermore, the post-test-1 and post-test-2 scores of the tutors were analyzed, and it was seen that the tutors had developed themselves after offering training in PAL to the tutees. Tutors who provide training advance themselves as well; therefore, providing training can also be instructive. According to the results of this study, the student in the tutoring position benefits from the training as much as the tutee does. Many researchers have reported similar results, indicating that PAL provides benefits for tutors and tutees (6, 7, 24, 32). Being a tutor seems to improve communication skills, contribute to teaching skills (6, 7, 28, 32, 43), and even

support the ability to give feedback (43). There is evidence in the literature that giving peer feedback is a skill that can be learned and can be upgraded over time (14). It may be beneficial for students to learn from peers through small group trainings, especially in veterinary schools where the ratio of the academic staff (educators) to students is limited and particularly in areas such as laboratories, clinical practices, and communication education.

In the literature, two students who are in the same class are called peers or true peers, and students in different classes are referred to as near-peers (7, 9). The peers in this study included both students in the same classes and those in different classes. The difference between peers and near-peers has been ignored in this study, which may be a limitation. However, one of the results of this study is that the academic year does not affect peer training, so the difference that is being ignored is not considered to be a disadvantage. Nevertheless, the researcher predicts that comparative analysis of peer-assisted and near-peer-assisted learning in future studies will contribute to the literature of the field.

It has been reported that most students preferred to receive feedback from peers as SCs (instead of real actors) (42). The qualitative data in the present study support the view that some students tend to be comfortable when role playing and receiving feedback from peers. In one such study in the literature (42), students reported the feedback from their peers who participated as SCs as being one of the strengths of the training. It has been reported that peers can be effective feedback sources (20).

According to a frequency assessment of the items in the Providing Feedback Guide, most students showed the ability to observe behaviors while giving feedback (see items 1 and 2). Related literature (20, 30, 36, 37) has also reported that effective feedback should focus on specific behaviors and tasks. The findings presented here are in accord with earlier findings in the literature. In addition, while giving feedback, most students could not identify the behavior requested in item 4 and could not explain the options about what should be done (item 7). This may be due to the fact that these items required more complex verbal communication skills to express and greater experience to assess the behaviors of peers in role play.

When the effect of the academic year and gender on PAL is evaluated, it is seen that the individual positive change in the scores of the tutors in each class (academic year) during the post-tests is remarkable. However, not enough information is available to reveal which class was the most successful in giving feedback. Revealing the effect of the academic year will require conducting comprehensive studies in the future, and this area is open to research. The fact that changes in the academic year and gender groups in tutees were not statistically significant shows that all participants were affected similarly by PAL (Table 3). This tends to be an advantage of incorporating peers into the training. It can be said that the use of PAL in this study led students to succeed, regardless of gender. While researchers in various studies in the communications field have emphasized that females generally perform better than males (19, 29, 34, 39); in a national study (44) conducted with male and female veterinary students, no gender difference was revealed in students' self-evaluation of their communication skills. Similarly, the gender results in the present study differ from the global trend in the communication literature.

As a consequence, it can be argued that all groups benefited from the feedback training. Tutees benefited from being trained by peers (tutors), and tutors also improved by training others. In other words, both tutors and tutees had the opportunity to improve themselves and increase their feedback skill levels due to teaching. In the study, which aimed to equip the students with the ability to give feedback with PAL, peer training proved to be effective for both tutors and tutees. Neither the academic year nor the gender of the students changed the effectiveness of this training. Within the framework of this research, the researcher recommends examining whether PAL is effective in gaining other skills in communication (effective listening, taking a medical history, giving bad news, coping with difficult clients) in future studies.

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Conflict of Interest

The author declared that there is no conflict of interest.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was approved by the Ethics Committee of Ankara University (Date: 24.09.2019; Number: 16/260).

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An integrative study of morphological and molecular characterization of *Diplectanum aequans* (Diplectanidae: Monogenea) infecting European sea bass *Dicentrarchus labrax* (Linnaeus, 1758) from Turkish coasts

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ABSTRACT

The diplectanid monogenean *Diplectanum aequans* (*D. aequans*) (Wegener, 1857) is one of the most important ectoparasites in the wild and cultured European sea bass *Dicentrarchus labrax* (*Dic. labrax*) (Linnaeus, 1758) worldwide. Presently, there is no information on the molecular data of *D. aequans* from Turkish coasts. In the present study, diplectanid monogeneans were collected from wild and cultured *Dic. labrax* in the Black Sea (FAO area 37.4.2) and Aegean Sea (FAO area 37.1.3) coasts of Türkiye, morphologically identified, and genetically characterized by sequencing of 28S rRNA and 18S rRNA fragments. The collected diplectanids were unambiguously identified as *D. aequans* based on detailed morphological features and sequence characterization of partial 28S rRNA and 18S rRNA genes. The overall prevalence and mean intensity of *D. aequans* were 100% and 15.1, respectively. This study reports first information of molecular (28S and 18S rRNA) evidence of *D. aequans* from *Dic. labrax* in the Turkish coasts. The new 18S and 28S rRNA sequences for *D. aequans* isolated from the Black Sea and Aegean Sea are genetically characterized. The 28S and 18S rRNA sequences of *D. aequans* can be used to resolve the phylogenetic positions of species found in the family Diplectanidae from the Black and Mediterranean Sea.

Introduction

The European sea bass, *Dicentrarchus labrax*, is the most commercially important fish species widely captured and cultured in the Mediterranean and the Black Sea. The larger producers of European sea bass are Türkiye, Greece, Italy, Spain, and Egypt in the Mediterranean countries, and Türkiye is a leader producer (9, 20). Monogeneans are common ectoparasitic flatworms of marine fish. The taxonomy of the monogenean family Diplectanidae (Platyhelminthes: Monogenea) comprises approximately 20 genera and >200 described species that attach to the gills of a wide diversity of marine fish (40).

Two nominal species of the Diplectanidae Monticelli, 1903 were described from the gills of European sea bass: *Diplectanum aequans* (Wegener, 1857) Diesing, 1858 and *D. laubieri* Lambert and Maillard, 1974 (18, 21), and exhibit strict host specificity (33). These two diplectanid species cause severe pathological damages in the gills and often death of the infected European sea bass (7, 10, 22, 35). Two diplectanids have also been reported in wild and cultured populations of European sea bass from the eastern Atlantic Ocean, English Channel, Aegean, Adriatic, Black, Mediterranean, and Red Sea (22, 35).

The ITS region, 18S, and 28S rRNA have been proven to be particularly useful for molecular characterization and the accurate identification of Diplectanidae species (4, 5, 25, 32). Although *D. aequens* has a wide geographic distribution, there has been limited information on its genetic diversity with only one publication from the Balearic Sea (Western Mediterranean Sea, 80 FAO area 37.1.1) (32). There had been no reports of characterizing the *D. aequans* from *Dic. labrax* using partial 28S and 18S gene fragments in the Black Sea (FAO area 37.4.2) and Aegean Sea (FAO area 37.1.3).

Our study aimed to gain new knowledge about the prevalence, intensity, and genetic diversity of *D. aequans* in wild and cultured European sea bass in the Black Sea and the Aegean Sea coasts of Türkiye using a combination of morphological and molecular analyses.

Materials and Methods

Sampling: A total of 40 wild and cultured European sea bass, *Dic. labrax*, were sampled from the Black Sea and Aegean Sea coasts of Türkiye. All fresh fish samples were purchased from fish markets and immediately transferred to the laboratory for parasitological examinations. Gills were removed and placed in 0.9 % saline solution and examined for diplectanid monogeneans under a stereomicroscope. Monogenean parasites were collected, counted, and preserved in 70 % ethanol. All specimens were individually mounted on slides in glycerol-gelatine and, then identified using morphological characters under a light microscope according to taxonomic keys (10, 18, 21, 26). Morphologically identified *D. aequans* were also measured, photographed using a light microscope with Leica application suite software and Leica MC 190 HD digital camera, and stored 96 % ethanol until DNA extractions. Five representatives (3 diplectanids; two from cultured and one from wild population from the Black Sea and 2 diplectanids; one from cultured and one from wild population from the Aegean Sea) were subjected to molecular analysis. Prevalence (P) and mean intensity (mI) were calculated according to Rózsa et al. (24) by using Quantitative Parasitology software (23).

PCR assays, DNA sequencing and phylogenetic analyses: Total genomic DNA (gDNA) was extracted from individual diplectanids using a commercial genomic DNA extraction kit (GeneJET Genomic DNA Purification Kit, Thermo Scientific, Lithuania EU) following the manufacturer's instructions. Concentration of extracted gDNA was measured using a spectrophotometer (Thermo Scientific, USA) at 260 nm, diluted to 10–50 ng/µl in TE buffer, and stored at −20 °C. The partial large subunit ribosomal RNA (28S rRNA) gene was amplified using the primers C1 (5'–ACCCGCTGAATTTAAGCAT–3') and D2 (5'–TGGTCCGTGTTTCAAGAC–3') (12). PCR reaction was performed in 50 µl volumes that contained 10–50 ng gDNA, 1X PCR Buffer with KCI (Thermo Scientific), 1.5 mM of $MgCl_2$ (Thermo Scientific), 200 µM of each dNTP (Thermo Scientific), 0.5 µM each of primers, 1.5 U of Taq DNA polymerase (Thermo Scientific), and nuclease-free water (Thermo Scientific). Cycle conditions were initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 60 s, 56 °C for 60 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min (12). The partial small subunit ribosomal RNA (18S rRNA) with internal transcribed spacer 1 (ITS1) region was amplified using L7 (5′–TGATTTGTCTGGTTT ATTCCGAT–3′) and IR8 (5'–GCTAGCTGCGTTCTT CATCGA–3') primer pairs (27, 31). PCR reaction was performed in a final volume of 50 µl containing 10–50 ng gDNA, 1X PCR Buffer with KCI (Thermo Scientific), 1.5 mM of $MgCl_2$ (Thermo Scientific), 200 µM of each dNTP (Thermo Scientific), 0.5 µM each of primers, 1.5 U of Taq DNA polymerase (Thermo Scientific), and nuclease-free water (Thermo Scientific). Reaction conditions were as follows: 4 min at 95 °C, then 35 cycles of 60 s at 92 °C, 60 s at 53 °C and 90 s at 72 °C followed by a final elongation of 10 min at 72 °C (27, 31). PCR products were visualized using a 1.5% agarose gel, and purified using a commercial kit (GeneJET PCR Purification Kit, Thermo Scientific). Purified products were bidirectionally sequenced with the same primers using an ABI PRISM 3130xl by Macrogen Company.

The quality of the sequences was checked with Phred scores ($Q \geq 20$), and sequences were assembled using Geneious R11 (16). The consensus sequences were blasted in GenBank for species identification (1). Subsequently, previously published sequences for species belonging to the Diplectanidae were selected from GenBank. Consensus sequences were aligned with those of Diplectanidae sequences using ClustalW (29) within MEGA X (17). Alignments were cleaned from ambiguous positions using Gblocks Version 0.91b (3). After editing the partial 28S and 18S rRNA with ITS1 region sequence alignments using Gblocks comprised 880 bases (90 % of the original 973 bases) and 402 bases (87 % of the original 457 bases), respectively. Pairwise estimates of evolutionary divergence (p–distance) between trimmed, aligned sequences were calculated as the percentage using the Kimura two-parameter model using uniform rates and a partial deletion of 95 % in MEGA X (17). All positions with less than 95 % site coverage were eliminated. That is, fewer than 5 % alignment gaps, missing data, and ambiguous bases were allowed at any position.

As there are more 28S rRNA sequences for *Diplectanum* specimens in the GenBank, we used only partial 28S rRNA sequences for the phylogenetic tree. There were a total of 804 positions in the final sequence dataset. Maximum-likelihood (ML) phylogenetic analysis was performed with PhyML (11) with automatic model selection using the AIC criterion (19) with topology

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assessed by bootstrapping with 100 replicates. We used a GTR + I + G model and *Tetrancistrum* sp. and *Cichlidogyrus* sp. as outgroups to do the ML analysis. Only bootstrap values above 70 were considered well supported (13).

Result

The P (%), mI and number of *Diplectanum* spp. infecting cultured *Dic. labrax* from the Black and Aegean Sea were 100 %, 26.40 and 264, and 100 %, 11.1 and 110, respectively. While the P (%), mI and number of *Diplectanum* spp. infecting wild *Dic. labrax* from the Black and Aegean Sea were 100 %, 9.90 and 99, and 100 %, 13.10 and 131, respectively. A total of 604 diplectanids were collected from gills of wild and cultured European sea bass in the Black and Aegean Sea coasts of Türkiye. The overall P and mI of diplectanids from the Black and Aegean Sea were 100 % and 15.1, respectively (Table 1).

All diplectanids were morphologically identified as *Diplectanum aequans* according to the identification keys. Ethanol-preserved our specimens (n = 10) were measured about 13.8 (11.3–17.8) mm in length and 0.32 (0.2–0.38) mm in width. Four eyespots were clearly visible in the cephalic part. There was a straight cirrus measured 170 (156–188) µ in length. The end of cirrus characteristically tapered and slightly ended as curved. We observed two squamodiscs (one dorsal and one ventral). A squamodisc was measured 131 (110–145) µ in diameter and typically exhibited 24–25 rows of sclerotized pieces (Figure 1).

Table 1. Epidemiological parameters of *Diplectanum* spp. in *Dic. labrax* from Turkish water.

Locality	n	P (%)	mI	Number of collected parasite
		Cultured		
Black Sea (FAO area 37.4.2)	10	100	26.40	264
		Wild		
	10	100	11.10	110
		Cultured		
Aegean Sea (FAO area 37.1.3)	10	100	9.90	99
		Wild		
	10	100	13.10	131
Overall	40	100	15.10	604

n: examined fish, P: prevalence, mI: mean intensity

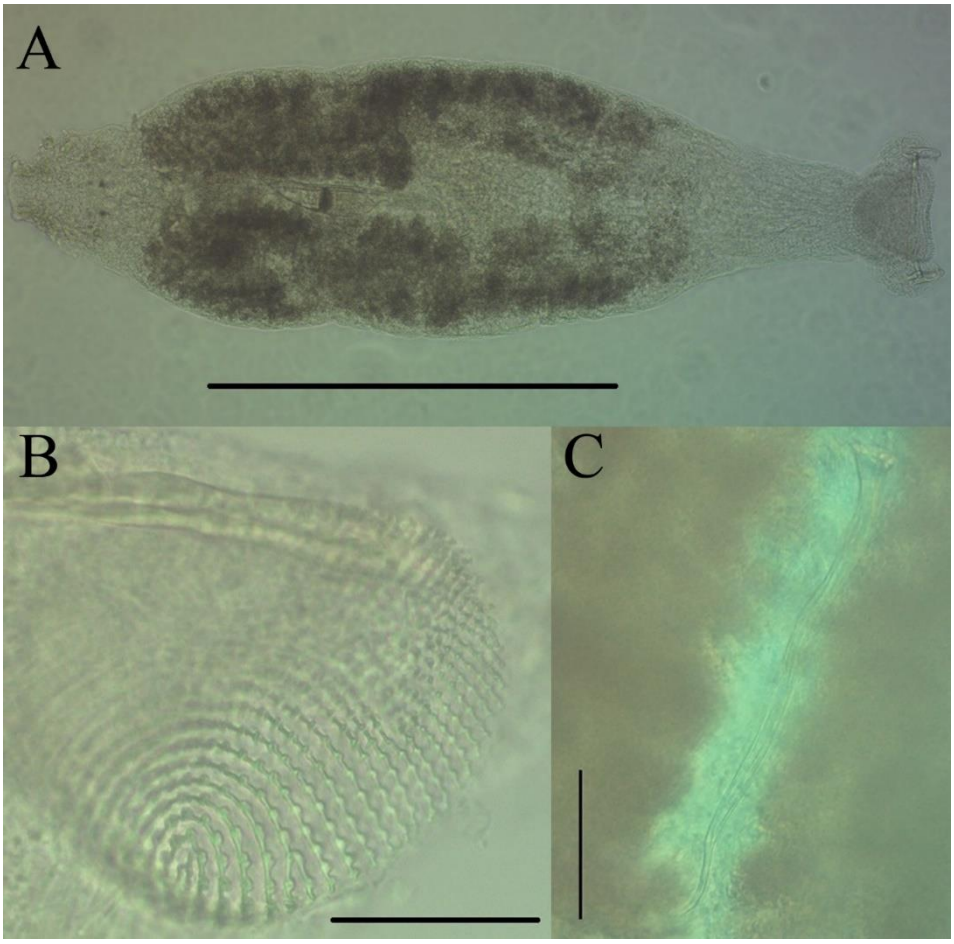

Figure 1. (A–C): Microphotographs showing main morphological features of *D. aequans* collected from *Dic. labrax* in the Turkish coasts: (A) whole body (scale bar = 500 µm), (B) detail of 24–25 rows of sclerotized pieces in a squamodisc (scale bar = 50 µm), (C) detail of cirrus (scale bar = 50 µm).

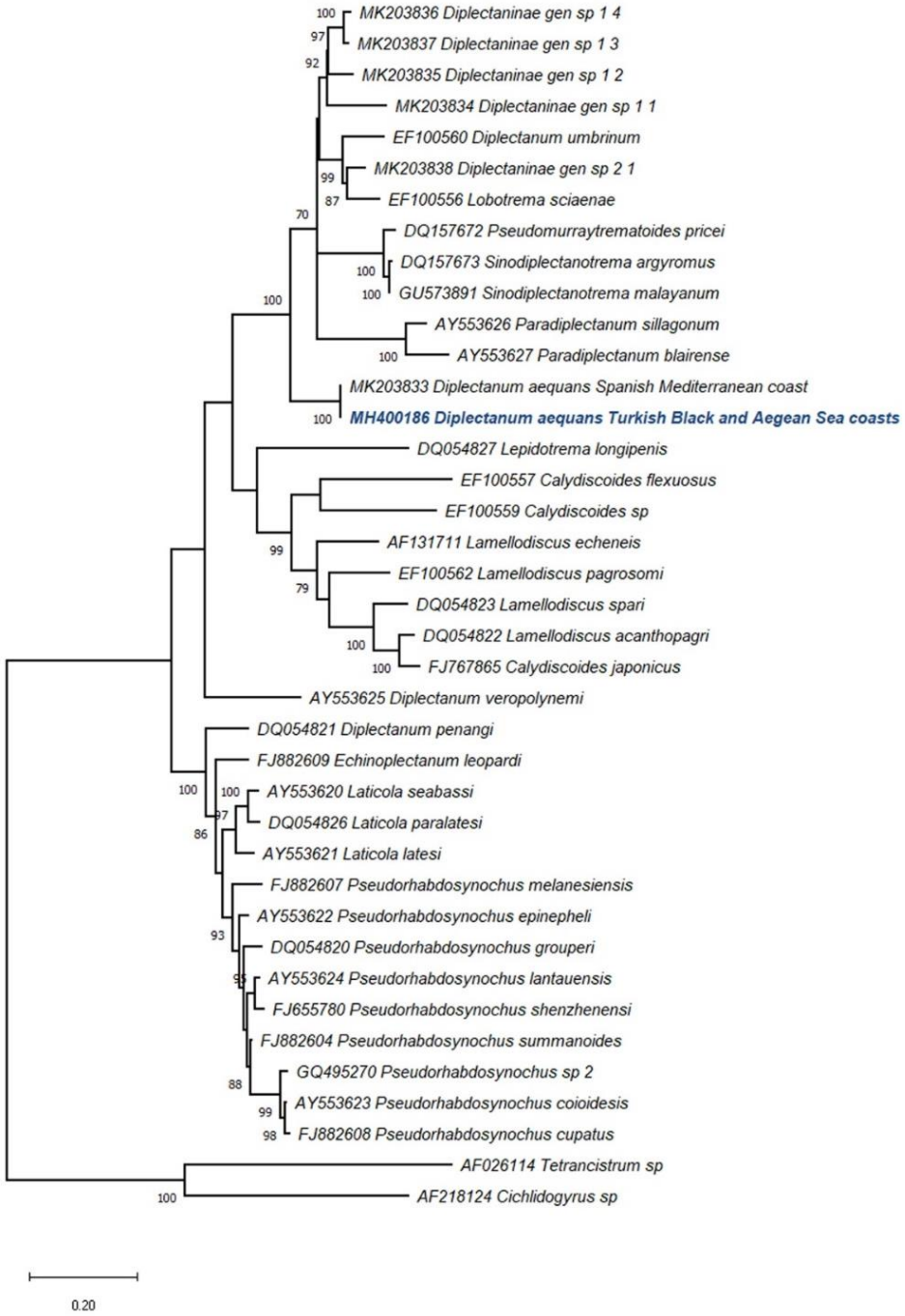

Figure 2. Phylogenetic tree generated by maximum likelihood analysis of the 28S rRNA sequences of Diplectanidae species. Only one representative sequence (coloured with blue and highlighted in bold) was used as all the five sequences were identical. Numbers above/below the branches are bootstrap confidence levels based on 100 replicates. Bootstrap values <70 are not shown. GenBank accession numbers are given beside species name. The scale shows genetic distance.

Five representatives were subjected to molecular analysis. The sequencing of partial large subunit ribosomal RNA gene and partial small subunit ribosomal RNA gene with ITS1 region of *D. aequans* produced two fragments of approximately 933 and 960 base pairs, respectively. Among representatives were not observed intraspecific nucleotide differences for two fragments. The sequences of 28S rRNA and 18S rRNA with ITS1 region of *D. aequans* from the Black Sea were deposited to GenBank with accession numbers MH400186 and MH400167.

Discussion and Conclusion

The diplectanids collected from wild and cultured *Dic. labrax* in the Turkish coasts were unambiguously identified as *D. aequans* based on detailed morphological

features previously reported (18, 21, 26), and sequence characterization of partial 28S rRNA and 18S rRNA data. The characteristic shape of the cirrus and the rows of sclerotized pieces in a squamodisc are the most diagnostic morphological structures to discriminate among valid two *Diplectanum* species in the European sea bass (10, 18, 21, 26). While *D. aequans* has the end of cirrus characteristically tapered and slightly ended as curved and arranged 18–31 rows of sclerotized pieces in a squamodisc, *D. laubieri* has the end of cirrus characteristically hook-shaped and made up 11-16 rows of sclerotized pieces in a squamodisc (10, 18, 21). Because the end of cirrus of our diplectanids tapering characteristically slightly end as the curve and typically comprise 24–25 rows of sclerotized pieces in a squamodisc, we did not hesitate to identify it as *D. aequans* in the present study (Figure 1). Most morphometric measurements of the same diplectanids which were preserved using the different fixation techniques may be insufficient for identifying the same species because different measurements could be obtained (5, 14). However, the shapes of sclerotized parts (cirrus and squamodisc) in diplectanids are preserved relatively well despite different fixation techniques and can still confidently be used for species morphological identifications (14). Therefore, we considered especially the shapes of the sclerotized parts rather than morphometric measurements in the morphological identification of diplectanids.

Molecular data provide us to understand the taxonomy, systematics, and phylogeny of Trematoda taxa. At least one conserved region (18S rRNA or 28S rRNA) and one spacer (ITS1, ITS2, or the entire ITS1–5.8S–ITS2) region of ribosomal DNA should be targeted for trematode taxonomy and systematics in the genetic analyses (2). The molecular methods combined with morphological identifications have been commonly used to identify species of diplectanids and estimate their phylogenetic relationships (4, 5, 25, 32). The 28S rRNA sequence of *D. aequans* in the present study matched 100 % with the 28S rRNA sequence of *D. aequans* (accession number MK203833) from the Spanish Mediterranean coast available in GenBank (32). Our diplectanid species had 85.38–87.04 % similarity with 28S rRNA sequences of Diplectaninae gen. spp. (accession numbers MK203834–MK203838) from the Spanish Mediterranean coast (32) (p–distance = 12.98–16.57 %), 83.73% with *D. umbrinum* larvae (accession number EF100560, unpub. data) (p–distance = 18.02%), 86.77 % with *Paradiplectanum* (*Diplectanum*) *sillagonum* (accession number AY553626) from the South China Sea (p–distance = 19.88 %) (34), 85.33 % with *P.* (*D.*) *blaiense* (accession number AY553627) from the South China Sea (p–distance = 21.38 %) (34), 85.71 % with *D. penangi* (accession number DQ054821) from the South China Sea (p–distance = 25.78 %) (37), and 84.05 % with *D. veropolynemi* (accession number AY553625) from South China Sea (p–distance = 25.78 %) (34).

In the present study, the length of the partial 18S rRNA and ITS1 fragments of *D. aequans* were comprised 457 and 503 bp, respectively. The partial 18S rRNA fragments of *D. aequans* reported herein from Turkish coasts showed 100% identity with the 18S rRNA of *D. aequans* (AJ276439 and AM943816) from the French Atlantic coast (6) and the Italian Mediterranean coast (28), respectively. The 18S rRNA sequence of our specimen was 96.96% identical to 18 rRNA of *P.* (*D.*) *sillagonum* (accession number AY553617) from the South China Sea (p–distance = 3.38 %) (34) and 95.78 % with *P.* (*D.*) *blaiense* (accession number DQ537356) (p–distance = 4.45 %). Comparison of partial 28S and 18S rRNA sequences confirmed that our diplectanid species in the Turkish coast are the same taxon as reported from the French Atlantic coast, the Italian Mediterranean coast, and the Spanish Mediterranean coast (6, 28, 32). This study also provides the first molecular confirmation of *D. aequans* sampled from cultured and wild *Dic. labrax* in the Black and Aegean Sea coasts of Türkiye.

Recently, it has been understood that *Diplectanum* species from the Spanish Mediterranean coast do not form a monophyletic group with previously sequenced diplectanids in the phylogenetic analyses using the 28S rRNA gene and therefore do not belong to a single genus, and *Diplectaum* species may represent three different genera as *Diplectanum* sensu stricto, Diplectaninae gen. clade B1 and Diplectaninae gen. clade B2 (32). The cladistic methods using comparative morphological characters were already supported the paraphyletic of *Diplectanum* genera (8). The phylogenetic results of 28S rRNA in congruence with those stated by Villar-Torres et al. (32), and our isolate was placed at the base of clade B included Diplectaninae gen. spp. from sciaenids (32) (Figure 2). Moreover, the polyphyletic status of the subfamily Diplectaninae has been reflected in the position of *Lobotrema* and *Murraytrema* (*Pseudomurraytrematoides*) genera as a sister group of *D. aequans* in clade "P" in the cladistic analysis (8). In the present study, our *D. aequans* phylogenetically represents a sister group of *Lobotrema* and *Murraytrema* (*Pseudomurraytrematoides*) genera and also supports the view of Domingues and Boeger (8) (Figure 2). Additionally, there are only available two 18S sequences from the French Atlantic coast (6) and the Italian Mediterranean coast (28), and one 28S rRNA and one entire ITS sequences from the Spanish Mediterranean coast (35) in the GenBank for *D. aequans*. This study is also the new record of 18S and 28S rRNA sequences for *D. aequans* sampled from the Black Sea in GenBank.

In conclusion, the current study reports molecular (18S rRNA and 28S rRNA) evidence of *D. aequans* from *Dic. labrax* in the Turkish coast for the first time. These molecular data can be used to resolve the phylogenetic position of Diplectanidae. Molecular data for species representing Diplectanidae are still currently lacking from Turkish waters. Further combining morphological and molecular studies are needed for resolving the phylogenetic relationship, taxonomy, and classification of the Diplectanidae in the coasts of Türkiye.

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Conflict of interest

The authors declare that they have no competing interests.

Author Contributions

CA and GZP conceived and planned the experiments. CA and GZP carried out the experiments. CA and GZP planned and carried out the simulations. CA and GZP contributed to sample preparation. CA and GZP contributed to the interpretation of the results. GZP took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

For this study, ethics committee approval was not needed because no handling of live marine teleost specimens was involved.

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An evaluation of Türkiye livestock congresses (1968-2000)

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ABSTRACT

Eight livestock congresses were held between 1968-2000 under the Turkish Veterinary Medical Association (TVMA) leadership to improve animal husbandry in Türkiye. In these congresses, besides problems in the livestock sector, issues related to the production and consumption of animal products were also discussed. The aim was for the organizers and participants to be composed of different fields related to animal husbandry and thus address the issue from every perspective. The establishment of an autonomous "Ministry of Livestock" was demanded in all congresses. Problems and solution proposals regarding animal husbandry in Türkiye were scientifically discussed with veterinarians, producers, industrialists, consumers, and government representatives. As a result, this study, which was carried out to evaluate the issues addressed in Türkiye Livestock Congresses and contribute to the determination of the problems and solutions of the livestock sector, can shed light on the resolution of today's livestock problems.

Introduction

The first international meeting of veterinarians was held in Hamburg, Germany, on 14-18 July 1863, at the initiative of Professor John Gamgee to ensure cooperation in the prevention of epidemic animal diseases (15). It is known that the Ottoman Empire was informed about this first meeting and decided to send a representative (Veterinary Major Ahmet Efendi) to the second meeting to be held in Vienna in 1865. Participation in various international congresses has been observed since the meeting in Vienna (33).

In the founding years of the Turkish Republic, the fight against animal diseases and improving animal breeding were among the most significant problems. There were only two laws, one directive and two serum establishments in animal health at this time. In parallel with the decisions of the Izmir Economy Congress (1923), a five-year program was prepared for the reorganization of veterinary medicine, and it was put into practice as of 1925 (1, 23). During this period, agreements were made with neighboring and non-neighboring states, and congresses on epidemic animal diseases were held to export animals and animal products from Türkiye (26).

Established to support the efforts to overcome the world economic crisis (1929), the “National Economy and Savings Society” pioneered the holding of the Industry Congress in 1930 and the First Agriculture Congress in 1931. In these congresses, primary industry and agricultural experts discussed the problems and prepared reports outlining the ways of development. "Animal Husbandry" was among the topics that made up the agenda of the First Agriculture Congress (24, 31).

The Turkish Veterinary Medical Association (TVMA), established by the law, numbered 6343 to implement and develop the veterinary profession in Türkiye, including the production and development of animal husbandry policies[1]. In addition to the various scientific meetings, it has organized for this purpose,

[1] Official Gazette dated 18.3.1954 and numbered 8661.

TVMA has organized eight congresses, the first in 1968 and the last in 2000, focused only on animal husbandry (21). This study aims to analyze these congresses' contents and reveal their contribution to animal husbandry in Türkiye.

Materials and Methods

This historical study aims to evaluate eight animal husbandry congresses held between 1968-2000 under the leadership of the Turkish Veterinary Medical Association. The study material consists of the printed book of seven congresses and the congress file of the seventh Congress. In the study, the data obtained through document analysis, which is used alone or as an auxiliary method in qualitative research (17), was evaluated. The text was written using the retrospective method used in historical studies.

Results

Livestock congresses organized by the TVMA are examined according to the organization date. Information on the organizations arranging these congresses is presented in Table 1 chronologically.

First Livestock Congress of Türkiye (1968) (4)**:** The First Livestock Congress of Türkiye was held between 13-15 February 1968 in Labor and Social Insurance Institution Hall, Ankara.

The congress was organized in five sessions lasting three days, and 11 papers and three commission reports (Production, Marketing, Organization) were presented and discussed. It was reported that 78% of the participants were small business owners. In addition, State Enterprises, State Economic Enterprises, Chambers of Agriculture, Chambers of Commerce and Industry, Commodity Exchanges, Higher Education Institutions, the press, responsible official authorities, army representatives, some governors, mayors, senators[2] and deputies, and relevant Ministers attended. President Cevdet Sunay and Prime Minister Süleyman Demirel sent a message to Congress. 72% of the congress participants, which had 603 delegates, were animal breeders, 22% were representatives of official institutions, 4% were lecturers, and 2% were senators and deputies. Minister of Agriculture Bahri Dağdaş, Minister of Rural Affairs Turgut Toker, Minister of Public Health and Welfare Vedat Ali Özkan[3] also attended the Congress. In addition, representatives of the Chamber of Agriculture, Chamber of Commerce, Animal Husbandry Cooperative, representatives of all sectors related to animal breeding and sales, pharmaceutical industrialists, technical personnel, and scientists from almost every province of Türkiye were in attendance.

Among the objectives of the congress are; 1- discussing the issues related to animal husbandry open to the public, 2- promoting the sector with all its aspects, 3- pioneering the solution of the problems.

Table 1. Organizations that organize congresses according to years.

Organizing Institutions	Turkish Livestock Congresses (Year of Organized)							
	I. (1968)	II. (1970)	III. (1972)	IV. (1974)	V. (1976)	VI. (1978)	VII. (1981)	VIII. (2000)
AUFVM	+	+	+	+	+	+	+	+
UTCCI-CE	+	-	-	+	+	-	-	-
TVMA-CC	+	+	+	+	+	+	+	+
VMS	+	+	+	+	+	+	+	+
VMU	+	+	-	-	-	-	-	-
ACV	+	+	+	+	+	+	+	+
UCAT	-	-	-	+	+	+	-	-
KK	-	-	-	-	-	+	-	-

AUFVM: Ankara University Faculty of Veterinary Medicine, UCCET: The Union Chambers and Commodity Exchanges of Türkiye, TVMA-CC: Turkish Veterinary Medical Association - Central Council, VMS: Veterinary Medical Society, VMA: Veterinary Medical Union, ACV: Ankara Chamber of Veterinarians, UCAT: Union of Agricultural Chambers of Türkiye, KK: Köy-Koop

[2] Senator: A member of the Senate of the Republic is the legislative body that constituted the upper wing of The Grand National Assembly of Turkey (GNAT) when the bicameral system was in force between 1961-1980 in Turkey.

[3] The speech of the Minister at the Congress was reported in a daily newspaper. (Cumhuriyet, "Beslenme yetersizliği çocuklarda %45 ölüme sebep oluyor" (14 Şubat 1968), 1, 7.)

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Table 2. Türkiye First Livestock Congress Organizing Committee.

Name	Institution
Prof. Dr. Selahattin Gürtürk (President)	Faculty of Veterinary Medicine
Advisor Ragıp A. Saguner (Secretary General)	Turkish Chamber of Commerce and Industry / Union of Stock Exchanges of Türkiye
Prof. Dr. Mihri Mimioğlu (Member)	Faculty of Veterinary Medicine
DVM Halil Örün (Member)	Veterinary Medical Association
Assoc. Prof. Dr. Cemal Omurtag (Member)	Veterinary Medical Society
DVM Alim Ersoy (Member)	Veterinary Medical Society
Assoc. Prof. Dr. H. Saim Kendir (Member)	Veterinary Medical Union
DVM Vedat Banoğlu (Member)	Veterinary Medical Union
DVM Osman Ünverici (Member)	Ankara Region Chamber of Veterinarians
DVM Kazım Ekenleroğlu (Member)	Ankara Region Chamber of Veterinarians

DVM: Doctor of Veterinary Medicine.

The congress emphasized that the livestock sector is the main factor in Türkiye's economy and development. However, it was reported that it had always been neglected and left behind in agricultural production. It was said that the sector had not been provided with an adequate organization in economic potential, nutrition, and health power. It was proposed to establish the "Ministry of Livestock, Fisheries, and Nutrition" and to include the following general directorates: "Animal Health Protection", "Livestock Development and Crediting", "Nutrition and Food Control", "Animal Breeding and Control", "Fisheries", "Animal and Animal Products Evaluation-Marketing and Control" and "Organization."

It was decided that the Congress would be held every two years.

The members of the organizing committee of the First Congress and the information of their affiliated institutions are presented in Table 2.

Second Livestock Congress of Türkiye (1970) (5)**:** As it was decided in the first Congress, the "Second Livestock Congress of Türkiye" was held between 23-27 March 1970, again in the Labor and Social Insurance Institution Hall, Ankara, with the participation of 940 delegates. At the opening, the Ministers of Agriculture, Health[4], Trade, and Labor and Bülent Ecevit gave a speech and emphasized the importance of animal husbandry. In the Congress, four commissions were established as 1-Organization; 2-Public Services Oriented to Livestock; 3-Economic Problems; 4-Technical Issues.

It has been emphasized that the share of livestock in national income is 30%; however, it could increase to 70-80%. It is stated that it can play an active role in preventing hidden unemployment by providing employment. It has been stated that adequate and balanced nutrition can be provided with animal products. The export share can increase. This sector with an enormous potential has to work with limited opportunities dispersed in different organizations. The participants unanimously agreed to request the establishment of a "Ministry of Livestock, Fisheries, and Nutrition," which will cover all institutions in the sector such as animal health, production, and consumption of animal foods, aquaculture, animal feed production. In addition, it was decided to open a new veterinary faculty. As chairman of the organizing committee, Prof. Dr. Mehmet Sandıkçıoğlu emphasized the importance of the issue in his closing speech as follows: "*Livestock breeding is a great potential for the Turkish economy. Utilizing this potential is only possible if people at various levels, from the shepherd at the head of the animal to the housewife in the kitchen, from the youngest official to the minister on the job, work with complete understanding and confidence and consciously and succeed*."

Third Livestock Congress of Türkiye (1972) (7)**:** The Congress was held on 19-21 April 1972 in the General Directorate of State Hydraulic Works (SHW) hall, Ankara. As in the first two congresses, 1100 delegates from all branches of the livestock sector, Minister of National Defense and Deputy, Prime Minister Ferit Melen, Minister of Agriculture Orhan Dikmen, and many deputies attended. Prof. Dr. Saim Kendir stated in the closing session of the Congress that two essential issues emerged. First, the producer was not organized in the livestock sector. The second was the lack of importance given to public services related to animal husbandry within the Ministry of Agriculture.

Regarding the first issue, it was stated that the breeders would be supported until they established their

[4] The speech of Vedat Ali Özkan, Minister of Health at the time, was published as a newspaper article. (Cumhuriyet " Hayvancılık Kongresi'ni Sağlık Bakanı Özkan açtı: Her yıl 30 bin kişiyi kedi ve köpek ısırıyor" (24 Mart 1970), 1, 7.)

organizations. Regarding the other issue, it was emphasized that animal husbandry is an advanced branch of agriculture and even the agriculture industry. The share allocated to animal husbandry should be increased, and a *"Ministry of Livestock"* should be established.

Fourth Livestock Congress of Türkiye (1974) (8)**:** The Fourth Congress was held on 6-8 May 1974 in the Faculty of Language, History, and Geography Conference Hall, Ankara. It is known that the participation is still high, with 982 delegates from 57 provinces. It is emphasized in the congress book that animal husbandry, which has been neglected for many years, has developed in recent years and that the congresses have contributed to the country's livestock and livestock policy. Thus, the Ministry of Agriculture was changed to the Ministry of Food, Agriculture, and Livestock.

The closing speech stated that animal husbandry is a part of the nation's natural resources. These congresses were organized to organize the Turkish animal breeders, raise awareness, increase animal production, and mobilize the country's most significant resource. In addition, Türkiye's animal wealth was stated, and the necessity of increasing animal production was emphasized. Based on the project, it was declared that there was no opposition to importing female material, but Türkiye should not become the animal market of other countries. The decision stated again that to solve animal husbandry's problems and ensure the integrity of its services, the "Ministry of Livestock, Nutrition, and Fisheries" must be established.

Fifth Livestock Congress of Türkiye (1976) (9)**:** The Congress was held in the SHW Hall, Ankara, with approximately 1000 delegates between 26-28 April 1976. Prof. Dr. Kendir stated in his opening speech that a small number of papers would be presented at the Congress so that breeders could share their problems. He also expressed the problems related to animal feeds and emphasized the problems caused by importing milk powder and butter. He stated that economic policies are not sufficient, despite the measures on livestock in the Development Plans. Korkut Özal, Minister of Food, Agriculture, and Livestock, attended the Congress. In his speech, Özal stated that they considered the work done in other congresses; he described himself as "*...not only in charge of an organization but also as the advocate of all livestock breeders in Türkiye...*". In addition, he made the following evaluations regarding the problems mentioned in the previous Congress: 1- The application of the minimum price in animal products was implemented with a decree on January 16, 1976, and in fact, this is a fundamental policy in meat livestock rather than determining the minimum price; 2-Meat-Fish Institution was included in the Ministry of Food-Agriculture and Livestock for a solution regarding the disorganization of livestock establishments; 3- Crediting the farmers; 4- In order to spread artificial seeds for the improvement of animal breeds and to increase the breeding qualities of livestock in the country, "core management" will be encouraged; 5- The number of feed factories has been increased.

In response to the minister's speech, the delegates took the floor and convincingly expressed their objections with numbers. Examples of these objections are the importation of butter and milk powder; the intensive dairy project's difficulties for the last three years were explained. It has been proposed to establish a "Ministry of Livestock" and gather livestock services there.

In the last session of the Congress, two important proposals were made by the delegates. The first is about the "Livestock Federation," and the other is the "Organization and form of organization." In addition, it has been reported that a poultry congress will be held every year as a continuation of the animal husbandry congress. And, it was stated that the "Poultry Association" would be established, and the "Poultry Journal" would be published. As a result of the negotiations held in the last session, establishing a broad-based "Livestock Federation or Association" was accepted. The committee that would make the preliminary preparations was elected by unanimous vote.

Sixth Livestock Congress of Türkiye (1978) (10)**:** The Congress was held on 16-18 May 1978 in the SHW Hall.

In the preface of the Congress booklet written by Sadi Aral, it was stated that in the livestock congresses held for the last ten years, all the components of the livestock industry, namely the producer, consumer were expressed and socio-economic problems in the rural areas mentioned. It has been emphasized that the solution proposals taken in the congresses were included in the development plans and government programs. Each Congress exceeds the dimensions of the previous one, and "*The Turkish animal breeder and breeder's own free and loud voice has reflected the public under the authority and responsibility of the Turkish Veterinarians community…*" It was emphasized that the problems related to feeds, credit and financing, health, marketing, and organization in animal production could not be solved by taking sufficient consideration by the authorities. The Congress stated that it would be evaluated whether these problems were taken into account by the political power. In addition to these problems, a new animal health policy should be determined; It has been stated that the producer sells his product for nothing, and the consumer cannot benefit from it because it is sold at a very high price. Aral reported that the "Ministry of Livestock," mentioned in the last five livestock congresses, has not been established yet.

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Seventh Livestock Congress of Türkiye (1981) (11)**:** The only Congress whose congress book was not published[5] was held in SHW Hall on 2-4 December 1981 under the coordination of TVMA, in cooperation with Ankara University Faculty of Veterinary Medicine, Ankara Region Chamber of Veterinarians and Veterinarians Association. The letter, dated 23.02.1981 and numbered 700/200 on the organization of the Congress, was sent to the relevant institutions with the signature of TVMA Central Council President Dr. M. Yücel Akıncı. "Ragıp Saguner, Muammer Bülent Birol and Muzaffer Çınkıloğlu" were assigned with the letter dated 15.04.1981 and numbered 700/213 for the organization of the Congress. According to the response letters from the institutions, Ankara Region Chamber of Veterinarians Ankara VHO representative Agah Dikmen, İsmail Tanık from the Veterinary Medical Association, Assoc. Dr. Ergun Özalp and Bünyamin Gerger as the Council Representative.

It was emphasized that the provincial administrators and Veterinary Affairs Directorates should be assisted in the selection of delegates in order to carry out beneficial studies and obtain positive results in the Congress. In order to explain the importance of the sector for the country to the public, it was requested to make use of the mass media at all levels and announce that all veterinarians present in the region are invited to the Congress[6].

TVMA also sent a letter[7] to 67 provincial governorships in Türkiye and requested that the delegates who would attend the Congress be selected partners or members of organizations such as livestock unions, associations, and cooperatives in animal husbandry and production.

The Minister of Agriculture and Forestry, Prof. Dr. Sabahattin Özbek, and Deputy Prime Minister Turgut Özal attended the meetings on the first day. However, as it is understood, there was no official participation in the following sessions of the Congress.

Assoc. Prof. Dr. Ersoy Canküyer pointed this situation as blow:

"*... animal producers from all over our country are present in the hall; the problems of animal husbandry are discussed with all aspects and dimensions here, but no officials related to animal husbandry have been found, except for the first session.*"

More than 500 delegates from all provinces were present at the Congress, and some breeders stated that "*... we animal breeders have been left to our fate ...*"

Breeders stated that veterinarians also had difficulties just like them, and they could solve these acting together. Halil Ayvazoğlu, the breeder and dairy business owner from Kars, stated that the academicians and the theorists of the subject and those faced with the problems should be talking at Congress.

In the economic commission report, in order for livestock to fulfill its economic function measures, and suggestions were listed such as below:

Incentive, fight against animal diseases, feed problem, acceleration of breeding by artificial insemination, use of credit, marketing, cooperatives, consumer protection, smuggled meat problem, resource problem due to lamb slaughter, the organization of breeders and producers. As a result, the necessity of establishing a Ministry of Livestock was emphasized.

In the Report of the Technical Commission, it was stated that the development plans had no response and implementation in the field. Animal husbandry problems were summarized under five headings as:

I. Animal breeding, II. Meadow, pasture, and forage, III. Protection of animal health, IV. Processing, evaluation, and marketing of products and V. The private and public sector organization.

It was also stated that animal husbandry improved remarkably in the last ten years despite all the negativities.

Eighth Livestock Congress of Türkiye (2000) (13)**:** The Congress, organized by the TVMA Central Council, was held in Ankara on 22-23 June 2000 at the SHW Hall with more than 700 delegates.

In this Congress, all problems were expressed, concrete solution proposals were presented to the public and private sector, and the development plans of the political power were guided.

TVMA Council President Nesrin Alpaslan emphasized that the Livestock Congresses did not sufficiently achieve their goals to embrace all relevant sectors and shed light on the development plans and government programs prepared for its development. She stated that credit, financing, feed, health, and organization deficiencies in animal production could not be solved. Also, it was emphasized that the solution is only "*In the union of animal producers and industry, who deal with their problems, find solutions, and demand the return of their work and labor*." At the Congress, papers on pets, cattle and sheep, horse breeding, aquaculture, beekeeping, and poultry breeding were presented in six sessions and

[5] It was determined that this file was given to the "Veterinary Medicine History Museum" by Dr. Mehmet Yücel Akıncı. (Dinçer, F. (2018). "Legendary President" Dr. Mehmet Yücel Akıncı (March 2, 1940 - January 5, 2018) Interior: On the first anniversary of his death, Dr. Mehmet Yücel Akıncı Memorial Booklet, p. 19-20, Ankara

[6] Turkish Veterinary Medical Association's letter dated 20.09.1981 and numbered 700/48.

[7] Dated 18.09.1981 and numbered 700/47.

one panel. At the end of the sessions, the participants presented their ideas.

In the speeches made on the first day of the Congress, the Ministry's lack of participation and indifference was criticized. It was emphasized that animal importation in 1994-1995 played an essential role in the worse course of animal husbandry. As a result of these criticisms, the Minister of Agriculture and Rural Affairs (1999-2002), Hüsnü Yusuf Gökalp, participated in Congress on the second day and gave the opening speech.

Papers, conferences, and panel information presented in eight congresses are given in Table 3. Congressional commissions are presented in Table 4. The Ministry of Agriculture, which was established in 1924, took its current form in 2018. The changes in the Ministry's name are presented in Table 5.

Table 3. Proceedings presented at the Congresses.

	Presenter	Presentation Title
I. (1968)	Prof. Dr. Cumhur Ferman / *	*Industry and Livestock in the Development of Türkiye*
	Prof. Dr. İsmail Türk / Faculty of Political Sciences	*Agriculture and Livestock in the Development of Türkiye*
	Hayri Başar / *	*Current Commercial Occupational Problems of Turkish Livestock*
	Ragıp A. Saguner / *	*New Views on Marketing of Animal and Animal Products*
	Üzeyir Eren / Ministry of Agriculture, General Directorate of Veterinary Affairs Consultant	*Our Livestock in Foreign Trade and Its Situation Against the Common Market*
	Assoc. Prof. Dr. Süleyman Kara / Animal Science and Research Institute	*Livestock in Türkiye's Nutrition*
	Prof. Dr. Selahattin Batu / Faculty of Veterinary Medicine	*Zootechnical Measures to be Taken in the Development of Animal Husbandry in Türkiye*
	Prof. Dr. Hasan Başkaya / Faculty of Veterinary Medicine	*Animal Health Services in the Development of Our Animal Husbandry*
	Prof. Dr. M. Mihri Mimioğlu / Faculty of Veterinary Medicine	*Parasitic Diseases as an Important Factor that Hinders the Development of Animal Husbandry in Türkiye*
	Prof. Dr. Latif Berkmen / Faculty of Veterinary Medicine Prof. Dr. Zeki Tolgay / Faculty of Veterinary Medicine	*The Importance of Animal Nutrition and Food Control and Technology in Community Nutrition*
	Prof. Dr. Sabri Dilmen / Faculty of Veterinary Medicine	*Feed and Animal Nutrition Problems in Turkish Livestock Industry*
II. (1970)	Ertuğrul Gökgün / Konya Chamber of Veterinarians	*Economic importance of animal husbandry, economic and social consequences of pasture destruction*
	İbrahim Kutlutan / Turkish Standards Institute	*Standardization in the problem of developing our livestock*
	* / Union of Agricultural Chambers of Türkiye	*Development of animal feed resources*
	Nevzat Uludağ / Lalahan Animal Science Research Institute	*Research and education problems in animal husbandry in Türkiye*
	Nevzat Uludağ / Lalahan Animal Science Research Institute	*Problems in Türkiye Animal Husbandry*
	* / Istanbul Commodity Exchange	*Concentration and modernization in the meat industry*
	Şükrü Sıdal / Union of Chambers of Agriculture of Türkiye	*Livestock movements marketing and smuggling*
	* / Lalahan Animal Science Research Institute	*Merino-mohair problems in Türkiye*
	Prof. Dr. Ömer Ertürk / Faculty of Veterinary Medicine	*The fight against epidemics and its importance in the development of livestock*
	Feridun Taşman / *	*Model and organization study in laying poultry for foreign marketing in Türkiye*
	Assoc. Prof. Dr. Osman (Nuri) Koçtürk / Turkish Veterinarians Union	*Animal protein sources and food control problem in Turkish people's diet*
	Aydemir Aşkın / Izmir Chamber of Commerce	*Exports of animal and animal products at domestic prices*

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III. (1972)	Prof. Dr. H. Saim Kendir / Faculty of Veterinary Medicine	*Meat production and problems in Türkiye*
	Assoc. Prof. Dr. Orhan Alpan / Faculty of Veterinary Medicine	*Milk production and problems in Türkiye*
	Assoc. Prof. Dr. Servet Şenel / Faculty of Veterinary Medicine	*Feed and animal production relations*
	Ziya Dalkılıç / Aktavuk Farm	*Egg production and problems in the Turkish economy*
	Dr. İsmet Baran / Faculty of Veterinary Medicine Dr. Fethullah Koç / Ankara Chamber of Veterinarians	*Aquaculture and problems*
	Dr. Faruk İmeryüz - Dr. Şefik Müftüoğlu / Lalahan Animal Science Research Institute	*Wool and mohair production problems in the Turkish economy*
	Ragıp Saguner / Deputy Secretary General of the Union of Stock Exchanges of Türkiye	*Livestock economy and policy*
	Halil Örün - M. Necip Yılmazer / General Directorate of Veterinary Affairs Branch Manager	*Training of the breeder and breeder*
IV. (1974)	Zeki Yücetürk / Secretary General of the Union of Turkish Agricultural Chambers	*Fundamental problems of livestock breeders in Türkiye*
	Mahmut Yasankul / National Productivity Center Agriculture Specialist	*Animal products production and feed relations*
	Necati Ölez / MFAL Deputy Head of Feed Registration and Control Affairs	*Marketing of Animal and Animal Products*
	Ömer Lütfi Hekimoğlu / UCAT Livestock and Aquaculture Branch Manager	*Common Market and Animal Husbandry of Türkiye*
	Prof. Dr. Afif Sevinç / Faculty of Veterinary Medicine	*Breeding and main problems of animal husbandry in Türkiye*
	Hidayet Karaer / MFAL General Directorate of Veterinary Affairs	*The negative effects of epidemic animal diseases on our economy and foreign trade and the measures to be taken*
	Assoc. Prof. Dr. İsmet Baran / Faculty of Veterinary Medicine	*Possibilities to utilize the potential of our fisheries*
	Dr. Sadi Aral / Faculty of Veterinary Medicine	*Organizing in the livestock sector*
V. (1976)	Ömer Lütfi Hekimoğlu - Dr. Yücel Akinci / UCAT Secretary General	*Economic problems of livestock in Türkiye and solutions*
	Prof. Dr. Orhan Alpan, Dr. Nevzat Uludağ, Dr. Fethullah Koç / Faculty of Veterinary Medicine	*Milk production problems and solutions*
	Mahmut Yasankul / National Productivity Center Agriculture Expert Ragıp Saguner / Trade Exchanges Association Rafet Yavuz / *	*Meat production and fattening problems and solutions*
	Assoc. Prof. Dr. Servet Şenel, Dr. Necati Ölez, Erdoğan Erünal	*Problems and solutions related to feed production*
	Assoc. Prof. Dr. Osman Koçtürk / Faculty of Veterinary Medicine	*The future of animal husbandry, national nutrition problems, the effects of economic and ecological change on the diet of our people in the industrialization process of Türkiye*
VI. (1978)	Prof. Dr. C. Nadi Aytuğ / Faculty of Veterinary Medicine	*Animal health services, Veterinary Medicine and public relations in animal production*
	Ömer Lütfi Hekimoğlu / UCAT Livestock Counselor Prof. Dr. Mahmut Akkılıç / Faculty of Veterinary Medicine	*Feed and production relations in livestock*
	Assoc. Prof. Dr. Rafet Arpacık / Faculty of Veterinary Medicine	*Animal breeding and breeding and health problems created by importing breeding animals*
	Assoc. Prof. Dr. Osman Koçtürk / Faculty of Veterinary Medicine	*Democratic people's cooperatives, Village-Urban settlement and livestock industry*
VII. (1981)	Prof. Dr. Orhan Alpan - Dr. Sadi Akgün / Faculty of Veterinary Medicine	*General Education and Basic Problems in Milk Production and Consumption in Türkiye*
	Assoc. Prof. Dr. F. Tahir Aksoy - Assoc. Prof. Dr. Nejat Aydın / Faculty of Veterinary Medicine	*General Structure of Modern Poultry and Problems of Turkish Poultry*
	Assoc. Prof. Dr. Sadi Aral - Assoc. Prof. Dr. Ersoy Canküyer / Faculty of Veterinary Medicine	*Butchery Animal and Meat Production Problems in Türkiye*
	Hakkı Tan / *	*Increasing the Efficiency of Public Services for Animal Husbandry*
	Dr. M. Muammer Bügü / *	*Animal Health*

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VIII. (2000)	Prof. Dr. Doğan Aslanbey (President) Dr. Ediz Hun (Parlamenter) - Dr. Gürbüz Ertürk (Veterinarian) - Dr. Burhan Hacıislamoğlu (ATAHI - Representative) - M. Akif Bilici	*Pets and Problems for Animal Lovers* (Session 1)
	Prof. Dr. Sadi Aral (President) Prof. Dr. Mehmet Evrim - Prof. Dr. Ahmet Altınel	*Cattle and Sheep Breeding* (Session 2)
	Turgut Gökçe / Ministry of Agriculture and Rural Affairs Dr. Sabri Keskin / TDMFIPA Levent Genç / UCAT Semih Tuna / Turkish Breeders Association	*Problems of Cattle, Sheep and Goat Breeders* - Panel
	Dr. Erol Demirtel (President) Assoc. Prof. Dr. Mustafa Çelebi - Nebi Aksal - Sadettin Atığ Assoc. Prof. Dr. Deniz Seyrek İntaş - Assoc. Prof. Dr. Kamil Seyrek - Bahadır Gödek	*Horse breeding and its problems* (Session 3)
	Prof. Dr. Gülten Köksal (President) Prof. Dr. Selçuk Seçer - Binhan Ganioğlu	*Fisheries and Problems* (Session 4)
	Volkan Gürpınar / *	*Beekeeping and Its Problems - Conference* (Session 5)
	Prof. Dr. Ahmet Ergün (President) Assoc. Prof. Dr. Erol Şengör - Veli Can Çelik	*Poultry Breeding and Its Problems* (Session 6)

*: Unspecified (presenter name or institution) UCAT: Union of Chambers of Agriculture of Türkiye, ATAHI: The Association of Turkish Animal Health Industry, TDMFIPA: Turkish Dairy, Meat, Food Industrialists and Producers Association, MFAL: Ministry of Food, Agriculture and Livestock.

Table 4. Commissions and Members of Congress (Titles and names of persons are mentioned in the congress books).

Congresses	Commissions*					
I. (1968)	1- Production Problems Commission Prof. Dr. Selahattin Batu Prof. Dr. Mehmet Aybay Emin Arıtürk Dr. Sait Abuşoğlu Nevzat Uludağ Asım Güven Muzaffer Özay Burhan Yamanoğlu Sadettin Karacabey	2- Marketing Issues and Industry Commission Burhan Maner Prof. Dr. İsmail Türk Ragıp Saguner Assoc. Prof. Dr. H. Saim Kendir Assoc. Prof. Dr. Orhan Alpan Üzeyir Eren Ömer Hekimoğlu Şehamettin Eğriçınar Mustafa Ceylan Kemal İyigün	3- Planning and Organization Commission Prof. Dr. Mihri Mimoğlu Salih Gül Assoc. Prof. Dr. Çahit Yalçın Alim Ersoy Osman Ünverci M. Haciömeroğlu Mustafa Başdurak Rasim Yücebaş Veli Seyisoğlu Ziya Ünal Adil Güven			
II. (1970)	1- Organizing Commission**	2- Public Services Commission for Animal Husbandry**	3- Economic Problems Commission**	4- Technical Issues Commission**		
III. (1972)	1-Meat Production Problems Commission Ömer L. Hekimoğlu Rafet Yavuz Necati Ölez Hüseyin Özçelik Adil Musa Savran Zaman Sarısu Nihat Eğilmez	2- Milk Production Commission Nevzat Uludağ Mahmut Yasankul Burhan Yamanoğlu Halil Ayazoğlu Necmi Pirimoğlu Abdullah Yüce Osman Özbek	3- Chicken and Egg Production Commission Mahmut Akkılıç Ertuğrul Gökgül Metin Berk Ziya Dalkılıç İbrahim Bağlı Cevat Seklice	4- Wool and Mohair Production Commission Nurettin Utkanlar Mehmet Örkiz Necmettin Ertuğrul Sabahattin Göktan Cahit Asilsoy Emir Tokoğlu Osman Yüksener	5- Fisheries Specialization Commission İsmet Baran Utku Ergül Nurettin Karataylı Sezai Karamuhaoğlu Mustafa Başbudak Haluk Erguven İmat Sevinçler	6- Feed and Animal Production Relations Commission Sadettin Karacabe Yavuz Mergen Yavuz Akdevelioğlu Nazif Balta Halis Bağrıaçık Celal Yeltekin Şemsettin Demirhan

IV. (1974)	1- Diseases and Breeding Commission Prof. Dr. Orhan Alpan Prof. Dr. Orhan Düzgüneş Meliha Gürelli Ekrem Advan D. Ali Güven Halil Ayvazoğlu Hidayet Karaer Ali Paşa Bak Yusuf Ekinci	2- Marketing Commission Prof. Dr. Cahit Yalçın Necati Ölez Prof. Dr. Turan Güneş Haydar Alpay Ziya Dalkılıç Osman Okumuş Osman Özbek Mehmet Özdemir İbrahim Boyacı	3- Organizing Commission Prof. Dr. İsmail Türk Assoc. Prof. Dr. Ayhan Eliçin Hasan Ertan Nihat Çelik Nuri Midillili Sami Türkmen İbrahim Efe Ali Şahap Gürelli Adnan Ertanık	4- Animal Production and Feed Relations Commission Assoc. Prof. Dr. H. Servet Şenel Assoc. Prof. Dr. Ahmet Erkuş Dr. Nevzat Uludağ Ziya Arıkök Yavuz Akdevelioğlu, M. Şahabettin Önal Osman Kınık	5- Price and Financing Commission Assoc. Prof. Dr. Mehmet Bülbül Dr. Yücel Akıncı İrfan Şahin İzzet Sabuncuoğlu Necmettin Pirimcioğlu Hüseyin Özçelik Z. Abidin Ünal Yılmaz Özbükücü Erol Şanal	6- Animal Products Industry Commission Prof. Dr. Zeki Tolgay Prof. Dr. Kemal Göğüş Rafet Yavuz Şerafettin Eğriçay Recep Çalı Nihat Eğilmez İzettin Yaşbek Mustafa İpekçi Osman Yükselener
V. (1976)	1- Economic Problems Commission Ömer Lütfi Hekimoğlu Meliha Gürelli Halil Ayvazoğlu Murat Hınıslıoğlu, Yüksel Erdoğan Osman Polat	2- Feed Production and Problems Commission Assoc. Prof. Dr. H. Servet Şenel Dr. Necati Ölez Erdoğan Erünal Azmi Bora A. Şahap Gürelli Çetin Özer Mehmet Toprak Taner Tekin Dr. Uğur Büyükburç Prof. Dr. Kamil Doğan Ahmet Can Adem Karaelmas Assoc. Prof. Dr. İhsan Özkaynak Osman Önder Cahit Ovalı Necmi Yarar Hakkı Çorakçı	3- Meat Production and Livestock Commission Baki Oral İbrahim Büyükkavas Kemal Özkütük Rafet Arpacık Mahmut Yasankul Rafet Yavuz Fikret Özkartal Macit Özhan Haydar Alpay Nihat Eğilmez Necmettin Gürseven	4- Milk Production Commission Sadettin Karacabey Prof. Dr. Orhan Alpan Dr. Nevzat Uludağ Dr. Fethullah Koç S. Hacıpaşaoğlu Ş.A. Çelik M. Onar A. Üstündağ N. Yılmaz A. Kesik V. Akyıldız E. Erdenışık		
VI. (1978)	1- Production Commission Baki Oral Arif Şahin Rafet Arpacık Ersoy Canküyer Mustafa Macaroğlu Halis Bağrıaçık Lokman Uyanık Mehmet Evrim	2- Marketing Commission Ömer Lütfi Hekimoğlu Şakir Tuncer Eşref Yamak Fikret Özkartal Çetin Eşcan Osman Özbek Fahri Kaynak Şahabettin Ünal Muzaffer Eken Sıddık Çanakçı	3- Organizing Commission Ali Güven Aydın Evren Halil Örün Adnan Ertanık İbrahim Topalak Halil Ayazoğlu Şahnazar Önen Aydoğan Atilla	4- Animal Health Commission C. Nadi Aytuğ Ahmet Mimbay Nejat Aydın Sabahattin Tuğay Seyfi Yeğenoğlu Recep Çalı Arif Şahin		
VII. (1981)	1- Technical Commission Orhan Alpan Şefik Müftuğlu Reşat Öznacar Fikri Özder Haydar Sancağ Mithat Bingöl Sabahattin Gezer Ali Yazıcı	2- Economic Commission Ersoy Canküyer Mehmet Taşdemir Hakkı Hınıslıoğlu Tahsin Diker Ahmet Küçükyıldız Sadi Aral Sıddık Çanakçı Savaş Unal	3- Anıtkabir (Mausoleum) Visiting Commission Mehmet Taşdemir Tahsin Diker Hayati Çağlayan Fuat Çiftçioğlu			
VIII. (2000)	It was determined that commissions were not formed in this congress.					

*: The title and institution information of the members are given in the congress book and file. **: It is not given in the congress book. In addition, this information could not be found in other references. UCAT: Union of Chambers of Agriculture of Türkiye, ATAHI: The Association of Turkish Animal Health Industry.

Table 5. Changes in the name of the Ministry of Agriculture between 1920 and 2018.

Year	Old name	New Name	Law No (OG Date / Number)
1920	-	Ministry of Economy	3 (02.05.1920 /?)
1924	Ministry of Economy	Ministry of Agriculture	432 (07.04.1924 / 68)
1928	Agriculture and Trade Ministries	Ministry of Economy	1200 (21.01.1928 / 793)
1931	Ministry of Economy	Ministry of Agriculture	1910 (30.12.1931 / 1989)
1974	Ministry of Agriculture	Ministry of Food, Agriculture and Livestock	4/92 PR Decree (26.01.1974 / 14780)*
1981	Ministry of Food, Agriculture and Livestock	Ministry of Agriculture and Forestry	2384 SK (17.02.1981 / 17254)
1983	Ministry of Agriculture and Forestry	Ministry of Agriculture, Forestry and Rural Affairs	Decree Law No. 183 (14.12.1983 / 18251)
1991	Ministry of Agriculture, Forestry and Rural Affairs	Ministry of Agriculture and Rural Affairs	Decree Law No. 441 (09.08.1991 / 20955)
2011	Ministry of Agriculture and Rural Affairs	Ministry of Food, Agriculture and Livestock	Decree Law No. 639 (08.06.2011 / 27958 Mkr)
2018	Ministry of Food, Agriculture and Livestock	Ministry of Agriculture and Forestry	No. 1 PR Decree (10.07.2018 / 30474)

*The text of the Decree was not found in the relevant Official Gazette, and it was determined that the name of the Ministry was mentioned for the first time in the publication title of the Official Gazette dated 12.02.1974 and numbered 14797, and since the OG dated 15.02.1974 and numbered 14800, the Minister (Ahmet Nusret TUNA) was named as the Minister of Food, Agriculture and Livestock.

Discussion and Conclusion

It is seen that each of the eight congresses (1968-2000) that constitute the subject of the study has a quality that reveals the situation of Türkiye's animal husbandry and keeps a projection for the future. In each Congress, papers describe Türkiye's animal husbandry with numerical data. For this reason, it is thought that the study can guide future research in the history of veterinary medicine and animal husbandry.

TVMA organized four "Conventions," the first of which was in 1998, apart from the Turkish Livestock Congresses. Although these conventions can be seen as the continuation of the livestock congresses, it was declared that "The First Convention of Turkish Veterinary Medicine is the first and most important meeting organized to discuss the past, present, and future of our profession throughout our professional history" (12). In this case, it can be said that conventions are not a continuation of congresses.

Boratav (16) said that, after 1962, economic policies were based on planning and public investments were made according to five-year plans. However, urbanization, which started in this period and exceeded even the level of industrialization, had a significant impact on agriculture and animal husbandry activities. The base price determination and support policies of the state, which were followed during the development plans period, contributed to the positive development in the economy. It can be said that the issues highlighted in these Congresses and the decisions taken in the Development Plans were also effective in the economic recovery of the country in this period. It has been emphasized that agriculture and animal husbandry are the two traditional sources of income for its economy. The ratio of products of animal origin among agricultural products is 30%. The importance of this situation in terms of Planned Development is emphasized. It has been reported that there will be a significant increase in value in both domestic and foreign trade if necessary, arrangements are made for the second development plan period. The development plans and the special commission reports also emphasized problems parallel to these issues. It was stated that the necessary steps for the targets were taken to a large extent. It has been reported that cattle and chicks imported for breeding purposes cause large-scale epidemics containing diseases not seen in domestic breeds. Therefore, animal importation is objectionable (19). However, when the reports of the development plans and special specialization commissions were examined, it was determined that the animal husbandry issue was discussed in more detail until the 4th Development Plan was prepared in 1983 (20). This situation recalls that there may be a relationship between the fact that no congress was held from 1981 to 2000 and that it was not transferred to those plans healthily. Why Congress could not be held for about 20 years is also a matter that needs to be addressed and discussed separately.

The commission reports drew attention to issues parallel to the Congress. When all Congresses are examined as a whole, it can be stated that the papers were

prepared to find solutions to the problems of the country's livestock due to their interconnectedness and importance. It was determined that the same detailed study was not carried out in the later development plans. It can be said that the papers presented at the Congresses and the determined principles are taken into consideration in the public opinion and the works of the State Planning Organization (SPO).

Issues such as lack of personnel in the fight against diseases, sustainable long-term projects for the development of animal husbandry, and improvement of pastures, which are mentioned in the Congresses, are included in the development plans commission reports as suggestions and measures. In the Commission reports of the Fourth Convention organized by TVMA in 2018, it was emphasized that "government programs, political party programs, Agricultural Law, Agricultural Strategy Document, EU Common Agricultural Policies, World Trade Organization rules" and policies "to give direction to agriculture and animal husbandry" were put forward. However, it was stated that "long-term and continuous policies" could not be fully reflected in production (30). Issues such as the fact that the supports in the field of animal husbandry are below the target, the negativities brought by imports, the opening of pasture and agricultural areas for development can be interpreted as a system or understanding that can cope with the problems faced by animal husbandry in the long term.

To be successful in the fight against diseases, it was emphasized that the quality of the practical training of veterinarians and allied health personnel should be increased (19). Despite all the shortcomings, it can be said that efforts are made to solve the problems with the suggestions exported in the development plans from the large-scale reports prepared during the congress periods. In the Congresses held between 1968-1981, it was mentioned that there was a shortage of personnel for carrying out veterinary medicine services in the field. Meanwhile, in the Veterinary Medicine Conventions organized by TVMA (1998, 2002, 2010, and 2018) and other professional meetings, the excessive increase in the number of veterinary faculties and the lack of quality in education and training problems are highlighted (27-30). It can be said that the first Five-Year Development Plan was prepared in 1963, and the scientific congresses organized by the ministry, the relevant sector representatives, and professional organizations tried to eliminate the deficiencies. Therefore, the eight Livestock Congresses contributed significantly to achieving these goals.

It is known that scientific studies on aquaculture in veterinary medicine started in the 1940s in Türkiye. Within the scope of Ankara University Faculty of Veterinary Medicine, an aquaculture course was included in the curriculum in 1948; in 1967, the "Aquaculture, Fisheries and Game Animals Chair" was established (18). In the following period, "Fishery Research Institute" was established in Samsun in 1969 (22). In addition, during the establishment of Bursa (Uludağ) University Veterinary Faculty (1978), the establishment of "Bursa University Fisheries Institute" was one of the priority issues (25). Simultaneously with all these studies, at first (1968), second (1970), third (1972), and fourth (1974) Congresses, it was suggested to establish the "Ministry of Livestock-Fisheries and Nutrition" which would include "General Directorate of Fisheries." "Water products, Fisheries and Game Animals Chair" was closed in 1981 with The Council of Higher Education (Yüksek Öğretim Kurulu YÖK). Its re-establishment came to the agenda in the early 2000s, in the process of harmonizing with the European Union laws. Addressing this situation in the congresses shows that the professional veterinary medicine organizations are aware of the importance of fishery products for Türkiye. Awareness-raising efforts at the government and the public level started long before the European Union (EU) directives.

The statement of Prof. Dr. Mehmet Sandıkçıoğlu in the closing speech of the Second Congress (1970) "... *from the shepherd herding the animal to the housewife in the kitchen* ..." overlaps with the statement of the EU today "*from the barn to table/farm to fork*" (14) and shows that the importance of the issue was understood about 50 years ago.

Again, the Second Congress (1970) also stated that a second faculty should be opened to train veterinarians that Türkiye needs. In the news published in 1961 in "Journal of the Turkish Veterinary Medical Association," it was reported that an attempt was made to establish the second faculty in İzmir (2). With the transition to the planned period in the economy in the same year, it was decided to open the second veterinary faculty in Elazığ in 1967, and the faculty started education in November 1970 (32). This situation can be evaluated as proof that Congresses consider the problems in all fields of veterinary medicine and keep the pulse of the society.

In the study, although it was stated that a Ministry of Livestock should be established to solve the problems in the field of animal husbandry in each congress outcome statement, an autonomous ministry was not established, as seen in Table 5. However, the word Livestock was included in the amendments made in 1974 and 2011. The reorganization in the field of veterinary services after 1980 and the abolition of the general directorates of veterinary affairs, the abolition of subsidies in the field of animal husbandry, and the termination of specialization training caused severe problems in both animal husbandry and veterinary services (3, 6, 19, 20). Between 1996-2000, which includes the Seventh Development Plan, there is a

statement that "*The Undersecretariat of Livestock will be established under the Prime Ministry to carry out all necessary studies for the development of animal husbandry, with special attention to.*" However, it has been determined that although the issues specified in the congresses were included in the Development plans, they were not implemented in the field, as in the case of the Undersecretariat of Livestock (Table 5).

When we look at the institutions organizing the Congresses, it is seen that almost all institutions involved in veterinary medicine and animal husbandry and the production and consumption of animal products participated in the organization of congresses in different periods (Table 1). This table shows us that these eight congresses held between 1968-2000 were embraced and supported by the public, supported by broad participation from different sectors. It has been determined that the last Convention held by TVMA in 2000 was attended by bureaucrats from the relevant ministries, not politicians. This situation may indicate that politicians' interest in the issue has decreased.

Evaluating the population growth and animal production data in the 8th Congress Declaration emphasized that 40 g of animal protein required per person per day could not be met shortly. According to the FAO, daily protein intake was determined as 38 g. In the 6th Development Plan Sub-Commission report published in 1988, the importance given to both veterinary medicine and animal husbandry was emphasized. It was stated that animal husbandry should be arranged to meet the needs of today and tomorrow (3, 6, 20).

In 7 congresses held until 2000, issues related to the breeding of farm animals were discussed. It is seen that pet animals and stray animals, whose breeding and ownership increased in Türkiye as in the world, were addressed in the eighth congress held in 2000, and the problems were evaluated within the framework of environment and animal ethics. It is accepted that unplanned, inadequate, and wrong policies are the basis of the problems experienced after 1980 in the field of livestock. It can be said that the eight congresses examined within the scope of the study contributed to the determination of current problems, the formation of the proper planning and policies for the future by bringing together the authorized and responsible persons in the scientific framework.

As a result, it can be said that the congresses evaluated within the scope of the study provided the opportunity to discuss the problems and solution suggestions related to animal husbandry in Türkiye with a broad audience including veterinarians, producers, industrialists, consumers, and state representatives, and produced and shared scientific and currently valid information to solve these problems.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

ŞS and AM conceived and planned the study. AY and ŞS designed the study. All authors conducted literature review and writing the manuscript. All authors contributed to the interpretation of the results. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

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The effects of *Pinus pinaster* extract supplementation in low protein broiler diets on performance, some blood and antioxidant parameters, and intestinal histomorphology

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ABSTRACT

This study was conducted to investigate the effects of *Pinus pinaster* extract (PPE) and encapsulated *Pinus pinaster* extract (EPPE) supplementation in normal and low protein broiler diets on performance, some blood and antioxidant parameters, and intestinal histomorphology. In the present study, PPE was covered with alginate in order to obtain EPPE. The present research was conducted during 41-days with 288 one-day-old male broiler chicks. Chicks were classified into two groups that had different protein levels, one of with normal, the other one with 10% low protein. Also, normal and low protein level groups were divided into one control and two trial groups. The amount of 100 mg/kg PPE was added to each trial group diet; and the same amount of EPPE was added to other trial group diets. Consequently, compared to PPE and EPPE groups with control, statistically significant differences were observed for body weight and body weight gain in terms of protein on 41-day results ($P<0.05$). For feed conversation ratio, better results were detected in PPE groups at 41 days ($P<0.05$). Besides that, statistically significant differences were found in breast meat thiobarbituric acid reactive substances (TBARS) values and intestinal histomorphology in PPE and EPPE groups compared to the control groups ($P<0.05$). In this study, the findings suggest that 100 mg/kg PPE and EPPE can be supplemented in normal and low protein broiler diets without any adverse effects and considered alternative aromatic substances for broiler rations.

Introduction

A standardised extract of *Pinus pinaster* is called Pycnogenol® most common commercially extract composed of a mixture of flavonoids (26). The chemical composition of Pycnogenol® mainly contains procyanidins and phenolic acids. The phenolic acids are derivatives of benzoic and cinnamic acids. Procyanidins, biopolymers of catechin and epicatechin, are composed of 70 -/+5 % of the content of Pycnogenol® (25, 32). It has been reported that Pycnogenol® shows antioxidant activity thanks to its rich natural polyphenols composition. Also, it has stated antimutagenic, anticarcinogenic effects, cardiovascular benefits, and enhancing microcirculation. Thus, it has been set out the protective role of Pycnogenol® against many diseases associated with oxidative stress (9, 18).

The proteins are one of the basic nutrients in the diet and deficiency, excess, or lack of a certain ratio with other basic nutrients in the ration of proteins are among the factors that may cause stress for poultry. In the case of low protein in the diets, the energy-protein balance is disrupted and this causes stress in poultry. Oxidative stress may lead to degenerative disorders, loss of performance and decreases in product quality. However, it is possible to reduce or eliminate these negativities with the addition of natural or synthetic antioxidant substances into the poultry rations for economic poultry nutrition (8, 19, 34). In addition, PPE with its antioxidant properties can be

supplemented as one of the antioxidant substances in this case.

Encapsulation is a technique preferred more commonly in recent times to preserve or stabilize of contents of active compounds for coating them with one or more other substances (17). For encapsulation, various types of polysaccharide-protein hydrogel carriers are used. One of the carriers is alginate obtained from brown algae (6). Coating with alginate is a commonly used encapsulation of microorganisms, enzymes, drugs, oils, and aromatic substances (7). Encapsulation of aromatic extracts allows to maintain the stability of phenolic contents, increase the bioavailability of compounds, and mask the strong odour and taste of aromatic extracts (20).

This study was conducted to investigate the effects of Turkish PPE and EPPE supplementation on broiler diets and present an alternative natural product to synthetic ones in animal nutrition. Additionally, in this study, Turkish PPE was coated with alginate for the first time in Türkiye to be used in animal feeding and evaluate the encapsulation of PPE.

Materials and Methods

Birds husbandry and diets: The duration of the present research was 41 days with 288 one-day-old male Ross 308 broiler chicks (initial weight 41.4 ± 0.05 g). Chicks were divided into six groups and then each group was divided into six subgroups that contained eight chicks. Besides, normal (23; 21.5; 19.5%) and low protein (20.7; 19.4; 17.5%) level groups were divided into one control and two trial groups. To conduct this study, ethics committee approval was taken, which was 2017-22-178 number from Ankara University Animal Experimental Local Ethics Committee. Ad-libitum feeding was applied to control and trial groups' diets during the trial, and the water requirements of chicks were met as ad-libitum.

The basal diets were formulated in normal and low protein groups at starter, grower and finisher periods, as shown in Table 1. While the control groups were fed only a basal diet, the amount of 100 mg/kg PPE, one of as normal and one of as coated, was added to the basal diets of the trial groups. EPPE was coated form of PPE, was obtained by encapsulation of PPE with alginate.

Table 1. Ingredients, chemical composition, and metabolic energy value of basal diets in normal and low protein groups at starter, grower and finisher periods.

Ingredients (%)	Normal Protein Group			Low Protein Group		
	Starter (0-10 days)	Grower (11-24 days)	Finisher (25-41 days)	Starter (0-10 days)	Grower (11-24 days)	Finisher (25-41 days)
Corn	51.02	47	50	56.40	50	52
Full Fat Soybean	18	16	15	13	12.2	12
Vegetable Oil	1	2	3	1	2.2	2.5
Soybean Meal	24.97	22.7	18.5	24.4	19	14
Wheat	0	8	9.3	0	12.05	15.1
Monocalcium Phosphate	2	1.7	1.6	2	1.7	1.6
Limestone	1.8	1.55	1.6	1.8	1.55	1.5
Sodium bicarbonate	0.1	0.1	0.1	0.1	0.1	0.1
Salt	0.3	0.3	0.3	0.3	0.3	0.3
DL-Methionine	0.3	0.2	0.2	0.34	0.25	0.25
L-Lysine	0.2	0.15	0.1	0.35	0.35	0.35
Vitamin Premix*	0.15	0.20	0.2	0.15	0.20	0.2
Mineral Premix**	0.1	0.10	0.1	0.1	0.10	0.1
Anticoccidial	0.06	0.0	0.0	0.06	0.0	0.0
Total	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Chemical Composition (Analyzed)						
Crude Protein, (%)	22.65	21.21	19.83	20.65	19.52	17.50
Metabolizable Energy***(kcal/kg)	3018	3101	3218	3000	3127	3211
Crude Fiber,(%)	7.19	6.59	6.07	6.33	5.45	5.25
Calcium, (%)	1.40	0.98	0.97	1.12	0.96	0.92
Phosphorus, (%)	0.65	0.50	0.47	0.57	0.50	0.46

*Vitamin premix in per kg of diets: Vitamin A: 11.000 IU, Vitamin D_3: 3.500 IU, Vitamin E: 100 mg, Vitamin K_3: 3 mg, Vitamin B_1: 3 mg, B_2: 6 mg, B_6: 4 mg, B_{12}: 0.02 mg, Niacin: 35 mg, Folic acid:1.5 mg, Vitamin H: 0.2 mg, Vitamin B_5: 15 mg.
**Mineral premix in per kg of diets: Manganese: 120 mg, Zinc:110 mg, Copper: 30 mg, Iron: 50 mg, Cobalt:0.5 mg, Iodine: 2 mg, Selenium:0.3 mg.
*** Metabolizable energy content of diets was calculated according to the equation of Carpenter and Clegg (5).

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For the experimental design, it is used a completely randomized design with 2x3 factorial arrangement of dietary protein level (normal and low) and PPE (no supplementation, PPE and EPPE).

***Encapsulation of pinus pinaster extract*:** PPE was encapsulated using Encapsulator Device (BUCHI B-390). After determining the best conditions for the encapsulation process, 1.5% powder of PPE was mixed and homogenized with 1% alginate solution. $CaCl_2$ was prepared as 1.7 M. After it was determined that the best option of the nozzle, frequency, bar pressure, and volt flow for this device, the mixture was passed through the encapsulator device, and sprayed into the $CaCl_2$ solution. When $CaCl_2$ solution was filtered, PPE microcapsules were obtained. These microcapsules were washed with deionized water and then dried.

Traits measured: During the trial body weight (BW) and feed intake (FI) for every chick were measured on 0, 10, 21, 31, and 41 days of the trial. Feed conservation ratio (FCR) was calculated as the amount of feed intake per kg body weight gain (BWG). At the end of the trial, immediately after slaughtering, carcass weight was measured. Carcass yield (%) was determined at the end of the trial with this formula. Carcass yield (%)= (body weight (g) on 41st day/carcass weight (g) on 41st day) X100. European Production Efficiency Factor (EPEF) was calculated per pen at 41 days with this formula. EPEF, %= [(Body weight (kg))* (Number of total alive chick/number of total chick at initial)*100)]/[(Trial period (day))*FCR)*100)].

***Sampling*:** On the 41st day of the trial, two birds per pen were randomly selected and slaughtered. Meanwhile, the slaughtering 12 blood samples were collected from each of the groups.

Measurement of biochemical parameters in serum samples: HDL (high-density lipoproteins), LDL (low-density lipoproteins), triglyceride, and total cholesterol levels were measured in serum samples collected at the end of the trial by an auto-analyzer (BT 3000, Biotechnica Instruments, Italy) using Commercial kits of Randox RX series (Randox Laboratories Ltd., London, United Kingdom).

Determination of breast meat malondialdehyde (MDA) value: Two pieces of breast meat samples were taken at slaughtering from each animal. Then TBARS method was used to determine the lipid oxidation level. In the beginning of MDA analysis, 10 gr of breast meats were taken and then 97.5 ml distilled water was added on each of them and mixtures were homogenized with ultra turrax. The mixtures were poured into Kjeldahl tubes then 2 ml (6N) HCl added and distilled. It was taken 5 ml from distillate and 0.02 M, 2-thiobarbütirik asit (TBA) added equal amount on distillate. The mixture was incubated in boiling water for 35 minutes.The absorbance was measured at 530 nm with a spectrophotometer (Shimadzu UV-1208). For calculation of TBARS values, the absorbance values were multiplied by K=7.8 and calculated in mg malondialdehyde/kg. It was calculated that TBARS values for samples stored at different time intervals were stored at + 4 ^{o}C for one day and three days after slaughtering (29).

Intestinal histomorphology: On the 21st and 41st day of trial, while slaughtering was carried out, intestinal samples were taken for the groups and preparations were prepared. In the preparation, villus height (VH) from the villus tip to bottom and crypt deep (CD) from villus bottom to the crypt were measured, then the ratio of villus height to crypt deep (VH/CD) was calculated. For each intestine section, 10 villi and crypts were measured using the camera system and Cellsens CS-ST-V1.8 standard software (3, 16).

Statistical analysis: All data were analyzed with Shapiro-Wilk and Levene Statistical tests. According to the results of these tests, a two-way analysis of Variance (ANOVA) statistical test was performed to detect the differences between average values of groups. In addition, to distinguish the significance of the differences between groups, the Tukey multiple range test was carried out. The statistical results were evaluated on the 95% confidence interval. The SPSS 22.0 (SPSS Inc., Armonk, NY) software was used.

Results

The growth performance values of groups are presented in Table 2. As shown in Table 2 for BWG, statistically significant differences were found between the groups in terms of protein at 11-21, 0-21, 22-41, and 0-41 days. In terms of PPE, it was determined that the increases between the 11-21 and 0-21 days also in these periods, the protein and PPE interaction values (Protein X PPE) were statistically significant ($P<0.05$). For feed intake values of groups, there were statistically significant differences in terms of protein between 22-31 and 0-41 days ($P<0.05$), no statistically significant differences were observed in terms of PPE for all the periods ($P>0.05$). Additionally, Protein X PPE values were not statistically significant either ($P>0.05$). For feed conversion rates of the groups, the differences were statistically significant in terms of protein for days 22-41 and 0-41 ($P<0.05$) and also it was determined significant differences in terms of PPE for 11-21, 0-21, and 0-41 days ($P<0.05$). However, no statistically significant values were observed in terms of the protein and PPE interaction for all the periods ($P>0.05$).

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Table 2. Body weight gains, feed intakes, and feed conversion rate values of the group.

Dietary Treatments		Body Weight Gain (g)					Feed Intake (g)					Feed Conversation Ratio (kg feed/kg weight gain)				
		Days														
		0-10	11-21	22-41	0-21	0-41	0-10	11-21	22-41	0-21	0-41	0-10	11-21	22-41	0-21	0-41
NP-C		185.48	591.60^{bc}	2155.13	777.08^{bc}	2932.21	261.86	1033.67	3434.80	1295.53	4730.34	1.41	1.75	1.60	1.67	1.62
NP-PPE		192.46	632.39^{b}	2183.40	824.85^{ab}	3008.25	278.12	1026.52	3295.98	1304.64	4600.62	1.45	1.63	1.51	1.58	1.53
NP-EPPE		189.89	675.35^{a}	2096.43	865.24^{a}	2961.67	285.39	1028.12	3304.85	1313.51	4618.36	1.51	1.52	1.59	1.52	1.56
LP-C		184.67	574.23^{c}	1802.41	758.89^{c}	2561.31	276.28	1004.76	3156.95	1281.05	4438.01	1.50	1.76	1.77	1.69	1.74
LP-PPE		187.92	611.41^{bc}	2015.78	799.33^{bc}	2815.11	269.88	1001.14	3235.77	1271.02	4506.78	1.44	1.64	1.61	1.59	1.60
LP-EPPE		186.40	588.83^{bc}	2010.21	775.23^{bc}	2785.44	268.21	954.79	3265.27	1223.00	4488.27	1.44	1.62	1.63	1.58	1.61
Protein	**Normal**	189.27	2144.99^{A}	2144.9^{A}	822.39^{A}	2967.38^{A}	275.46	1029.43	3345.22	1304.56	4649.7^{A}	1.45	1.63	1.56^{A}	1.59	1.57^{A}
	Low	186.33	1942.80^{B}	1942.80^{B}	777.82^{B}	2720.62^{B}	271.46	986.90	3219.33	1258.36	4477.6^{B}	1.46	1.67	1.67^{B}	1.62	1.65^{B}
PPE	**NI**	185.07	582.91^{y}	1978.77	767.98^{y}	2746.76	269.08	1019.21	3295.88	1288.29	4584.17	1.46	1.75^{x}	1.68	1.68^{x}	1.68^{x}
	PPE	190.19	621.90^{x}	2099.59	812.09^{x}	2911.68	274.00	1013.83	3265.88	1287.83	4553.70	1.44	1.63^{xy}	1.56	1.59^{xy}	1.57^{y}
	EPPE	188.15	632.09^{x}	2053.31	820.23^{x}	2873.56	276.80	991.45	3285.06	1268.26	4553.32	1.47	1.57^{y}	1.61	1.55^{y}	1.59^{xy}
SEM		2.29	4.83	28.07	6.082	29.97	3.02	11.64	35.45	12.72	42.10	0.01	0.02	0.02	0.02	0.02
		Significance (P-value)														
Protein		0.52	< 0.001	0.001	0.001	0.001	0.548	0.078	0.086	0.079	0.049	0.87	0.351	0.03	0.36	0.02
PPE		0.66	0.001	0.224	0.003	0.079	0.577	0.592	0.941	0.766	0.943	0.71	0.004	0.09	0.008	0.03
Protein X PPE		0.94	0.010	0.157	0.042	0.353	0.106	0.649	0.328	0.456	0.597	0.16	0.59	0.49	0.81	0.63

NP-C: Normal Protein Control, NP-PPE: Normal Protein *Pinus pinaster* extract, NP-EPPE: Normal Protein Encapsulated *Pinus pinaster* extract, LP-C: Low Protein Control, LP-PPE: Low Protein *Pinus pinaster* extract, LP-EPPE: Low Protein Encapsulated *Pinus pinaster* extract, NI: Not included
SEM: Standard error of the mean.
a,b,c: Mean values within the same column carrying different superscripts are significantly different at P<0.05.
x,y: Mean values within the same column carrying different superscripts are significantly different at P<0.05.
A,B: Mean values within the same column carrying different superscripts are significantly different at P<0.05.

For carcass yield and EPEF, it was shown numerically better values PPE and EPPE groups than control both normal and low protein groups, respectively (Table 3). However, there were no statistically important differences in PPE and EPPE groups (P>0.05).

For biochemical parameters in serum samples, the findings showed that there were no significant differences between the groups' values of HDL, LDL, total cholesterol, and triglycerides (P>0.05) in terms of PPE. However, it was shown a certain significant decrease (P <0.05) in LDL levels in low protein groups (Table 4). But there were no statistically significant values were shown in terms of the protein and PPE interaction (P>0.05).

Table 3. Carcass yield and European Production Efficiency Factor (EPEF) values of the groups.

Dietary Treatments		Carcass yield (%)	EPEF
NP-C		72.49	444.62
NP-PPE		72.96	482.79
NP-EPPE		73.20	467.87
LP-C		71.84	361.05
LP-PPE		73.53	433.93
LP-EPPE		74.24	426.78
Protein	**Normal**	73.20	465.09
	Low	72.82	407.25
PPE	**NI**	72.16	402.83
	PPE	73.25	458.36
	EPPE	73.62	447.32
SEM		0.25	0.001
		Significance (P-value)	
Protein		0.442	1.0
PPE		0.063	1.0
Protein X PPE		0.31	1.0

NP-C: Normal Protein Control, NP-PPE: Normal Protein *Pinus pinaster* extract, NP-EPPE: Normal Protein Encapsulated *Pinus pinaster* extract, LP-C: Low Protein Control, LP-PPE: Low Protein *Pinus pinaster* extract, LP-EPPE: Low Protein Encapsulated *Pinus pinaster* extract, NI: Not included, SEM: Standard error of the mean.
The differences between the mean values of the groups are not statistically significant (P>0.05).

Table 4. Levels of some biochemical parameters in blood serum and plasma samples of the groups.

Dietary Treatments		HDL (mg/dl)	LDL (mg/dl)	Triglyceride (mg/dl)	Total cholesterol (mg/dl)
NP-C		69.80	65.26	44.25	60.67
NP-PPE		86.52	74.25	52.25	69.17
NP-EPPE		75.07	61.11	50.67	59.58
LP-C		79.40	54.19	50.17	65.92
LP-PPE		71.45	40.06	48.83	58.75
LP-EPPE		71.58	41.05	51.08	59.92
Protein	**Normal**	77.12	66.88^{A}	49.06	63.14
	Low	74.14	45.10^{B}	50.03	61.53
PPE	**NI**	74.58	59.73	47.21	63.29
	PPE	78.99	57.16	50.54	63.96
	EPPE	73.32	51.08	50.87	59.75
SEM		2.15	2.22	0.94	1.7
			Significance (P-value)		
Protein		0.49	0.001	0.61	0.64
PPE		0.53	0.28	0.23	0.56
Protein X PPE		0.08	0.12	0.14	0.17

NP-C: Normal Protein Control. NP-PPE: Normal Protein *Pinus pinaster* extract, NP-EPPE: Normal Protein Encapsulated *Pinus pinaster* extract, LP-C: Low Protein Control, LP-PPE: Low Protein *Pinus pinaster* extract, LP-EPPE: Low Protein Encapsulated *Pinus pinaster* extract, NI: Not included, SEM: Standard error of the mean.
The differences between the mean values of the groups are not statistically significant (P>0.05).
A,B: Mean values within the same column carrying different superscripts are significantly different at P<0.05.

As shown in Table 5, there were no significant differences in breast meat MDA level on the 1st-day results, but on the 3rd day's results, statistically significant decreases were found in terms of protein, PPE, and interaction value (Protein X PPE) (P<0.05). Besides this, groups of EPPE levels were determined numerically lower than PPE groups (Table 5). Also, it was detected the lowest MDA level on the 3rd day's results in normal protein encapsulated *Pinus pinaster* extract group (NP-EPPE).

As shown in Table 6, on the 21st day, while it was found statistically significant differences for jejunum VH and CD in terms of protein and PPE (P<0.05), there were no statistically significant differences in ileum values (P>0.05). Additionally, Protein X PPE values were not statistically significant (P>0.05) except the value of jejunum CD (P<0.05).

As shown in Table 7, on the 41st day, while it was observed statistically significant differences for jejunum VH in terms of PPE (P<0.05), there were detected statistically significant differences in terms of both PPE and protein for jejunum CD (P<0.05). However, no statistically significant differences were shown in the ratio of VH/CD for both jejunum and ileum (P>0.05). In addition, for ileum CD values, statistical significance was found in terms of PPE (P<0.05).

Table 5. Levels of MDA values on the first and third day in breast meat of the groups.

Dietary Treatments		TBARS Values (mg/kg)	
		1st day	3rd day
NP-C		0.46	1.02^{a}
NP-PPE		0.31	0.68^{c}
NP-EPPE		0.25	0.63^{c}
LP-C		0.49	1.09^{a}
LP-PPE		0.40	0.86^{b}
LP-EPPE		0.33	0.67^{b}
Protein	**Normal**	0.34	0.77^{A}
	Low	0.41	0.90^{B}
PPE	**NI**	0.47	1.05^{x}
	PPE	0.35	0.74^{y}
	EPPE	0.29	0.60^{y}
SEM		0.07	0.10
		Significance (P-value)	
Protein		0.21	0.02
PPE		0.3	0.05
Protein X PPE		0.25	0.01

NP-C: Normal Protein Control, NP-PPE: Normal Protein *Pinus pinaster* extract, NP-EPPE: Normal Protein Encapsulated *Pinus pinaster* extract, LP-C: Low Protein Control, LP-PPE: Low Protein *Pinus pinaster* extract, LP-EPPE: Low Protein Encapsulated *Pinus pinaster* extract, NI: Not included, SEM: Standard error of the mean.
a,b,c: Mean values within the same column carrying different superscripts are significantly different at P<0.05.
x,y: Mean values within the same column carrying different superscripts are significantly different at P<0.05.
A,B: Mean values within the same column carrying different superscripts are significantly different at P<0.05.

Table 6. Effects of intestinal histomorphology of jejunum and ileum on the 21st day in groups.

Dietary Treatments		Jejunum			Ileum		
		Villus Height (µm)	Crypt Depth (µm)	Villus Height/ Crypt Depth Ratio	Villus Height (µm)	Crypt Depth (µm)	Villus Height/ Crypt Depth Ratio
NP-C		1174.69	156.33^{ab}	7.57	726.42	126.05	6.30
NP-PPE		1181.70	146.94^{abc}	8.05	754.83	127.61	5.72
NP-EPPE		1288.67	162.5^{a}	7.94	779.22	134.00	5.64
LP-C		1071.83	135.08^{c}	7.58	710.00	120.19	6.15
LP-PPE		1164.11	141.61^{bc}	7.96	736.33	134.97	5.68
LP-EPPE		1186.69	162.36^{a}	7.33	748.91	137.08	5.21
Protein	**Normal**	1215.02	155.26^{A}	7.86	753.49	129.22	5.89
	Low	1140.88	146.35^{B}	7.62	731.75	130.75	5.68
PPE	**NI**	1123.26^{y}	145.71^{y}	7.58	718.21	123.12	6.23
	PPE	1172.90^{xy}	144.28^{y}	8.00	745.58	131.29	5.70
	EPPE	1237.68^{x}	162.43^{x}	7.63	764.07	135.54	5.43
SEM		12.40	1.72	0.11	14.02	2.26	0.15
				Significance (P-value)			
Protein		0.006	0.015	0.312	0.44	0.73	0.45
PPE		0.003	0.001	0.251	0.42	0.09	2.28
Protein X PPE		0.29	0.047	0.5	0.98	0.49	0.14

NP-C: Normal Protein Control, NP-PPE: Normal Protein *Pinus pinaster* extract, NP-EPPE: Normal Protein Encapsulated *Pinus pinaster* extract, LP-C: Low Protein Control, LP-PPE: Low Protein *Pinus pinaster* extract, LP-EPPE: Low Protein Encapsulated *Pinus pinaster* extract, NI: Not included, SEM: Standard error of the mean.
a,b,c: Mean values within the same column carrying different superscripts are significantly different at P<0.05.
x,y: Mean values within the same column carrying different superscripts are significantly different at P<0.05. A,B: Mean values within the same column carrying different superscripts are significantly different at P<0.05.

Table 7. Effects of intestinal histomorphology of jejunum and ileum on the 41st day in groups.

Dietary Treatments		Jejunum Villus Height (µm)	Jejunum Crypt Depth (µm)	Jejunum Villus Height/ Crypt Depth Ratio	Ileum Villus Height (µm)	Ileum Crypt Depth (µm)	Ileum Villus Height/ Crypt Depth Ratio
NP-C		1267.30	154.41^{b}	8.26	1117.24	143.83	6.05
NP-PPE		1334.47	171.77^{a}	7.79	1151.60	153.55	7.63
NP-EPPE		1392.16	190.11^{a}	7.33	1150.50	146.19	7.57
LP-C		1256.33	154.19^{b}	8.16	877.82	135.64	8.26
LP-PPE		1320.08	168.83^{b}	7.83	1159.50	147.30	7.83
LP-EPPE		1322.13	160.17^{b}	8.26	1101.99	159.11	7.23
Protein	**Normal**	1331.31	172.10^{A}	7.79	1139.78	147.86	7.08
	Low	1299.51	161.17^{B}	8.08	1046.44	147.35	7.77
PPE	**NI**	1261.82^{y}	154.31^{y}	8.21	997.53	139.73^{y}	7.15
	PPE	1327.27^{x}	170.31^{x}	7.82	1140.89	150.43^{xy}	7.72
	EPPE	1357.15^{x}	175.29^{x}	7.80	1126.25	152.65^{x}	7.40
SEM		10.77	1.79	0.10	31.30	2.03	0.23
			Significance (P-value)				
Protein		0.15	0.005	0.15	0.15	0.90	0.14
PPE		0.04	0.001	0.16	0.22	0.032	0.6
Protein X PPE		0.46	0.003	0.08	0.12	0.08	0.07

NP-C: Normal Protein Control, NP-PPE: Normal Protein *Pinus pinaster* extract, NP-EPPE: Normal Protein Encapsulated *Pinus pinaster* extract, LP-C: Low Protein Control, LP-PPE: Low Protein *Pinus pinaster* extract, LP-EPPE: Low Protein Encapsulated *Pinus pinaster* extract, NI: Not included, SEM: Standard error of the mean.
a,b: Mean values within the same column carrying different superscripts are significantly different at P<0.05.
x,y: Mean values within the same column carrying different superscripts are significantly different at P<0.05.
A,B: Mean values within the same column carrying different superscripts are significantly different at P<0.05.

Discussion and Conclusion

In this study, statistically significant differences between the groups of BWG in terms of protein at 0-41 days (P <0.05) were observed. For all periods except 0-10th days interval, higher BWG in normal protein groups than low protein ones (P<0.05) (Table 2) were determined. In agreement with our study, Sigolo et al. (28) stated that growth performance was negatively affected in their study, conducted by reducing the protein level in broilers by 2.5% from the recommended levels. Aftab et al. (2) investigated the effects observed when protein levels indicated in NRC (1994) were reduced by 10% using balanced rations of amino acids at 0-21, 21-42, and 42-56 days of broiler chickens. For this purpose, they gave rations containing 20.7, 18.0, and 16.2% crude protein at 0-21, 21-42 and 42-56 days, respectively. However, they stated that there was a decrease in BW, BWG and carcass yield in all low protein rations compared to the control group. Likewise, in our study, when the protein level decreased 10% in low protein groups, a decrease was found compared to the normal ones for BWG and carcass yield. Cardinal et al. (4), in their studies, comparing growth performance and intestinal health in broiler groups with standard protein, the protein level reduced by 6% and protease added standard and low protein groups; reported that in the low protein group, BW, BWG and FCR were significantly adversely affected in 1-42 days compared to the standard protein group. These findings were consistent with the findings we obtained in our study that were determined in terms of protein.

In another study, Hilliar et al. (13) conducted to evaluate the effects of low protein (LP) diets supplemented with approximately 3% of glycine, serine and threonine amino acids in broilers. It was stated that the results showed LP group, also LP and supplemented with amino acids groups had lower final BW than the standard protein group. It was notified that the standard protein group FCR is better than LP; moreover, it was better than low protein supplemented with amino acids groups during the trial. Zhou et al. (37) was designed a study to evaluate the effects of dietary serine supplementation on performance in laying hens fed low protein (LP) diets. The trial included a control diet with standard protein (16.49% CP) and 4 low protein diets (14.05% CP) supplemented with 0, 0.114, 0.306, and 0.498% L-serine, respectively. At the end of the study, it was notified the supplementation of serine to LP diets improved performance and led to an optimal egg production with serine level of 0.498%. In addition, total protein and globulin contents were significantly increased (P<0.05) with serin supplementation at the levels of 0.306% and 0.498%. These results are in line with our findings about reducing protein level in broiler diets. While it was stated the supplementation of amino acids was used for compensation of the adverse effect of reducing protein level in broiler diets in above-mentioned studies, PPE and EPPE aromatic substances

were used to fortify compensation of this effect in our study.

Also, it was notified in a study when plant extracts from rosemary, olive leaves, pine bark concentration of 2.5 and 5.00 g/kg, and polyphenolic compound quercetin 0.25 and 0.50 mg/kg concentration were added in broiler diets, no effect was observed on BWG and FCR for the olive leaves, pine bark extracts as well as quercetin (27). However, it was reported to be observed better BWG, FI and FCR when added 600 and 2400 mg/kg PPE in broiler diets during 21 days before slaughtering by Herranen et al. (12). In our study at 0-41 days period, the numerically highest body weight gain was found respectively in NP-PPE and then NP-EPPE groups. In terms of PPE, statistically significant increases on days 11-21 and 0-21 for BWG and better FCR that showed significant effects on days 11-21 and 0-41($P<0.05$) were found.

In another study to investigate the effects of chitosan nano-encapsulating mint, thyme, and cinnamon essential oils in broiler diets added at 0.025, 0.04, and 0.055%, respectively, starter, grower, finisher period notified that encapsulated forms of essential oils were significantly improved ($P<0.05$) for BWG and FCR compared to free forms (23). Also, it was reported that in the comparison of powder and encapsulated form of garlic and *Phyllanthusniruri L.* mixture, the encapsulated form had more powerful effects on BWG and FCR than powder form (22). Additionally, Haafez et al. (11) reported that encapsulation of aromatic substances affected FCR positively compared to powder form. Mourtzinos et al. (21) and Zhang et al. (36) stated that the encapsulation process increases the bioavailability of the product. Also, in our study between 11-21 and 0-21 days of age, better BWG and FCR values were observed in EPPE groups ($P<0.05$) than PPE and EPPE values. In these periods, the protein and PPE interaction values were statistically significant also ($P<0.05$). It was observed that the addition of coated extract in 0-21 days provided statistically significant positive effects for BWG in normal protein groups and better FCR values in both normal and low protein groups. These findings were in harmony with the general approach of increase in the bioavailability using encapsulation of aromatic substances.

It was stated that conducted a study to evaluate the effects of supplementation of 5-10% Moringa olifera leaves meal (MLM) and 50-100 ml Moringa olifera extracts (MLEx) in standard and low protein (LP) diets in broilers. It was notified in this study the lowest BW was detected in the LP control group, whereas the best BW value was in the LP+100 ml MLEx group. Also, it was reported the best FCR was found in the LP+50 ml MLEx group and the best TBARS value in the LP+100 ml MLEx group (1). The above mentioned results of adding aromatic substances (MLM and MLEx) to LP diets to improve growth performance are in line with the findings of our study.

Guo et al. (10) reported that with the supplementation of 0, 1, 3, 5% pine needles powder in broiler diets, decreases were found in triglyceride levels for the groups of 3 and 5% and in cholesterol levels for groups 1 and 5% in blood serum when compared to control. Meanwhile, in the present study, there were no significant differences compared to control when it was used 100 mg/kg of PPE and EPPE.

MDA is one of the most important and toxic substances that occurred by lipid peroxidation of unsaturated fatty acids with oxygen. Due to based on detection of MDA levels in blood plasma and poultry meat, the TBARS method is commonly used as an indicator of lipid oxidation level (14). Guo et al. (10) also declared to detection a decrease in blood TBARS level compared to control when 5% of pine needles powder was added into the broiler diets. It was reported that when fermented pine needles powder was used, 0.3% for the starter period and 0.6% for the grower-finisher period in broiler diets, it was observed better MDA level and antioxidant activity (35). Ramay and Yalçın (24) explained to observe a linear decrease in MDA levels in breast meat stored for 1 and 10 days when broilers were fed a linseed oil-based basal diet supplemented with 0.25, 0.5, 0.75, 1% pine needles powder. In parallel to earlier studies, it was found statistically significant decreases in the 3rd-day breast meat MDA values in PPE and EPPE groups than control ($P <0.05$). When compared MDA values in PPE and EPPE groups, a numerical decrease was observed in EPPE groups in our study ($P>0.05$).

Villus height (VH) and crypt depth (CD) are related to the digestive capacity of the small intestine. Laudadio et al. (15) conducted a study with equal energy and three different protein levels that were high 22.5%, medium 20.5%, low 18.5% HP for comparison of intestinal VH and CD values; they found significant numerical increases in values of medium and high protein groups than low protein one. Similar to our study, higher VH and CD values were found in normal protein groups than low protein ones. In another study, it was reported that conducted to evaluate the effects of low protein (LP) diets supplemented with arginine, glutamine, methionine, and threonine in Eimeria-infected chickens. The results revealed that the intestinal health of chickens challenged with a mild coccidia infection can be improved when fortified in 0.75% of arginine, glutamine, methionine, and threonine to LP diets (30). Van Nevel et al. (33) reported that the low VH/CD ratio indicates that the intestinal turnover rate decreases. Thus, it may lead to an increase in growth rate by consuming less energy for vital activities.

Also, Tufarelli et al. (31) emphasized that the increases of villi height improve total absorption area in the intestine. This situation provides a positive effect on digestive enzymes and transporting nutrients on the villi surface. In our study, it was found a lower VH/CD ratio in the normal protein groups than in the low protein groups. Moreover, in terms of PPE, the jejunum VH/CD ratio was found lower in the PPE and EPPE added groups on the 41st-day results. When compared to control, addition to PPE and EPPE provide higher VH and deeper CD in jejunum at 21 and 42 days age. It was thought that the increasing VH-CD and improvement of performance could be caused by growing so that it could be a positive increase, but no data were found about the effects of PPE on intestinal histomorphology in the broiler, so it was thought to be investigated with further studies.

In light of the findings obtained in this study, it was observed that with supplementation of 100 mg/kg PPE or EPPE obtained from Türkiye in normal and 10% low protein broiler rations; no adverse effect was observed in the groups. Also, positive effects on performance, intestinal histomorphology, and better TBARS levels with antioxidant properties were detected. In conclusion, it was estimated that 100 mg/kg PPE or EPPE would be used as an alternative aromatic substance for broiler diets. Also, encapsulation can be preferred to obtain better antioxidant activity and intestinal capacity on stress conditions in the field. Besides that, further studies are needed to determine the range of usage amount to provide maximum effects for dietary supplementations in broiler diets.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Author Contributions

GÖ and SK conceived, planned and carried out the experiments. GÖ took the lead in writing the manuscript. GÖ and SK provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was approved by the Ankara University Animal Experiments Local Ethics Committee (2017-22-178).

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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Determination of the effect of milk fat on the inactivation of *Listeria monocytogenes* by ohmic heating

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ABSTRACT

Research in recent years has focused on innovative technologies that provide pathogen inactivation without damaging the structural properties of foods. Ohmic heating (OH) is an innovative technology, that provides an effective microbial inactivation with massive and rapid heating. This study aims to determine the effects of milk fat on the inactivation of *Listeria monocytogenes* by OH with a low voltage gradient. *L. monocytogenes* (ATCC 13932) inoculated 3.1%, 1.5%, and 0.1% fat-milk samples were heated up to 62°C by OH and conventional heating (CH) process. OH treatment lead to the inactivation of *L. monocytogenes* in both 1.5% and 0.1% groups and led to approximately 5.30 log decrease, however, there was a 3.10 log decrease in the 3.1% group at 6 min. CH lead to a few reduction as 0.21, 0.29 and 0.39 log in 3.1%, 1.5% and 0.1% fat-milk respectively. In OH, the sublethal injury ratio was higher than CH in all milk groups. However, OH did not statistically change color and pH values at the 6th min of the process, had a significant effect on hydroxymethylfurfural value only in 3.1% fat-milk. In conclusion, the increased fat content may have important inhibitory effects on pathogen inactivation in OH. Thus, the OH conditions should be chosen carefully to sufficient inactivation of pathogens in milk with high-fat content.

Introduction

Listeria monocytogenes is a harmful foodborne pathogen found in milk and dairy products, resulting in foodborne diseases and deaths (4). Due to its ubiquitous feature, *L. monocytogenes* can spread widely to the environment and maintain its vitality even under adverse conditions such as cooling, freezing, heating, and drying (26).

Inadequately heat-treated foods cause the majority of foodborne infections in many parts of the world. Several preservation methods, including thermal and non-thermal treatments, have been applied for many years to inactivate foodborne pathogens and maintain food safety (19). Thermal treatment, which has long been utilized to prevent foodborne infections, has a fundamental constraint in terms of quality degradation caused by severe heat. Research in recent years has focused on innovative technologies that provide pathogen inactivation without damaging the structural properties of foods. Ohmic heating (OH) is an innovative technology that provides an effective microbial inactivation with massive and rapid heating. In this technic, heat is generated when an alternative current passes directly through foodstuff (9, 10). OH has been gained much attention in the food industry due to its rapid, uniform heating distribution to provide food safety with minimal changes in structure, nutrition, or sensory attributes in foods (1, 2, 20, 21, 24).

Many researchers have recently researched the use of ohmic heating to inactivate foodborne bacteria (13, 18, 22). Previous studies showed that OH is affected by extrinsic factors such as voltage and frequency and intrinsic factors such as features of lipids, proteins, carbohydrates and their quantities in food. Both of them significantly affect the electrical conductivity and pathogen inactivation (12, 13, 18).

Although OH has been widely used in the processing of milk products, to the best of our knowledge, research

on the combined effect of milk fat and low voltage gradient on pathogen inactivation is quite limited. This study aimed to investigate the effect of milk fat and low voltage gradient on the inactivation of *L. monocytogenes* by OH and the determination of the quality changes.

Materials and Methods

Bacterial strain and cultural condition: American Type Culture Collection (ATCC) standard *L. monocytogenes* 4b (ATCC 13932) were used in this study. Stock cultures were transferred in Tryptic Soy Broth (BK046HA, BIOKAR) and incubated aerobically overnight at 37°C before experiments. Bacterial cultures were centrifuged at 5000 rpm for 5 min at 4°C, and the pellets were washed twice in 9 ml 0.9% saline with thorough mixing by a vortex. After centrifugation, the bacterial pellets were re-suspended in the same buffer. The bacterial suspensions were serially diluted in 0.1 % peptone water and plated on COMPAS Listeria agar (BK192HA, BIOKAR) with the spread plate technique for the enumeration. Plates were incubated for 24-48 h at 37°C before counting colonies. All bacterial suspensions were confirmed to be contained approximately 10^7 bacterial cells mL^{-1}.

Sample preparation and inoculation: A total of 72 UHT milk samples packed in 200 mL aseptic tetra pack containers were purchased from a local market in the province of Burdur, Türkiye. The whole (3.1%, n=24), semi-skimmed (1.5%, n=24), and skimmed (0.1%, n=24) UHT milk kept at +4 °C until the experiments were carried out. Before inoculation, milk samples were plated onto Nutrient Agar (105450, Merck) at 37 °C for 24-48 h, and there was no colony detected. The harvested pellet of *L. monocytogenes* strains was re-suspended in 9 mL 3.1%, 1.5%, and 0.1% UHT milk samples, respectively. The final concentration of *L. monocytogenes* cells in milk was confirmed by spreading the dilutions on plates and it was approximately 10^7 CFU/mL.

Experimental equipment: OH was carried out by a laboratory-type OH unit consisting of food stuff-grade stainless steel electrodes (304 L), a K-type thermocouple, a microprocessor (ERAK-TEK, Konya), a personal computer, a power supply (VARSAN, İstanbul) providing AC, 50Hz, 10 A and output voltage range 0-250 V, a magnetic stirrer and a heating unit. Time and temperature changes were recorded during the heating process through the microprocessor linked to the personal computer (Figure1). The heating unit consisted of a glass vessel of 300 mL capacity and two food stuff-grade stainless steel electrodes (electrode gap 5 cm). The K-type thermocouple was inserted into the unit's center to monitor temperature during the OH process.

Two hundred mL milk samples inoculated (0.05%,1 mL) with *L. monocytogenes* were subjected to 10 V/cm and 50 Hz in the OH treatment. All experiments were started from the initial state at 23.8 °C, and continued until the temperature at the center of the milk reached 62°C (it takes approximately 6 min and 20 min for OH and CH, respectively). This temperature was considered the end of the heating process. For the conventional heating treatment, a 200 mL sample was heated using a digital hot water bath (WB14, Memmert, Germany) (set temperature: 62 ºC) by the same heating procedure. During the heating process, 1 mL of milk sample, at the center of the heating unit was taken out, respectively, by one-off sterile injector at appropriate intervals (2 min), and immediately cooled in an ice-water bath.

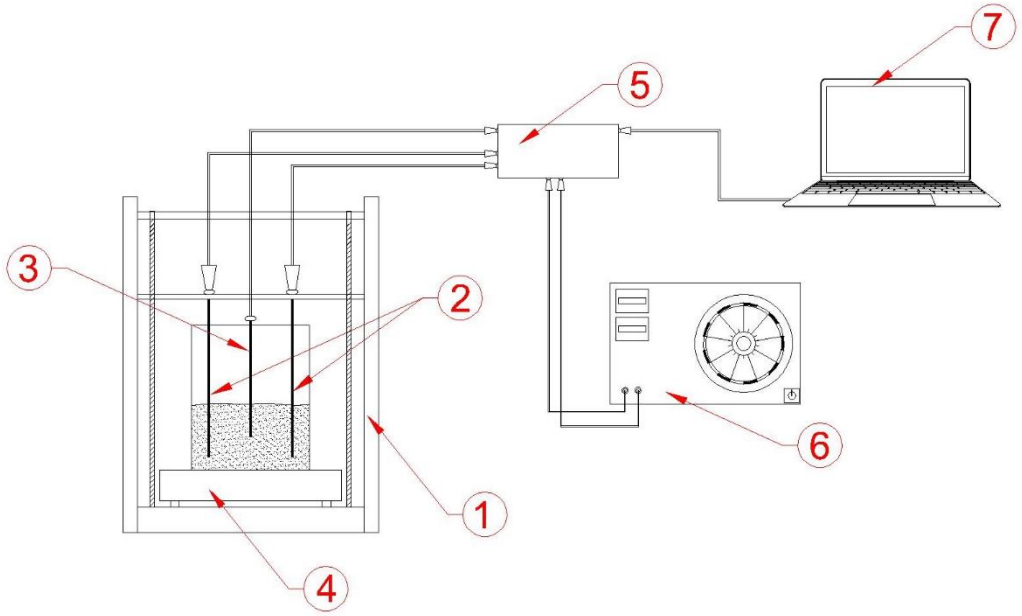

Figure 1. Schematic diagram of the OH system. 1: Ohmic cell, 2: Electrode, 3: Thermocouple sensor, 4: Magnetic stirrer, 5: Microprocessor, 6: AC power supply, 7: Computer.

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Enumeration of viable and injured cells: Spread plate method was used to enumerate the *L. monocytogenes* population after the heating treatment. One mL of cooled milk samples were serially diluted in 0.1% peptone water, and 0.1 mL samples were spread plated onto selective Compass Listeria Agar (CL) to count noninjured and were spread onto Compass Listeria Agar + TSA (CLTSA) to count both injured and noninjured bacterial cells. All plates were incubated for 24–48 h at 37°C before counting colonies. The sublethal ratio was calculated according to the following equation:

$$\text{The sublethal ratio (\%)} = 100 - [(\text{CFU/mL CL})/(\text{CFU/mL CLTSA})] \times 100$$

Where CFU/mL CL was the counts on Compass Listeria Agar; CFU/ mL CLTSA was the counts on Compass Listeria Agar +TSA (22).

Color, pH, and hydroxymethylfurfural value: The color of the samples was measured using a Konica Minolta CR400 colorimeter (Konica Minolta, Osaka, Japan). The hydroxymethylfurfural (HMF) content of the samples was determined according to the method described by Keeney and Bassette (11). The absorbance of the samples was calculated at 443 nm using a spectrophotometer (Thermo-MultiScan GO, ThermoScientific™). The standard curve was prepared using the HMF standard (Sigma, 1-10 µM/mL), and the results were expressed as µM/L. The pH was measured with a pH-meter (WTW Lab-pH Meter inoLab® pH 7110) at room temperature. Measurements were carried out in triplicate.

Statistical analysis: The mean values were obtained from triplicate trials. The results were analyzed by one-way analysis of variance (ANOVA) and T-test using SPSS software (Version 21.0; SPSS Inc., IBM Corporation, USA). Significant differences between the treatments were examined by Duncan's test and the results were expressed as mean ± standard deviation ($P<0.05$).

Results

The temperature changes of the samples inoculated with *L.monocytogenes* treated with OH and CH were presented in Figure 2. The initial temperature was 23.8-24.0 ºC ($P>0.05$). All OH-processed samples showed a similar linearly increasing trend in temperature. In all milk samples required approximately 6 min to reach 62 ºC. In the CH-treated samples, the rate of temperature increase dropped progressively, taking 20 min, to reach 62 ºC. Temperature rise was more rapid in OH treatment than CH.

Survival rates of *L. monocytogenes* cells during OH and CH in milk with different fat rates are shown in Table 1. In the OH experiment, the initial counts of *L. monocytogenes* cells were almost the same in all experimental milk samples. Also, the counts of *L. monocytogenes* remained stable ($P>0.05$) throughout the first 4 min of OH treatments while the temperature was below 50ºC (Figure 2). After the 4th min of the OH experiment, microbial counts of 1.5% and 0.1% fat-milk samples had already been reduced and the reductions of both experimental groups were greater than those in 3.1% groups ($P<0.05$). At the end of the 6th min, *L. monocytogenes* were inactivated in both 1.5% and 0.1% groups and led to approximately 5.30 log CFU/mL decrease ($P<0.05$). However, there was a 3.10 log CFU/mL decrease in the 3.1% group at the same process time ($P<0.05$).

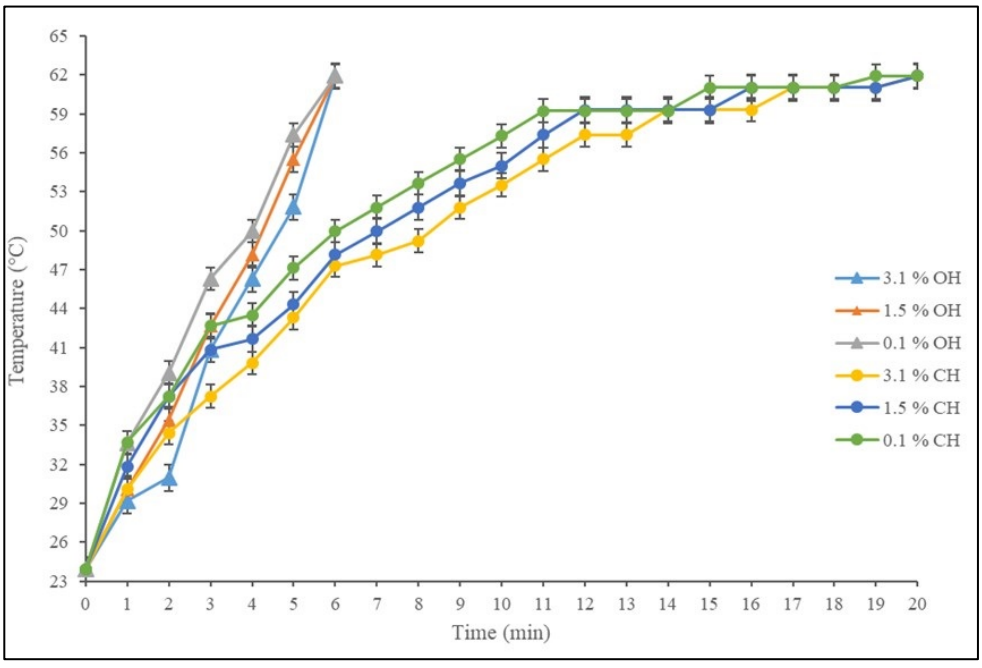

Figure 2. Time-temperature profiles of ohmic and conventional heating of milk samples with different fat contents. OH: Ohmic heating, CH: Conventional heating.

Table 1. Inactivation of *L.monocytogenes* cells by OH and CH in milk with different fat content at 10 V/cm voltage gradient.

Experiment	Time (min)	Colony counts (log CFU/mL)		
		3.1%	1.5%	0.1%
OH	**0**	5.32 ± 0.14^{x}	5.33 ± 0.03^{x}	5.32 ± 0.02^{x}
	2	5.35 ± 0.14^{x}	5.32 ± 0.01^{x}	5.32 ± 0.01^{x}
	4	5.33 ± 0.11^{x}	5.25 ± 0.05^{y}	5.22 ± 0.05^{yB}
	6	2.21 ± 0.28^{yaB}	ND^{zbB}	ND^{zbB}
CH	**0**	5.30 ± 0.12^{x}	5.30 ± 0.12^{x}	5.32 ± 0.07^{x}
	2	5.34 ± 0.08^{x}	5.34 ± 0.03^{x}	5.31 ± 0.03^{x}
	4	5.34 ± 0.18^{x}	5.31 ± 0.12^{x}	5.32 ± 0.02^{xA}
	6	5.09 ± 0.09^{yaA}	5.01 ± 0.10^{yabA}	4.93 ± 0.08^{ycA}

Values were means ± standard deviation of three replicates. ND: Not detected.
[x-z ,A-B] Values with different superscripts within columns differ significantly (P<0.05).
[a-c] Values with different superscripts within raws differ significantly (P<0.05).
OH: Ohmic heating, CH: Conventional heating.

In the CH experiment, the initial counts of *L. monocytogenes* cells were the same in all groups. During the CH, there was no change between the experimental groups at the 2nd and 4th min of experiments (P>0.05). At the end of the 6th min, there were a few reductions as 0.21, 0.29, and 0.39 log CFU/mL in 3.1%, 1.5%, and 0.1% groups, respectively (P<0.05), and the microbial reduction in 0.1% was higher than the others. Moreover, the reduction of *L. monocytogenes* microbial counts was significantly different (P<0.05) between the OH and CH treatments at each time point.

In this study, it was determined that the sublethal injury ratio increased with time (Figure 3). However, OH had a higher injury ratio than CH treatments at the same time. In this investigation, the maximum damage ratios of OH-treated *L. monocytogenes* in 3.1%, 1.5% and, 0.1% fat milk were 38.48%, 21.63%, and 20.50%, respectively, while the values for CH-treatments were 31.7%, 15.7%, and 17.14%, respectively. Due to the inactivation of *L. monocytogenes* at the 6th min of OH treatment in both 1.5% and 0.1% groups, the rate of sublethal injury could not be calculated.

Color values of milk samples were presented in Table 2. The initial *L** values were similar in 3.1% and 1.5% fat milk (90.74±0.89 and 89.29±2.05; P>0.05), while the initial *L** value of 0.1% fat milk (86.35 ±1.27; P<0.05) is statistically different. This difference observed in the initial color values could be due to the differences in fat content of the samples (3). However, generally, all OH-treated samples' *L**, *a** and *b** values did not significantly differ according to treatment times (P>0.05). CH-treated samples showed a significant increase in 3.1% and 0.1% groups in *L** and *a** values (P<0.05) according to treatment times, respectively, but not in *b** value (P> 0.05).

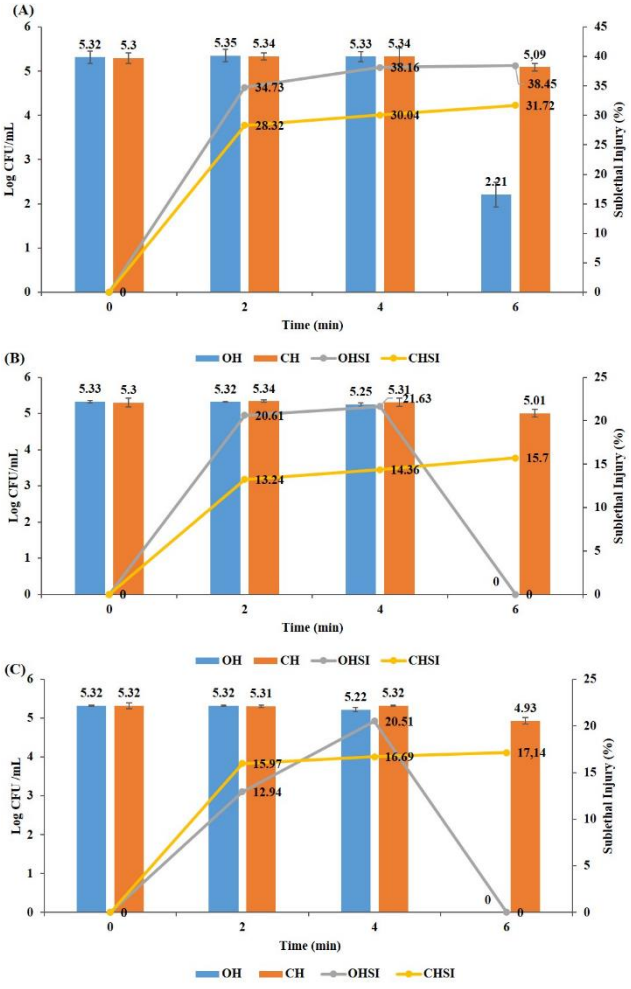

Figure 3. Inactivation of *L.monocytogenes* in milk samples by OH and CH. (A) 3.1% (B) 1.5%, (C) 0.1% fat milk. The columns represented the colony forming units, and the lines represented injury ratio (%). OH: Ohmic heating, CH: Conventional heating, OHSI: Ohmic heating sublethal injury ratio, CHSI: conventional heating sublethal injury ratio.

Table 2. Color values of milk samples.

		Milk Group (fat %)								
		3.1%			1.5%			0.1%		
	Time (min)	*L**	*a**	*b**	*L**	*a**	*b**	*L**	*a**	*b**
OH	0	90.74±0.89^{x}	-2.48±0.07^{x}	7.62±0.25^{y}	89.29±2.05^{x}	-3.52±0.23^{y}	7.33±2.26^{y}	86.35±1.27^{y}	-3.35±0.37^{y}	5.61±0.86^{y}
	2	92.73±0.21^{x}	-2.46±0.11^{x}	8.54±0.53^{x}	89.17±1.18^{y}	-3.50±0.39^{y}	6.58±1.99^{xy}	86.11±1.79^{z}	-3.54±0.42^{y}	5.18±1.58^{y}
	4	91.32±0.99^{x}	-2.40±0.11^{x}	7.84±1.02^{x}	89.38±1.38^{y}	-3.54±0.30^{y}	6.00±0.99^{xy}	85.63±1.03^{z}	-3.55±0.07^{y}	5.47±1,42^{y}
	6	91.39±0.67^{x}	-2.42±0.06^{x}	7.66±0.80^{x}	89.92±0.87^{x}	-3.43±0.26^{y}	6.78±1.53^{xy}	86.34±1.35^{y}	-3.50±0.15^{y}	5.53±1.33^{y}
CH	0	90.74±0.89^{bx}	-2.48±0.07^{x}	7.62±0.25	89.29±2.05^{x}	-3.52±0.23^{y}	7.33±2.26	86.35±1.27^{y}	-3.35±0.37^{by}	5.61±0.86
	2	92.53±0.64^{ax}	-2.53±0.04^{x}	8.09±1.35^{x}	90.69±0.94^{x}	-3.55±0.30^{y}	5.97±1.42^{y}	84.28±1.85^{y}	-3.71±0.09^{ay}	5.76±0.79^{y}
	4	93.05±0.26^{ax}	-2.60±0.02^{x}	8.55±1.22^{x}	90.81±0.85^{y}	-3.54±0.24^{y}	6.07±1.53^{y}	85.13±1.25^{z}	-3.70±0.07^{ay}	5.89±0.85^{y}
	6	92.67±0.43^{ax}	-2.52±0.03^{x}	8.14±1.23^{x}	90.56±0.84^{y}	-3.55±0.25^{y}	6.03±0.80^{y}	85.83±1.75^{z}	-3.70±0.13^{ay}	6.22±0.29^{y}

Values were means ± standard deviation of three replicates.
$^{x-z}$ Values with different superscripts within raws differ significantly (P<0.05).
$^{a-c}$ Values with different superscripts within columns differ significantly (P<0.05).
OH: Ohmic heating, CH: Conventional heating.

Table 3. HMF and pH values of the milk samples.

			Milk Group (fat %)		
		Time (min)	3.1%	1.5%	0.1%
HMF (µM/L)	OH	0	8.09±0.23^{ya}	6.34±0.32^{b}	7.72±0.54^{a}
		2	8.25±0.71^{y}	6.98±1.34	8.34±0.37
		4	8.30±0.30^{y}	7.06±1.53	8.99±2.00
		6	9.17±0.33^{xa}	7.11±0.71^{b}	9.06±0.66^{a}
	CH	0	7.95±0.97^{a}	6.34±0.32^{b}	7.72±0.54^{a}
		2	8.73±0.91^{a}	6.45±0.61^{b}	8.14±0.68^{a}
		4	9.96±1.27^{a}	7.06±1.53^{b}	8.19±1.09^{b}
		6	8.54±1.41	7.07±1.39	8.25±1.37
pH	OH	0	6.54±0.07^{b}	6.67±0.02^{a}	6.53±0.02^{b}
		2	6.61±0.16	6.56±0.05	6.57±0.02
		4	6.57±0.14	6.55±0.05	6.54±0.03
		6	6.59±0.20	6.52±0.11	6.55±0.02
	CH	0	6.54±0.07^{b}	6.52±0.10^{a}	6.47±0.08^{b}
		2	6.61±0.17	6.55±0.10	6.48±0.08
		4	6.57±0.18	6.49±0.12	6.47±0.11
		6	6.56±0.19	6.49±0.13	6.46±0.14

Values were means ± standard deviation of three replicates.
$^{x-z}$ Values with different superscripts within columns differ significantly (P<0.05).
$^{a-c}$ Values with different superscripts within raws differ significantly (P<0.05).
OH: Ohmic heating, CH: Conventional heating.

Hydroxymethylfurfural and pH values of the milk samples treated with OH and CH were presented in Table 3. There was a difference in the HMF content of all groups at the initial of the OH process. It was determined that the OH process had a significant effect on HMF values only in 3.1% fat milk, and the highest HMF value is reached at the 6th min of the OH process (9.17µM/L) compared to other times (P<0.05). However, there was no difference between the process times in 1.5% and 0.1% fat milk groups (P>0.05). There was no statistically significant difference in the CH process in processing times in all milk groups (P>0.05). However, a significant difference was determined between the groups at the initial 2nd and 4th min of the CH process (P<0.05).

Before the heating treatments, the initial pH values of the 3.1%, 1.5% and 0.1% fat-milk were determined as 6.54±0.07, 6.67±0.02 and 6.53±0.02, respectively (Table 3). Although the initial pH values were statistically different between the sample groups, there was no significant difference between the groups according to the processing time during both processes of OH and CH (P>0.05).

Discussion and Conclusion

The inhibitory effect of milk fat content on the inactivation of *L. monocytogenes* by OH with a low voltage gradient was evaluated in the present study. The counts of *L. monocytogenes* cells in milk groups remained relatively stable during the first 4 min when the temperature was below 50°C of both OH and CB treatments (Figure 2 and Figure 3), which could be attributed to the fact that 50°C treatment was regarded as the initial stage to cause protein denaturation and cell components (6). Therefore, CH treatment is insufficient to inactivate *L. monocytogenes* cells below 50°C considerably. After 6 min of the treatment (over 50°C), OH could inactivate bacterial cells in 1.5% and 0.1% fat-milk sample groups, probably due to the combined effect of both electroporation and heat generation during the OH treatment (8, 17, 18).

Electroporation occurred during the OH increases the cell permeability and may induce permanent cell damage by lead to leaking biological components such as amino acids, proteins, nucleic acids, and coenzymes. In this circumstance, the bacterial population may evolve into either a dead or sublethally injured subpopulation (23). In the present study, the percentage of sublethal injured and inactivated cells rose with increasing temperature in OH treated all milk groups. The sublethal damage ratios of CH-treated milk groups were lower than OH-treated samples. Similarly, Shao et al. (18) reported that OH showed a higher injury ratio in 6 min treatment compared to that of WB (approximately 60% and 30%, respectively).

In this study, reductions of *L. monocytogenes* counts were significantly different, corresponding to fat content and processing time. While fat content does not affect the inactivation of microorganisms during CH, the microbial inactivation with the OH process was affected by the properties of the food material processing conditions (temperature, frequency, and electric field strength). However, fat works as an insulator, and high-fat content is associated with lower electrical conductivity (13, 23).

The nutritional components of food, such as salts, sugars, proteins, and fats, increase bacteria's heat resistance (7). Espina et al. (7) reported that the nutritional components of apple juice had a protective effect on the thermal inactivation (54°C) of *E. coli* O157:H7. Kim et al. (13) also reported that both lactose and fat content had an inhibitory effect on the inactivation of *L. monocytogenes* by OH. Similarly, Kim and Kang (12) found that OH was more effective than CH at inactivating *L. monocytogenes* in skim milk and cream. The results of our study were also consistent with previous reports.

In the present study, color and pH values did not significantly change during the OH treatments. Also, there was no significant difference between OH and CH-treated milk groups. Similarly, Park et al. (16) found that the color and pH values of OH-treated samples did not differ from non-treated samples. Moreover, Shao et al. (18) reported that the color and pH values of OH-treated milk samples had no significant differences from that of CH treated and control samples.

Hydroxymethylfurfural is known as a marker of Maillard reaction (15). The HMF content of the samples remained stable during the CH treatment; however, initial heating of 4 min was stable and then increased slightly at 6 min in OH treatment. Initial HMF content of milk groups was significantly different from each other. The differences in HMF values observed from the initial heating processes may be due to brand differences in the milk used. In a study, the HMF content of whole, semi-skimmed, and skimmed milk from different brands were in the range of 4.90±0.27-12.74±0.63, 2.50±0.30-8.89±0.18, 2.16±0.37-7.49±0.11 µM/L, respectively (25). In another study, the total HMF content of commercial UHT and sterilized milk samples with different fat content was in the range of 3.46–5.75 µM/L and 15.52–21.38 µM/L, respectively (14). Morales and Jiménez-Pérez (14) concluded that the amount of fat and total HMF values were negatively correlated in UHT and sterilized milk, which was confirmed with our study results. Results achieved in the present study, the initial HMF content of all samples was much higher before the heating treatments. The result of the present investigation was in line with the above-reported work, probably because of the prolonged heating times of different commercial milk brands were used.

In conclusion, due to the non-thermal impacts, OH demonstrated a higher inactivation efficacy to CH treatment in a shorter heating time on *L. monocytogenes* in low-fat milk. Besides, there was no significant change between OH- and CH-treated samples regarding color and pH. These findings revealed that OH might efficiently inactivate *L.monocytogenes* with no significant difference in milk quality compared to CH treatment. However, the increased fat content protects bacteria from thermal damage in OH treatment. Thus, the OH treatment conditions should be chosen carefully to sufficient inactivation of pathogens in milk with high-fat content. These results may be useful in providing a reference for using OH, as a time and energy-saving thermal processing, in milk sterilization.

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Conflicts of Interest

The authors declare that there is no conflict of interest.

Author Contributions

SÖ and HAK designed the experiments. SÖ carried out the analyses. HAK contributed to interpreting the results and took the lead in writing the manuscript. Both authors provided critical feedback and helped shape the research, analysis, and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

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Investigation of the presence of some antibiotics in Raw Goat milk collected from Ankara, Kırıkkale and Çankırı provinces

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ABSTRACT

Antibiotics used in food producing-animals may cause residual problems in food in terms of public health. This situation can lead to serious problems in terms of human health. Raw milk is one of the foods that are likely to contain antibiotics, even in trace amounts. This study aimed to determine the residue levels of commonly used antibiotics of raw goat's milk samples offered for sale in Ankara, Çankırı and Kırıkkale. One active ingredient was selected from the five most commonly used antibiotic groups in animals and it determined the residue levels of these substances in milk. For this purpose, within one year and in two different periods, 150 raw goat milks analyzed in terms of antibiotics using HPLC method. The values for enrofloxacin, sulfamethoxazole, tylosin, penicillin G and oxytetracycline were 7.9, 9.7, 11.5, 5.4 and 7.3 minutes for retention times, 1.47, 0.8; 7.51; 2.69 and 8.89 µg/L for limit of detection (LOD) and 4.47, 2.44, 22.78, 8.16 and 26.96 µg/L for limit of quantification (LOQ), respectively. No antibiotic residues were detected in the goat milk samples. It is predicted that the higher resistance of goats to diseases compared to other milk-producing animals, and therefore the lower use of antibiotics in these animals, leads to this result. The findings obtained as a result of this study are valuable in terms of public health. It is important that no antibiotic residues are found in the analyzes.

Introduction

Milk is a biological substance produced by mammals in their mammary glands following pregnancy. It contains almost all the nutrients. In fact, the main purpose of secretion or production of the milk is to ensure the immunological adaptation of the newborn to the outside world and to meet the basic nutritional needs of the infant. Among milks, goat milk has special importance for human as it is the closest milk to human breast milk. The fact that it has much less allergic effects and trans-fat content compared to cow's milk, and its high digestibility increases its importance even more. The lower ratio of trans fatty acids compared to cow's milk also reduces the risk of heart disease (4, 6). Recently, the interest in goat milk and its products has increased with the demonstration of its beneficial aspects to human health. In addition to the use of goat milk as dairy products such as sterilized and pasteurized drinking milk, yogurt, cheese, ice cream and dairy desserts, goat milk is involved in the manufacture of cosmetic products such as hand and bath soaps, hand and face moisturizers that can be used by atopic patients (1, 3, 24, 26). Goat milk is in the group of casein milks. Considering its composition, it has been reported that the dry matter is around 12.5% on average and this total dry matter contains on average 4% fat, 3.3% protein, 4.1% lactose and 0.8% ash (18). The composition of goat's milk differs according to the country and breed where it is grown (3, 10, 12).

The drugs used for growth promotion improved feed conversion efficiency and for the prevention and treatment of diseases in animals cause residue problems by accumulating in the tissues or organs of animals. The presence of drug residues in foodstuffs poses a significant risk to consumer health and well-being (13). Due to the

reasons described above, the use of drugs can sometimes be unavoidable. In such cases, residue levels in animal products should be kept below the levels specified by the authorities by constantly monitoring. Residues in foods exceeding the permissible amount pose a potential toxicological hazard to consumers (2, 5, 11, 22). This study aimed to determine the presence of some antibiotics in raw goat milk collected from Ankara, Kırıkkale and Çankırı provinces of Türkiye.

Materials and Methods

In this study, milk samples were collected from goat raw milk (or collection containers) of goat breeding farms in Ankara, Çankırı and Kırıkkale provinces of Türkiye twice in 2019 (March and September were preferred according to lambing time of goats). Samples were taken from five different farms in each province and five different goats from each farm. 500 mL of raw goat's milk was packed in leakproof, disposable glass containers. Samples were taken in accordance with the National Residue Monitoring Program (23). The samples were brought to the laboratory under cold chain and kept at -20°C until analysis.

All measurements were performed using a Shimadzu Ultra Fast Liquid Chromatograph (UFLC) (Shimadzu, Japan) system (LC-20AD, Shimadzu) equipped with a quaternary pump, a vacuum degasser, a column compartment, an auto sample, and a diode-array detector, and controlled by the LabSolutions chromatography software. The analytical column was HPLC Column, Intersil ODS4, 5 µm 4.6×250 mm. Other equipment such as pH meter (HANNA Instruments HI 2211), electronic weighing balance (Sartorius), centrifuge (NF 815), ultrasonic cleaner (Probetec) and vortex (Heidolph) were also used in this study. Standard solutions and samples prepared for analysis were injected into the instrument in 50 µL. Methanol, acetonitrile and ammonium dihydrogen phosphate solutions used as mobile phases in the method were defined to the device according to the program shown in Table 1.

Extraction: 2 mL of milk was placed into a 15 mL centrifuge tube and 5 mL of acetonitrile was added to ensure the denaturation of proteins and mixed in a vortex for 1 minute. Then, 0.25 g Sodium chloride (NaCl) was added to the tube and vortexed for 1 minute. After homogenization for 5 minutes in an ultrasonic bath, it was centrifuged at 3000 rpm for 5 minutes. Then, it was filtered through a 0.45 µm nylon filter and 50 µL was applied to the system (9, 27). The parameters of the HPLC device used for the analysis are given in Table 2.

Preparation of Standards: Main stock solutions for each active substance (Enrofloxacin, Sulfamethoxazole, Tylosin, Penicillin G and Oxytetracycline) were prepared by dissolving the amount equivalent to 10 mg of standard substance in 10 mL of distilled water (1 mg/mL). Working solutions of 20, 40, 80, 100, 200, 400, 800, and 1000 µg/L were prepared from the main stock solution.

Validation of the Method: Accuracy, linearity and working range, selectivity, precision, limit of detection and limit of measurement were accepted as the preferred performance criteria in determining the validation of the method (5, 7, 21, 25).

Table 1. Gradient conditions.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.01	70	30
3	65	35
5	45	55
6.5	45	55
9	0	100
10	0	100
11	65	35
12.5	65	35
14	70	30

Table 2. HPLC parameters.

HPLC Parameters	
Colon	Intersil ODS4 (250 x 4.60 mm, 5 µm)
Colon temperature	40°C
Mobile phase	Mobile Phase A: Methane: acetonitrile: water (1:3:1) Mobile Phase B: NH_4PO_4 20 mM, pH: 2
Flow rate	0.8 mL/min
Wavelength	280 nm
Detector	Diode-Array Detection (DAD)
Injection volume	50 µL
Analysis time	16 min

Results

Method Validation

Accuracy: Recovery values were calculated as 96.98% for enrofloxacin, 94.08% for sulfamethoxazole, 106.95% for tylosin, 102.71% for penicillin G and 105.24% for oxytetracycline (Table 3).

Linearity and Working Range: R^2 values were calculated as 0.998 for enrofloxacin, 0.999 for sulfamethoxazole, 0.999 for tylosin, 0.998 for penicillin G and 0.998 for oxytetracycline (Table 4). The calibration curves obtained for each antibiotic are shown in Figure 1.

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Table 3. Recovery values.

Antibiotics	Dose (µg/L)	Recovery (%)	Mean (%)
Enrofloxacin	40	92.17	96.98
	80	100.49	
	100	98.28	
Sulfamethoxazole	40	88.63	94.08
	80	91.74	
	100	102.45	
Tylosin	40	92.92	106.95
	80	91.4	
	100	104.63	
Penicillin G	40	107.57	102.71
	80	96.61	
	100	103.96	
Oxytetracycline	40	118.5	105.24
	80	99.35	
	100	97.74	

Table 4. Correlation coefficients.

Standarts	R^2	Equation
Enrofloxacin	0.998	Y=306912x+11790.2
Sulfamethoxazole	0.999	Y=523358x+18609.3
Tylosin	0.999	Y=73973x-930.373
Penicillin G	0.998	Y=100681x+24013.3
Oxytetracycline	0.998	Y=121810x-4075.2

Selectivity and Precision: Chromatograms of the blank sample (Figure 2A) and the standard loaded samples (Figure 2B) reveal the selectivity of the method. Retention times of antibiotic standards were determined as 7.9 minutes for enrofloxacin, 9.7 minutes for sulfamethoxazole, 11.5 minutes for tylosin, 5.4 minutes for penicillin G and 7.3 minutes for oxytetracycline HCI. It was also seen that no peak of any compound was detected at the same retention time. Each antibiotic was evaluated as an internal standard for the remaining active ingredients. For this reason, it was not necessary to use a different active substance for the internal standard.

Reproducibility: Reproducibility study results of the method, % recovery and %RSD values are given in Table 5.

The limit of Detection (LOD) and Limit of Quantification (LOQ): LOD and LOQ results; 1.47 µg/L and 4.47 µg/L for enrofloxacin, respectively; 0.8 µg/L and 2.44 µg/L for sulfamethoxazole; 7.51 µg/L and 22.78 µg/L for tylosin; 2.69 µg/L and 8.16 µg/L for penicillin G, and 8.89 µg/L and 26.96 µg/L for oxytetracycline, respectively, are demonstrated in Table 6.

Determination of Antibiotic Presence in Goat Milk: After validation parameters of the method were made, 150 goat milk samples collected from Ankara, Çankırı and Kırıkkale provinces were analyzed. According to the results of the analysis, no antibiotic presence was detected in the samples.

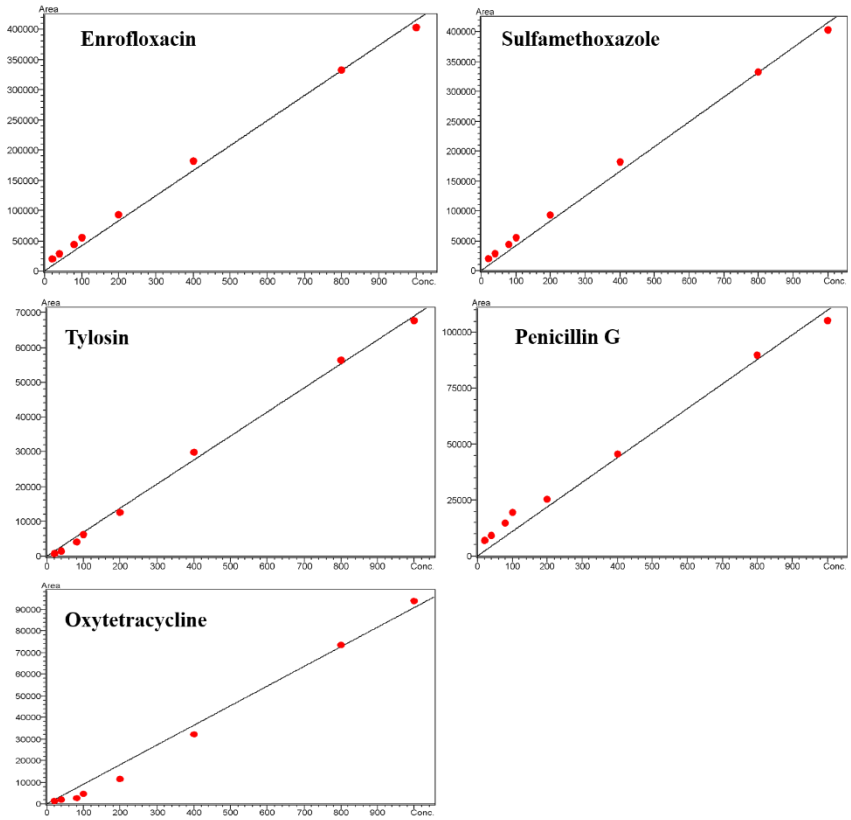

Figure 1. Calibration curves for tested antibiotics.

Table 5. The reproducibility of the method.

Antibiotic	Day	Dose (µg/L)	Repetitions	Mean (%)	Recovery (%)	Standard deviation	RSD (%)	Relative Error (%)
Enrofloxacin	1	40	3	36.52	91.3	0.29	0.79	8.7
		80	3	79.64	99.55	0.65	0.81	0.06
		100	3	97.95	97.95	0.27	0.27	2.05
	2	40	3	35.93	89.82	0.67	1.86	10.17
		80	3	75	93.75	1.22	1.62	6.25
		100	3	97.74	97.74	0.55	0.56	2.26
	3	40	3	38.29	95.72	1.46	3.81	4.27
		80	3	76.04	95.05	0.73	0.96	4.95
		100	3	102.25	102.25	2.75	2.68	-2.25
Sulfamethoxazole	1	40	3	35.35	88.37	0.26	0.73	11.63
		80	3	73.39	91.73	0.002	0.002	8.26
		100	3	102.43	102.43	0.03	0.02	-2.43
	2	40	3	34.92	87.3	0.42	1.2	12.7
		80	3	72.15	90.18	0.04	0.05	9.81
		100	3	102.43	102.43	0.04	0.03	-2.43
	3	40	3	35.05	87.65	0.24	0.68	12.37
		80	3	72.15	90.18	0.04	0.33	9.81
		100	3	102.74	102.74	0.30	0.29	-2.74
Tylosin	1	40	3	37.5	93.75	0.62	1.65	6.25
		80	3	71.83	89.78	1.11	1.54	10.21
		100	3	105.77	105.77	1.18	1.11	-5.77
	2	40	3	39	97.5	2.97	7.61	2.5
		80	3	78.77	98.46	3.27	4.15	1.53
		100	3	105.03	105.03	2.36	2.24	-5.03
	3	40	3	37.45	93.62	0.29	0.77	6.37
		80	3	77.84	97.3	3.21	4.12	2.7
		100	3	104.92	104.92	0.61	0.58	-4.92
Penicillin G	1	40	3	43.66	109.15	0.54	1.23	-9.15
		80	3	76.9	96.12	2.01	2.61	3.87
		100	3	104.64	104.64	1.54	1.47	-4.64
	2	40	3	43.23	108.07	1.22	2.82	-8.07
		80	3	74.33	92.91	1.30	1.74	7.08
		100	3	106.09	106.09	2	1.88	-6.09
	3	40	3	41.24	103.1	1.16	2.81	-3.1
		80	3	82.89	103.6	1.92	2.31	-3.6
		100	3	111.35	111.35	1.56	1.4	-11.35
Oxytetracycline	1	40	3	45.75	114.37	2.43	5.31	-14.37
		80	3	81.30	101.62	2.04	2.5	-1.62
		100	3	98.38	98.38	1.10	1.11	1.62
	2	40	3	43.99	109.97	2.62	5.95	-9.97
		80	3	77.92	97.4	1.62	2.07	2.6
		100	3	99.84	99.84	2.71	2.71	0.16
	3	40	3	42.95	107.37	1.26	2.93	-7.37
		80	3	76.70	95.87	1.21	1.57	4.12
		100	3	96.03	96.03	0.32	0.33	3.97

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Table 6. LOD and LOQ values.

Antibiotic	LOD (µg/L)	LOQ (µg/L)
Enrofloxacin	1.47	4.47
Sulfamethoxazole	0.8	2.44
Tylosin	7.51	22.78
Penicillin G	2.69	8.16
Oxytetracycline	8.89	26.96

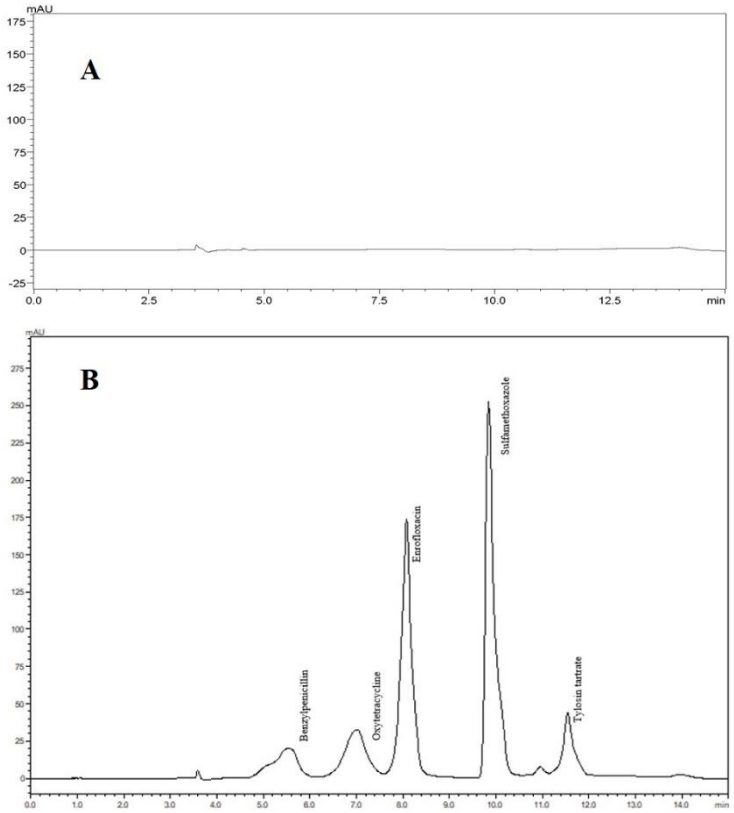

Figure 2. Chromatograms of blank (A) and antibiotic standards (B).

Discussion and Conclusion

Medicines used for various purposes in animals may leave residues in animal products due to erroneous and misguided use, thereby creating negative consequences for human health and resulting in economic losses. In Türkiye, drugs and chemical substances, including antibacterial drugs in animal products are monitored by the "National Residue Monitoring Program," launched by the Ministry of Agriculture and Forestry.

One of the most important steps in performing drug analysis in matrices with complex structures (such as milk) is the process of extraction. Liquid-liquid extraction is actually a versatile sample preparation technique specified in many analytic methods. This technique has the disadvantages of being tedious for incorporating several stages and taking too much time to perform. Also, it was reported that too many poisonous and expensive chemicals are used in this process, and it has the potential to lead to environmental pollution (17, 18, 20). In this study, the chemical substances used were fewer and these chemicals were used in lower quantities compared to other methods, and therefore, this may be seen as an advantage for this study.

In the present study, the presence of antibiotic residues in goat milk was examined and its significance for public health was assessed. Additionally, the data related to the method employed in this study and analysis results were compared to similar methods and results.

Oruç and Sonal (16) investigated residues of oxytetracycline, penicillin G and sulfadimidine in 25 raw milk samples in Bursa using the HPLC method and reported no antibiotic residues in the samples. The results of this study were similar to the data of our study.

Nina et al. (15) used microbial tests and immunoassay method in the preliminary survey of 1,259 raw milk samples collected over a period of three years in

Croatia to identify the presence of certain antibiotics including chloramphenicol and reported the antibiotic residue in 37 samples. In the same study, the validation of the positive samples using the HPLC method demonstrated that only three samples contained residues above the permitted limit values (2 µg/kg penicillin, 19 µg/kg amoxicillin and 1.671 µg/kg tetracycline).

Navratilova et al. (14) studied 150 raw cow milk samples collected from the South Moravia and Vysočina regions of Czechia using the HPLC method and reported fluoroquinolone residues in 87.3% of the samples. The difference between the results of this study and the current study may have resulted from the fact that the samples analyzed were collected from different countries and in different periods.

Boultif (8) used the ELISA and HPLC methods to look for any residue of oxytetracycline and penicillin G in 120 milk samples in Algeria and reported oxytetracycline residues in 22 milk samples. The difference between the results of this study and the current study may have resulted from the fact that the samples analyzed were collected from different countries and in different periods.

In the study, the residual presence of enrofloxacin, sulfamethoxazole, tylosin, penicillin G and oxytetracycline in raw goat milk collected from Ankara, Kırıkkale and Çankırı provinces was investigated by HPLC method. No antibiotic residue could be detected in 150 milk samples. Considering that goats are more resistant to diseases than other animals whose milk is used, and therefore antibiotic use in goats is considered as at a lower level than other animals, it is predicted that this result has been achieved.

In the current study, the adaptation and validation of the test method for determining enrofloxacin, sulfamethoxazole, tylosin, penicillin G, and oxytetracycline in raw goat milk was performed. The method validated in the study was found to be fast, easy, practical and reliable for the analysis of the samples, and the chemical substances used were kept at a minimum, ensuring an analysis of more samples at a shorter time and with lower costs, which were considered the advantages of the method selected. Likewise, the ability to analyze five different active substances with a simple application following a single process of extraction highlights the usability of the method. In this regard, this method may be useful in ensuring that analysis for residue monitoring can be performed rapidly, and this may allow it to be used in a more widespread manner.

When the reasons for drug residues in animal source foods are examined, the failure to comply with the waiting period before slaughtering generally stands out. Therefore, it is critical to inform the breeders who use drugs on animals of this requirement. Likewise, awareness raising activities targeting veterinary physicians, animal breeders, facilities producing or selling veterinary drugs, public organizations and institutions and consumers are important as well. In this context, rational drug use, compliance with principles of preventive medicine and, in particular, use of prescription drugs is obligatory.

Considering the results of this study, it can be said that it is good news for public health that no residue from the antibiotics in question could be determined in the goat milks collected from the specified regions. It was concluded that the national residue monitoring programs, the activities in which the importance of lack of residues is stressed for breeders, the antibiotic awareness week, and the efforts and programs implemented as part of the One Health Approach were effective in obtaining these results. It should be remembered that such activities and programs should be maintained in the future as the residue problem is a matter that continues to be relevant at all times and that has an international dimension. Even though such analyses are performed within the framework of the National Residue Monitoring Program, the number of analyses performed on samples of other types of milk than cow milk, such as goat milk, water buffalo milk and other milks produced less compared to cow milk, but enjoying increased popularity should be supported. This study was conducted on a sizable number of goat milk samples and did not find any risk factor for public health in them in terms of antibiotics examined.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Author Contributions

LA and ATM conceived and planned the experiments. ATM and YA carried out the experiments. LA, ATM and YA planned and carried out the simulations. LA and ATM contributed to sample preparation. LA, ATM and YA contributed to the interpretation of the results. ATM took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study did not present any ethical concerns.

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Physical properties and bacterial viability of functional ice cream enriched with kefir

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ABSTRACT

In this study, different ratios (A-0%, B-25%, C-50%, D-75%) of kefir were used in the ice cream mix in order to obtain functional ice cream enriched with probiotic bacteria. There was no difference between the chemical and physical properties of the samples ($P<0.05$), except for acidity and overrun values ($P>0.05$). Kefir containing samples showed probiotic properties during 90-day storage when the probiotic bacterial counts were considered. In terms of texture and flavor properties, sample D had the lowest scores, while B and C had similar scores compared to sample A in sensory evaluation. As a result, B and C were identified as probiotic products with acceptable properties during 90-day storage.

Introduction

Ice cream is a product that is produced worldwide and consumed by individuals of all ages. It is a highly nutritious product due to the ingredients used. The composition of ice cream can be changed easily in comparison to the other dairy products, giving it a special place among other functional products that are increasing in production and consumption (38).

Enrichment of ice cream by probiotics and/or prebiotics has been the subject of majority of research. In these studies, the production is usually carried out by adding pure probiotic culture and/or prebiotic compounds directly to the ice cream mix (4, 18, 30).

Probiotics are microorganisms that have a positive effect on the intestinal system when consumed in a certain amount (17). Probiotics in the intestinal microflora improve the immune system, protect the body against various diseases and they also have anticarcinogenic and serum cholesterol-lowering effects (28). Lactic acid bacteria are the most commonly used bacteria group for enriching foods with probiotic microorganisms. Prebiotics, on the other hand, are substances that directly enter the intestinal system when taken into the body and stimulate the growth of probiotic bacteria (17). They were reported to have positive effects on the digestion of sugars, protective effects against heart disease risk and inhibiting effects on pathogenic microorganisms (28). Prebiotic substances are generally classified as inulin, oligofructoses and fructooligosaccharides (27).

Enriching ice cream with probiotics is a more appropriate way compared to other dairy products. This was explained by the higher pH of ice cream compared to those of fermented milk products since the survival of probiotic bacteria in low pH environments is low (1).

Kefir, one of the richest products in terms of probiotic microorganisms, is a fermented dairy product in

which starter cultures or kefir grains consisting of ~83-90% lactic acid bacteria and ~10-17% yeast and acetic acid bacteria are used in the production (20, 39). These cultures usually include different strains of *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Acetobacter* genera and also lactose fermenting (*Kluyveromyces marxianus*) and non-fermenting yeasts (*Saccharomyces unisporus*, *Sacch. cerevisiae* and *Sacch. exiguus*) (15, 36). Since most of these microorganisms have probiotic properties, the evaluation of kefir in different ways has become an increasingly interesting issue.

The aim of this study is to enrich ice cream with probiotic bacteria and to obtain a functional product with increased nutritional value. For this purpose, kefir, was added to ice cream mix in different proportions and ice cream production was carried out. The study is considered to be important for obtaining an ice cream with probiotics that is not available in the market and has a higher nutritional value than both ice cream and kefir.

Materials and Methods

Kefir and ice cream preparation: For the preparation of kefir, raw cow's milk (Ankara University Faculty of Agriculture Research and Application Farm, Ankara, Türkiye) was subjected to heat treatment at 90 °C/10 min and cooled to 25 °C. Immediately after the cooling process, kefir culture (CHOOZIT® Kefir DC, LYO 1000 L, Danisco, Germany) was inoculated (according to the ratio specified on the package, 5 g/1000 L) and the samples were incubated at 25 °C until pH 4.5-4.6 and kept at +4 °C for ~24 h until being used in ice cream mix preparation.

All ice cream mixes were formulated to contain 10% fat (derived from 65% fat cream; Ankara University Faculty of Agriculture Research and Application Farm, Ankara, Türkiye), 12% milk solids-not-fat (derived from cream and skim milk powder; Izi Sut A.S., Türkiye), 15% sucrose (derived from sugar obtained from local market) and 0.5% stabilizer-emulsifier mixture (Cremodan Sim Veg, Danisco, Germany). Mixing rates of kefir and ice cream mixes were shown in Table 1. In the preparation of the mixes, the amount of fat and milk solids-not-fat to be covered from kefir to be added to each sample was calculated separately, and these values were subtracted from the amount of the main substance desired to be in the final product, and the remaining amount was calculated from 2250 ml, 1500 ml and 750 ml mixes, respectively. The prepared mixes were heat treated at 80 °C for 20 min and homogenized with ultraturrax (DIAX 900, Heidolph, Schwabach, Germany) for 5 min. Subsequently, the mixes were promptly cooled to ~25 °C and then aged for approximately 19 h at + 4°C. At this point, the aged mixes were inoculated with previously prepared kefir culture in the amounts specified in Table 1 and the final mixture was re-homogenized with ultraturrax for 10 min. 3 L of ice cream mixes were frozen in a batch freezer (Triomaxx, Ada, Jiangmen, China) for 15 min and the samples were packaged and hardened at -25 °C for 20 h. All ice cream formulations were produced in duplicate.

Table 1. Mixing rates of kefir and ice cream mixes used in ice cream production.

Sample code	Kefir ratio (%)	Kefir used in mixture (ml)	Ice cream mix used in mixture (ml)
A (Control)	0	0	3000
B	25	750	2250
C	50	1500	1500
D	75	2250	750

Determination of lactic acid, total solid, fat, total protein and ash contents: Lactic acid contents of the ice cream samples were determined by using the titration method and the results were calculated as percent lactic acid (8). Fat contents were determined by Gerber method (6), total dry matter and ash contents were determined by gravimetric method according to AOAC (7) and Goff et al. (14), respectively. Kjeldahl method was used to determine the total protein content by multiplying the total nitrogen content by the factor of 6.38 (6).

Rheological measurements: The rheological properties of the samples were determined by Malvern Kinexus Pro+ rheometer (Worcestershire, UK) with a cone and plate geometry (diameter: 40 mm, cone angle: 4°). The consistency index (*K*) and flow behavior index (*n*) of the samples were determined by dynamic rheometry at 2 mm gap, 0.1-300 s^{-1} shear rate at 5 °C. The data obtained from the analysis were adjusted to the Herschel-Bulkley model based on the following equation:

$$\tau = \tau_o + K \dot{\gamma}^n \qquad (1)$$

where τ is the shear stress (Pa), τ_o is the yield stress (Pa), K is the consistency coefficient (Pa s^n), $\dot{\gamma}$ is the shear rate (s^{-1}) and n is the flow behavior index.

All measurements were performed at least in duplicate.

Overrun measurement: Overrun was measured by comparing the weight of a certain volume of ice cream mix and the same volume of ice cream. Overrun results were calculated by using the weights recorded according to the equation below (14):

Overrun (%) = [(Weight of mix-Weight of ice cream) / (Weight of ice cream)] x 100 (2)

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Melting characteristics: The method specified by Mendez-Velasco et al. (22) was applied in order to determine the meltdown rates of ice cream. Samples were removed carefully from the containers and their weight were recorded. Ice creams were placed on a stainless steel wire with 2.5 mm^2 holes and a glass beaker placed underneath to collect the melted part. The analysis performed at room temperature (~22 °C) and the first dripping time of each sample was recorded in min. In addition, the weight of drained material through the wire was recorded every 20 min for 120 min, and the meltdown rates of ice cream were calculated according to the following formula:

Meltdown rate (%) = (Weight of drained material / Weight of ice cream) x 100 (3).

In addition, the average melting rates were calculated considering the amounts of the dripped portion after a total of 120 min and expressed in g/min. Also the weight of the drained material of ice cream after 120 min was recorded and the percent mass retention was calculated by using the following equation (40):

Mass retention (%) = 100 – Drained material after 120 min (%) (4).

Hardness measurement: The hardness values of the samples were determined by using a texture analyzer (TA.Xt Plus, Stabel Micro Systems®) equipped with a 5 mm diameter cylindrical stainless steel probe (Part Code: P/5, Stable Micro Systems®). The samples were kept at -15 °C for 24 h before analysis. Three measurements were recorded from three different containers for each sample and the average of these measurements was calculated. The parameters for analysis specified by Akalin et al. (4) were as follows: penetration distance = 15 mm, force = 5.0 g, probe speed during penetration = 3.3 mm s^{-1}, probe speed pre- and post penetration = 3.0 mm s^{-1}.

Bacteriological analysis: Ice cream samples (10 g) were diluted in 90 ml sterile Ringer solution (Merck, Darmstadt, Germany) and homogenized in a Stomacher (Bag Mixer 400 VW, Interscience, France) for 2 min. Subsequent serial dilutions were prepared in 9 ml sterile Ringer solution and poured onto plates of the various selective and differential agars in duplicate. M17 agar (Merck, Darmstadt, Germany) and MRS agar (de Man Ragosa Sharpe Agar, Merck, Darmstadt, Germany) were used for the enumeration of *Lactococcus* spp. and *Lactobacillus* spp. respectively. All plates were incubated at 37 °C for 24-48 h. The applied incubation conditions were aerobic and unaerobic for M17 agar and MRS agar respectively. Colonies were enumerated after the incubation and the results were expressed as log cfu/g.

Sensory evaluation: Approximately 25 g of ice cream were scooped into 50 ml plastic containers and kept at -15 °C for ~2 h before evaluation. For the sensory evaluation of ice cream samples, a scoring test with 7 experienced panelists from the academic staff of Ankara University Department of Dairy Technology were applied. The test form suggested by Meilgaard et al. (21) were modified and used for sensory analysis. Panelists were asked to evaluate the samples over 5 points in terms of appearance, texture and flavor characteristics. The panelists evaluated the four samples in the same session. Drinking water and unsalted crackers were provided to clean the mouth before and between the samples.

Statistical analysis: Total solid, fat, total protein, ash contents, rheological characteristics, overrun values, melting characteristics and hardness values were determined only on the 1st day of the storage. The other analysis were performed on the 1^{st}, 30^{th}, 60^{th} and 90^{th} days of the storage. All analysis were performed in duplicate for each parameter.

Analysis of variance (One-way ANOVA) method was used to evaluate the differences between kefir ratios (0, 25, 50, 75%) in terms of total solid, fat, total protein, ash, rheological characteristics, overrun, first dripping time, average melting rate, mass retention and hardness parameters. The Repeated Measures ANOVA method was used to determine whether the differences between the level means of the kefir ratios (0, 25, 50, 75%), time (40, 60, 80, 100, 120 min) factors and their interaction on the meltdown rates are statistically significant. The differences between the kefir ratios (0, 25, 50, 75%) and storage time (1, 30, 60, 90 days) and their interaction in terms of titratable acidity, bacterial counts and sensory characteristics were evaluated using the Factorial ANOVA method. Tukey multiple comparison test was used to separate means of data when significant differences ($P<0.05$) were observed. IBM SPSS Statistics 20 software was used for statistical analysis and the results were expressed as mean ± standart error.

Results

The chemical composition of ice cream samples: The chemical composition of ice cream samples is given in Table 2 ($P>0.05$). The samples produced in this study were classified as fatty ice cream according to the Turkish Food Codex Communique on Ice Cream (35) since the total solid and fat contents of fatty ice cream should be at least 36% and 8% respectively.

Rheological characteristics: In Table 3, consistency index (*K*) and flow behavior index (*n*) values of the samples are given. All of the ice cream mixes were compatible with the "Herschel-Bulkley" behavioral model (correlation value - $R^2 > 0.99$) and no difference was found between the K and *n* values of the samples interpreted according to this model ($P>0.05$).

Table 2. The chemical composition of the samples (n=2).

	Samples*			
	A	**B**	**C**	**D**
Total solid (g/100 g)	36.87 ± 0.019	36.68 ± 0.019	36.61 ± 0.015	36.81 ± 0.027
Fat (g/100 g)	10.50 ± 0.000	10.25 ± 0.250	10.25 ± 0.250	10.25 ± 0.250
Total protein (g/100 g)	3.56 ± 0.035	3.56 ± 0.060	3.57 ± 0.055	3.54 ± 0.050
Ash (g/100 g)	0.87 ± 0.011	0.83 ± 0.015	0.85 ± 0.002	0.85 ± 0.009

*A: 0% kefir - Control, B: 25% kefir, C: 50% kefir, D: 75% kefir.

Table 3. The consistency index (*K*), flow behavior index (*n*) and hardness values of the samples (n=2).

	Samples*			
	A	**B**	**C**	**D**
K (Pa.s)	0.23 ± 0.006	0.23 ± 0.002	0.23 ± 0.009	0.23 ± 0.003
n	0.72 ± 0.011	0.72 ± 0.024	0.72 ± 0.013	0.72 ± 0.008
Hardness (g)	15 539 ± 190	16 096 ± 315	15 628 ± 886	15 945 ± 390

*A: 0% kefir - Control, B: 25% kefir, C: 50% kefir, D: 75% kefir.

Overrun: As it is seen from Figure 1, the overrun values of the samples C and D with the highest kefir content were found to be the highest, while sample A without kefir was determined as the sample with the lowest overrun (P<0.05).

Melting characteristics: It was determined that there was no difference between the first dripping times, average melting rates, and remaining mass retentions at the end of 120 min (P>0.05) as it is seen from Table 4. Similarly, no difference was found between the meltdown rates of the samples at 40, 60, 80, 100 and 120 min (P>0.05) (Figure 2).

Hardness: Hardness values of the ice cream samples are given in Table 3. According to the results, kefir addition did not affect the hardness values of the samples (P<0.05).

Lactic acid: Use of kefir in ice cream mix affected the lactic acid content of the final product during 90-days of storage (Table 5). As the amount of kefir in mix increased, the lactic acid content of the product was also increased at all storage days.

Bacterial counts: As expected, *Lactococcus* spp. and *Lactobacillus* spp. counts increased for each day of storage (P<0.05) as the kefir amount increased (Table 5). In addition, *Lactococcus* spp. counts were higher compared to *Lactobacillus* spp. counts at all storage days. However, bacterial viability decreased for both probiotic bacteria during 90-days of storage (P<0.05), but all of the samples still maintained their probiotic properties since they contained at least 10^7 cfu/g (7 log cfu/g) of probiotic bacteria.

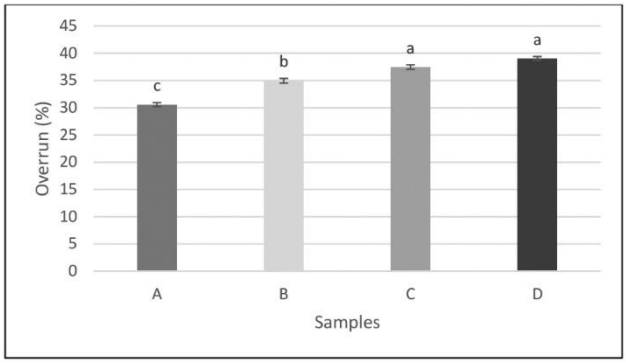

Figure 1. Overrun values of the samples.
A: 0% kefir - Control, B: 25% kefir, C: 50% kefir, D: 75% kefir.
Values with the different letter are significantly different (P<0.05).

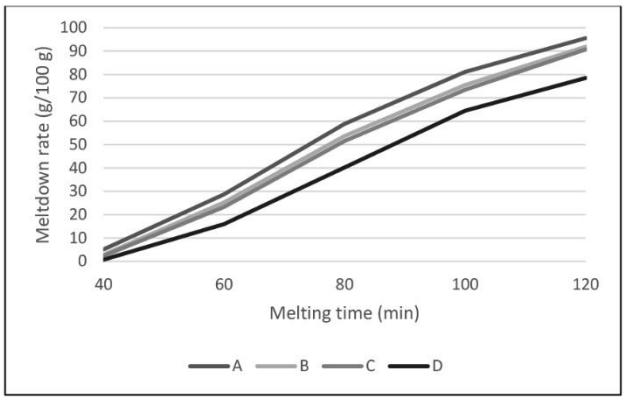

Figure 2. Meltdown rates of the samples at 40, 60, 80, 100 and 120 min.
A: 0% kefir - Control, B: 25% kefir, C: 50% kefir, D: 75% kefir.

Table 4. First dripping time, average melting rate and mass retention values of the samples (n=2).

	Samples*			
	A	**B**	**C**	**D**
First dripping time (min)	32.00 ± 2.000	33.00 ± 1.00	34.00 ± 1.000	40.00 ± 2.000
Average melting rate (g/min)	0.80 ± 0.030	0.79 ± 0.030	0.79 ± 0.025	0.66 ± 0.040
Mass retention (%)	4.48 ± 2.320	8.13 ± 3.230	9.32 ± 3.740	21.53 ± 6.050

*A: 0% kefir - Control, B: 25% kefir, C: 50% kefir, D: 75% kefir.

Table 5. Lactic acid content, *Lactococcus* spp. and *Lactobacillus* spp. counts of the samples during storage (n=2).

	Storage (Day)	Samples*			
		A	**B**	**C**	**D**
Lactic acid (g/100 g)	1	0.26 ± 0.005^{d}	0.41 ± 0.010^{c}	0.58 ± 0.010^{b}	0.69 ± 0.005^{a}
	30	0.25 ± 0.010^{d}	0.41 ± 0.005^{c}	0.58 ± 0.005^{b}	0.68 ± 0.010^{a}
	60	0.25 ± 0.000^{d}	0.41 ± 0.005^{c}	0.58 ± 0.015^{b}	0.69 ± 0.005^{a}
	90	0.25 ± 0.010^{d}	0.42 ± 0.005^{c}	0.58 ± 0.005^{b}	0.68 ± 0.005^{a}
Lactococcus spp. (log cfu/g)	1	ND^{A}	8.27 ± 0.040^{Ac}	9.02 ± 0.07^{Ab}	9.34 ± 0.075^{Aa}
	30	ND^{A}	8.16 ± 0.045^{ABc}	8.74 ± 0.060^{Bb}	9.15 ± 0.080^{ABa}
	60	ND^{A}	8.00 ± 0.050^{Bc}	8.58 ± 0.110^{BCb}	8.92 ± 0.090^{BCa}
	90	ND^{A}	7.92 ± 0.090^{Bc}	8.47 ± 0.0750^{Cb}	8.81 ± 0.055^{Ca}
Lactobacillus spp. (log cfu/g)	1	ND^{Ac}	5.07 ± 0.055^{Ab}	5.79 ± 0.095^{Aa}	5.99 ± 0.085^{Aa}
	30	ND^{Ad}	3.99 ± 0.065^{Bc}	4.67 ± 0.055^{Bb}	5.11 ± 0.050^{Ba}
	60	ND^{Ad}	3.71 ± 0.080^{Cc}	4.27 ± 0.085^{Cb}	4.64 ± 0.075^{Ca}
	90	ND^{Ad}	3.54 ± 0.060^{Cc}	4.06 ± 0.055^{Cb}	4.43 ± 0.045^{Ca}

*A: 0% kefir - Control, B: 25% kefir, C: 50% kefir, D: 75% kefir.
Values with the different lower case letter within the same row and upper case letter within the same column are significantly different (P<0.05).
ND: Not detected.

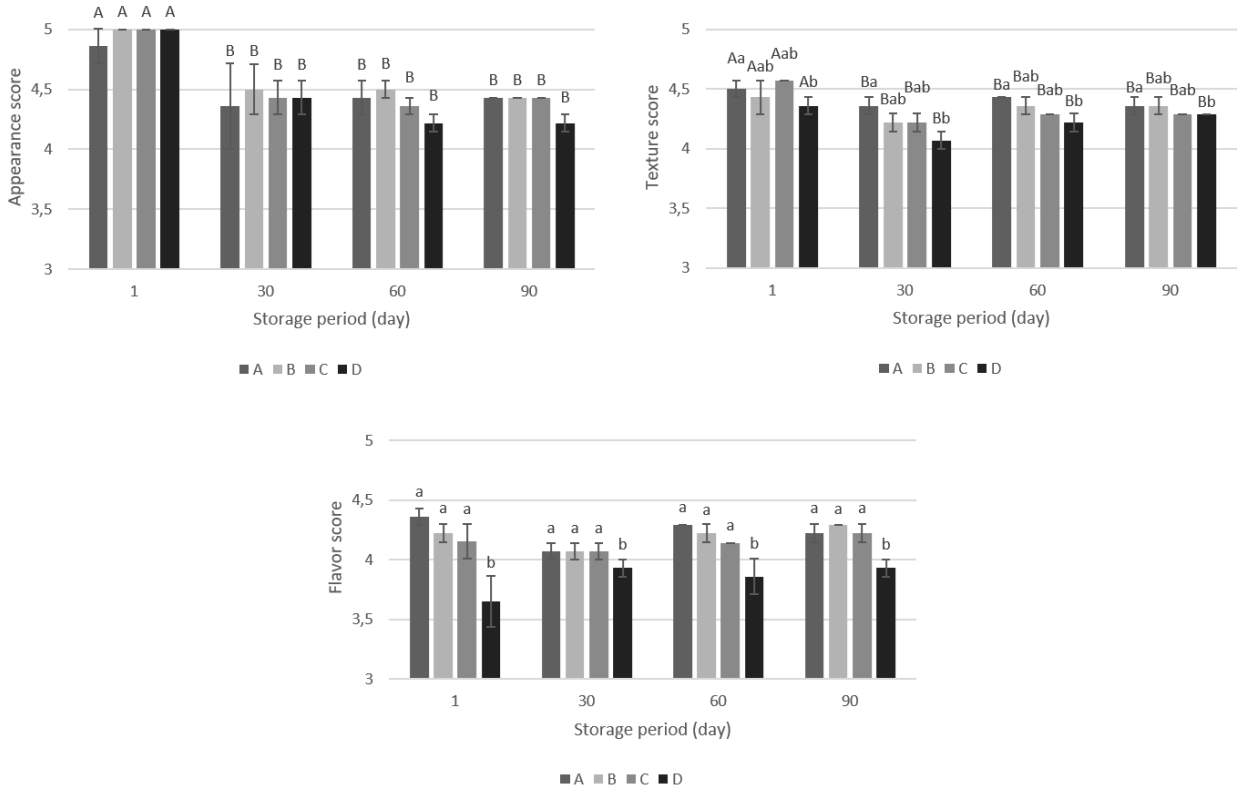

Figure 3. Sensory evaluation results of the samples during storage (n=2).
A: 0% kefir - Control, B: 25% kefir, C: 50% kefir, D: 75% kefir.
Values with the different lower case letter within different samples in the same storage day and upper case letter within different storage days for the same sample are significantly different (P<0.05).

Sensory evaluation: Scoring test results performed by experienced panelists are given in Figure 3. The ratio of kefir did not affect the appearance of the samples ($P>0.05$), however, the effect of storage was found to be significant in terms of the same property ($P<0.05$). All of the samples had the highest scores on the 1st day of storage, and the scores did not statistically change after the 30th day until the end of storage. Unlike the appearance, both the ratio of kefir and the effect of storage duration were statistically significant on textural characteristic ($P<0.05$). Sample D, which has the highest kefir ratio, had the lowest scores for texture property during the storage where samples B and C were not different from the control sample. All samples had the highest scores on the 1st day of storage, and the scores did not change on the 30th, 60th and 90th days. Considering the flavor characteristic, there was no difference between samples A, B and C ($P>0.05$), however, sample D got the lowest scores for each day of storage ($P<0.05$).

Discussion and Conclusion

All of the ice cream samples were standardized in terms of total solid and fat contents. Therefore, there was no statistical difference between the chemical composition of ice cream samples ($P>0.05$) (Table 2).

Similarly, there was no difference in the rheological properties of the samples examined. It is well known that the rheological properties of foods greatly affect the acceptability of the product by consumers (3). The rheological properties of ice cream are generally related to the dry matter content and the components with hydrocolloid properties such as stabilizers in the mix composition (14). Since the same type and amount of stabilizer was used in all ice cream samples in this study and no difference was found between the dry matter contents of the samples, it is an expected result that there was no difference in the rheological properties examined.

All of the ice cream mixes showed non-Newtonian flow characteristics since the *n* values were found to be below 1. Goff et al. (13) stated that the flow behavior index of a typical ice cream mix should be ~0.7. As it is seen from Table 3, the *n* values of the samples were determined close to this value as they should be.

The weight of ice cream per unit volume is one of the important physical properties that affect the quality of the product. The overrun of the product varies depending on the air given to the mix during the freezing process. It has been stated that the overrun of high-quality ice creams should be between 15% and 50% (33). According to this definition, the ice creams obtained in this study are of high quality (Figure 1). The main reason that the overrun values obtained in the study were not very high is that it is very difficult to exceed 35-40% overrun values of ice creams produced in batch type freezers (2).

The overrun value of ice cream can be affected by the state and denaturation level of the proteins in the product composition as well as the acidity and the freezing point of the product. Salem et al. (29) reported that the addition of different types of probiotics has an effect on the overrun of ice cream, and this is due to the change in the nature of the proteins and the freezing point of the product affected by the increase in acidity depending on the probiotic used. Therefore, in this study, it is thought that the destabilization of casein, which has an important role in the stabilization of air bubbles in the structure of ice cream, with the increase in acidity due to the addition of kefir, may have an effect on the overrun value of the product. It is probably due to fact that the emulsifying ability of casein increases with destabilization and therefore the overrun of ice cream increases due to the decrease of the interfacial tension (14).

The increase in overrun value with the increase in kefir ratio might also be related to the ability of microorganisms in kefir to produce Exopolysaccharides (EPS) during fermentation. It was stated that EPS can contribute to the formation of a matrix that can increase the amount of entrapped air and keep oxygen more efficiently. EPS are polysaccharides and contribute to keeping the air in the system of ice cream since they have the foam stabilizing ability (18).

Melting properties are one of the most important physical properties of ice cream, and the rapid melting of the product is undesirable particularly for consumers. Environmental conditions affect the melting properties of ice cream. When the ice cream is left to melt, the warm air in the environment penetrates into the ice cream and the ice crystals in the product begin to melt. The water occurred by the melting of ice crystals spreads into the unfrozen serum phase and as a result, the solution formed begins to flow from ice cream (24). In addition to environmental conditions, production conditions, type of milk, the composition of the mix, dry matter content, rheological properties, type and amount of stabilizer used are highly influential on the melting properties of ice cream (14, 19, 24, 30).

In this study, all production parameters including the composition of ice cream mixes and the rheological properties were the same. It is therefore no differences in melting behaviour of the ice cream samples were observed ($P>0.05$) (Figure 2). In addition to this, it has become clear that the use of kefir in ice cream production did not affect the melting properties of the product. Agreeing with the result of previous studies (12, 30), the addition of probiotics did not affect the melting properties of ice cream. Additionally, all of the samples can be classified as good quality ice cream in terms of meltdown properties, since Arbuckle (5) stated that a good quality ice cream

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should remain at room temperature for ~10-15 min without melting.

Hardness value refers to the strength required to create a certain deformation in ice cream (9) and it is a significant physical property for the acceptability of the product by consumers.

There was no difference in hardness values among the ice cream samples produced in this study ($P<0.05$) (Table 3). The hardness value of ice cream is closely related to the total dry matter content (14). Similar results were reported (19), indicating that there was no difference between the hardness values was due to the lack of difference between the soluble solid and fat in the product as in this study. Another reason could be speculated that the same stabilizer was used in the same amount in all of the samples yielded the similar results. As a matter of fact, it is known that the type and amount of hydrocolloid used in ice cream production affect the hardness of the product (32). Similarly, mix viscosity has a significant effect on the hardness value of ice cream as it is a measure of the viscosity of the unfrozen phase of the product (24). Therefore, the fact that there was no difference between the consistency index values obtained in this study was also effective on the hardness values.

The effect of the kefir ratio used on the acidity values during the storage period of ice creams was found to be significant ($P<0.05$). As in this study, ice cream mixes containing 12% non-fat dry matter are expected to have a titration acidity (lactic acid%) of ~0.2 (14) and sample A had approximately this value (Table 5). In addition, as the amount of kefir in ice cream increased, the acidity value of the product was found to be higher at all storage days (Table 5). This is related to the use of kefir, which consists various microorganisms that have a high ability to metabolize the lactose into lactic acid (37). There are other studies (26, 34) reporting that use of probiotic bacteria in ice cream increases the acidity of the product. The increased acidity of ice cream samples containing kefir can protect the product against spoilage microorganisms during storage, thus yielding longer shelf life of the product. Neverthless, increased titratable acidity of ice cream, may adversely affect the sensorial acceptability of the product (18).

Normally the acidity of the fermented products is expected to increase during storage due to post-acidification (31). However, this study showed that the acidity values of the samples, even those containing kefir, did not change as the storage time progressed. This situation is likely to be caused by the slowing down of the metabolic activity of lactic acid bacteria present in the product with the storage of ice creams at very low temperatures (-25 °C). Farias et al. (11) and Turgut et al. (34) also reported that the lactic acid contents of probiotic ice creams did not change during the storage period.

Lactococcus spp. and *Lactobacillus* spp. counts were both increased as the amount of kefir in ice cream mix was increased. It was seen that *Lactococcus* spp. counts detected in the samples were higher in each day of storage compared to *Lactobacillus* spp. counts. This situation might be related to the culture used in kefir production, as well as due to the fact that *Lactobacillus* strains are more sensitive to low temperatures (19, 25). In addition, it is known that the growth of anaerobic *Lactobacillus* strains decreases with the increase of oxygen in the external medium (11). *Lactobacillus* spp. cannot synthesize ATP by respiration and their oxygen-scavenging systems are reduced or disappeared completely. As a result, oxygen is incompletely reduced to hydrogen peroxide and toxic oxygen metabolites (O_2^-, OH^- and H_2O_2) accumulate in the cell; and finally leads to cell death (10, 23). Therefore, it is thought that the amount of air given to the mix during freezing process and the fact that this air is kept in the product matrix during the storage period also negatively affect the growth of *Lactobacillus* spp.

Reduction in the both of bacterial strains counts during the storage period ($P<0.05$) is possible to be caused by the destruction of the microorganisms due to low temperature storage conditions. Osmotic pressure, which also changes with the decreasing temperature, causes dehydration in the bacterial cells, leading to cell damage (30). Ice crystals forming in ice cream could be another factor causing cell lysis by destroying the cell walls or membranes of microorganisms. The cells may be damaged as a result of mechanical stresses of ice crystals that may form inside the cell (23). Furthermore, toxic metabolites that may occur during storage may also cause cell lysis (31).

According to the International Dairy Federation, in order for food products to have probiotic properties, they must contain at least 10^7 cfu/g (7 log cfu/g) of probiotic bacteria during the storage period (16). The survival of probiotics in a food product varies depending on many factors such as acidity, the presence of other microorganisms in the medium, and bacterial metabolites; these bacteria can generally maintain their stability in ice during storage (26). As it is seen in Table 5, kefir containing samples (B, C and D) did not lose their probiotic properties during the storage. The number of probiotic bacteria were above the minimum required level at all times during the storage, although it tended to decrease towards the end of the storage. It was determined that only the control sample (A) did not contain *Lactococcus* spp. and *Lactobacillus* spp. Similarly, Ahmad et al. (2), Parussolo et al. (26) and Salem et al. (29) reported that although the ice cream samples containing various *Lactococcus* and *Lactobacillus* strains had a gradually decreasing bacterial count during storage, they still retained their probiotic properties.

In sensory evaluation, kefir addition did not affect the appearance property of the samples. However, the texture and the flavor characteristics were affected with the amount of kefir added. While there was no difference between samples A, B and C in terms of the mentioned properties, sample D with the highest kefir content had lower scores than the other samples. This was probably due to the fact that sample D had the highest acidity value. It was already mentioned that the increase in acidity in ice cream has a negative effect especially on the flavor properties since the product normally does not have a high acidity value (10). In addition, panelists detected fermented taste in kefir-containing samples, and this flavor was most intense in sample D. This was one of the reasons why sample D getting the lowest score. In similar studies (4, 19, 34), it was reported that the flavor scores of probiotic ice creams decreased with the increase in acidity. As it is seen from Figure 3, it has been determined that the storage time had no effect on the flavor scores of the samples ($P>0.05$). As a matter of fact, no difference was observed in acidity values of the samples during storage.

All in all, all of the ice cream samples were found acceptable considering the chemical and physical properties. In addition, all of the samples maintained their probiotic properties during the 90-day storage period. However, due to the increase in acidity with the increase of kefir ratio, it was observed that sample D, which had the highest kefir ratio (75%), had lower scores from the panelists compared to the other samples in terms of texture and flavor properties. It was determined that samples B and C were not different from the control sample on each day of storage considering the same properties. Therefore, it is possible to produce probiotic ice cream with generally acceptable properties with the production method applied in samples B and C.

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Conflict of interest

The authors declared that there is no conflict of interest.

Author Contributions

NK performed conceptualization, the production, all of the analysis, data interpretation and writing-editing the manuscript. BB contributed the microbiological analysis. RAD performed data analysis and interpretation. AG performed conceptualization and reviewing the manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

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Effect of ascorbic acid on collagen and some inflammatory mediators in rats

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ABSTRACT

Musculoskeletal injuries as a kind of trauma that the human body is exposed to, adversely affect the quality of life and workforce of individuals due to restriction of movement function. This study aimed to evaluate the effects of dose-dependent ascorbic acid (AA) administration on the repair process after gastrocnemius muscle injury in rats. In this study, 5-month-old 66 male Wistar Albino rats were used and rats were randomly divided into 6 groups of 11 each [control, muscle injury, healthy (with 5 mg/10 mg/kg/day AA-treated group), injury (with 5 mg/10 mg/kg/day AA-treated group)]. A linear incision was made in the gastrocnemius muscle of thirty-three animals included in the muscle injury groups. AA (5-10 mg/kg/day) was administered to the four groups intraperitoneally just after surgery once a day. Animals were sacrificed twenty-one days later. Blood and tissue samples were used for cytokine, collagen, and histological measurements. It was found that a dose of 5 mg/kg/day AA administration reduced serum IL-6 and muscle tissue TNF-α levels, and increased liver tissue IL-10 levels. Muscle tissue collagen levels were not statistically different between the groups in parallel with our histological results. In our study, it was demonstrated that vitamin C has effects on inflammatory mediators during muscle tissue repair to explain the mechanism detailed molecular analyzes are needed.

Introduction

Musculoskeletal injuries affect lots of people worldwide and can cause pain and physical disability (30). They adversely affect the quality of life and labor force due to the limitation of movement function (7). Wound healing is a dynamic process and involves interactions between the extracellular matrix, many various cell types, and chemical mediators (12). The tissue repair process consists of four overlapping stages: (i) hemostasis, (ii) inflammation, (iii) proliferation, and (iv) remodeling (29). The tissue repair process is affected by many factors including age, gender, systemic diseases, nutrition, medicine, oxygenation, and radiation (13).

Ascorbic acid (vitamin C, AA) is a water-soluble micronutrient that is important for immune system functions and has an antioxidant effect (8). It is also involved in the formation process of collagen, which supports bones, teeth, tendons, muscle fibers, and blood vessel walls (21). The results of previous studies have shown that AA modulates cytokine production, stimulates keratinocyte differentiation, and increases neutrophil migration and T lymphocyte proliferation (6, 9, 10, 14). The data suggest that AA may positively affect the healing of damaged tissue. On the other hand, there is no consensus on the safe and effective dose range of AA administration after tissue injuries in the literature. Some

studies have reported that high doses of AA positively affect the wound healing process (23, 27). However, studies are reporting that low-dose AA is more effective than high-dose (11, 15).

Cytokines are important regulators of the tissue repair process. IL-6 and TNF-α are cytokines involved in the early stage of the inflammatory response (2). It is known that IL-10 exerts an anti-inflammatory effect and inhibits the release of pro-inflammatory cytokines (16). A previous study by Bowie and O'Neill (5) reported that AA inhibited TNF-mediated NF-kB activation. A further animal study found that AA reduced the inflammatory response by inhibiting IL-6 and TNF-α expression (26).

In this study, we aimed to examine the effects of dose-dependent AA administration on collagen synthesis and inflammatory parameters in the case of skeletal muscle injury.

Materials and Methods

This study was approved by the Experimental Animals Local Ethics Committee at the Ankara University with decision number 2019-9-96. All experimental procedures were performed according to institutional guidelines, in compliance with national and international laws and guidelines for the use of animals in biomedical research.

In this study, 66 adult male Wistar Albino rats were used. As a result of the power analysis using the G*Power 3.0.10 software (University of Kiel, Germany), 11 animals for each group were included in the study at 80% power and 5% significance level. Since evaluations will be made by determination of the cytokine levels and due to the potentially high standard deviations of the cytokine measurements, the number of animals was decided as 11 per group. Animals were randomly divided into six groups (n = 11/group): (i) control, (ii) muscle injury, (iii) healthy rat with 5 mg/kg/day AA (Healthy 5AA), (iv) healthy rat with 10 mg/kg/day AA (Healthy 10AA), (v) muscle injury with 5 mg/kg/day AA (Injury 5AA) and (vi) muscle injury with 10 mg/kg/day AA (Injury 10AA). AA (100468 L (+)-Ascorbic Acid, Merck, Germany) was administered intraperitoneally just after surgery and then once a day until the euthanasia procedure. Distilled water was applied intraperitoneally to rats in the muscle injury group. Animals were maintained in 12-h light and dark cycle. Food and water were provided *ad libitum*. For surgery, rats were anesthetized intraperitoneally with 100 mg/kg ketamine and 10 mg/kg xylazine. A linear incision was made in the right gastrocnemius muscle of the animals with a No. 11 surgical blade, 5 mm deep and 5 mm long. The wound was closed and sutured under sterile conditions with 3/0 absorbable suture material (18).

Twenty-one days after AA administration, animals were sacrificed with an overdose of ketamine and xylazine. Blood samples, liver, and gastrocnemius muscle were taken. Blood samples were centrifuged for 10 min at 2800 rpm to obtain serum. Blood and tissue samples were stored at -80 °C until collagen and cytokine measurements were performed.

Measurement of serum and tissue cytokines and muscle tissue collagen: Tissues were weighed for cytokine measurement in tissues, homogenization solution was added according to tissue weight and the measurement was made by optimizing the amount of tissue. Protein isolation solution was prepared using RIPA lysis buffer and protease inhibitors (pepstatin, leupeptin, aprotinin, 2 µg/ml) (Sigma, USA), and tissue samples were homogenized. Muscle tissue collagen I levels, serum and tissue IL-6, IL-10, and TNF-α measurements were performed using commercially available ELISA kits (Bioassay Technology Laboratory, China) according to instruction.

Histological analysis: The gastrocnemius muscle pieces were fixed with 10% neutral buffered formalin for 1-2 days for evaluation under a brightfield microscope (Zeiss Axio Scope A1, Oberkochen, Germany), after the dissection. Following washing under the tap water and dehydration with ethanol series, the muscle pieces were incubated in xylene until the clearing. After the tissues were embedded into the paraffin mixture, transverse sections were cut (5-7 µm) by sliding microtome (RM 2125RT, Leica, Germany) and stained using Hematoxylin-Eosin (H&E) (Figure 1) (4).

Histological evaluation was based on measuring the diameter of muscle fibers under the brightfield microscope (Figure 2). Three photos were captured in each skeletal tissue section and the diameters of ten muscle fibers on each of photos were measured in all animal. All measurements on digital images were recorded using the Axiovision Rel 4.8 software (Germany) and evaluated statistically.

Statistical analysis: IBM SPSS Statistics 25 (Armonk, New York, USA) was used for statistical analysis. Data were presented as mean ± standard deviation. ANOVA was used for the analysis of homogeneous variables. Post hoc Tukey's test was used when the F values were significant. Kruskal-Wallis test was used for the data analysis without homogeneous distribution. Spearman test was used for correlation analysis and $P<0.05$ was regarded as statistically significant.

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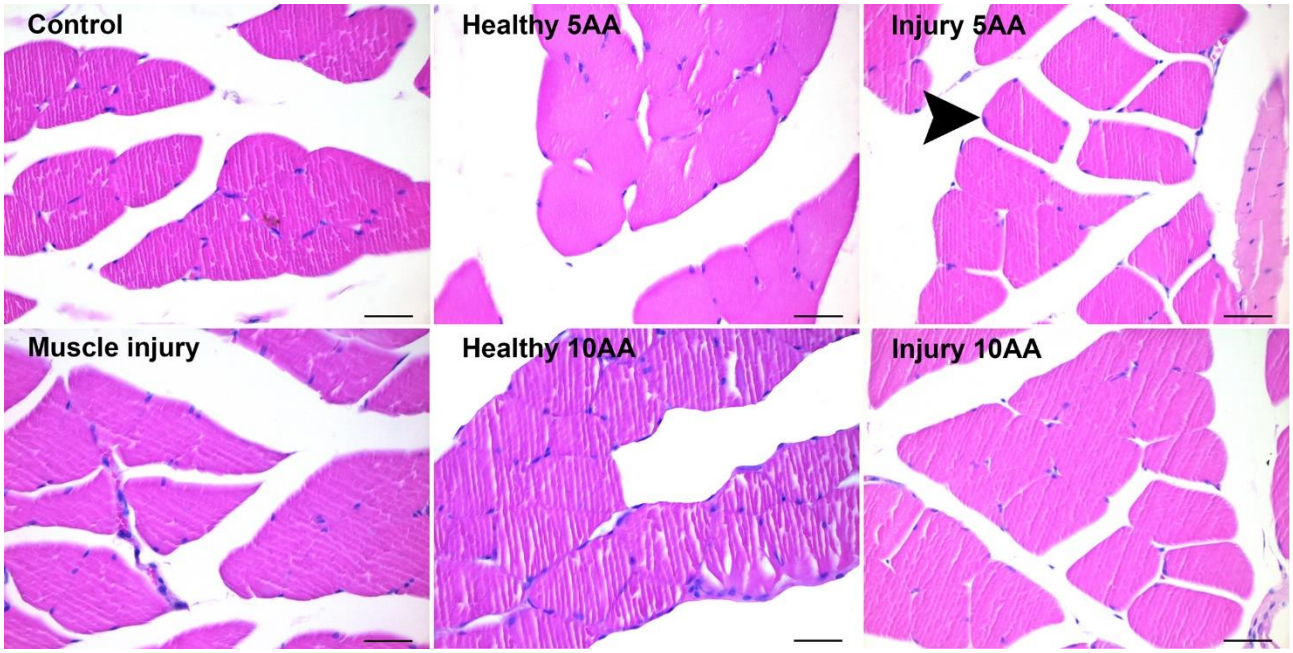

Figure 1. The muscle fibers of control, muscle injury, and the experimental groups (Arrowhead: Muscle fiber). Staining: H&E, (X40), Bar= 50 µm.

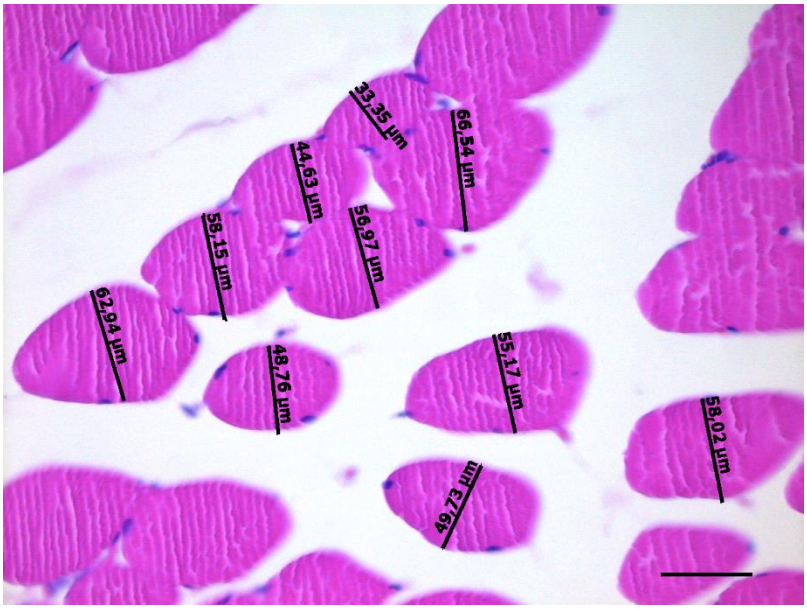

Figure 2. The measurement of muscle fibers on images. Staining: H&E, (X40), Scale bar= 50 µm.

Results

Although there was no significant difference between the groups in terms of weight gain (P>0.05), rats tended to weight gain, and the weight gain of the muscle injury group was found to be lower than other groups.

Levels of IL-6, IL-10, and TNF-α examined in serum and tissues of groups to understand inflammatory responses are shown in Table 1.

Collagen type I levels were not different between the groups in parallel with our histological results (Table 2).

Correlation analysis was performed to determine the relationship between cytokine levels and collagen type I levels in muscle tissue. According to these results, muscle IL-6 levels were correlated negatively with IL-10 levels (R = -0.350, P<0.01). There was a positive correlation between serum IL-6 levels and serum IL-10 (R = 0.325, P<0.01) and serum TNF-α levels (R = 0.638, P<0.01). Muscle tissue collagen type I levels were correlated with serum IL-6 (R = 0.377, P<0.01) and IL-10 (R = -0.248, P<0.05) levels. Our results showed that muscle tissue TNF-α levels were correlated with serum TNF-α (R = 0.544, P<0.01) and IL-6 (R = 0.327, P<0.01) levels.

The data obtained as a result of the examination of muscle fiber diameters are shown in Table 3. Muscle fiber diameters were not different between the groups.

Table 1. IL-6, IL-10, and TNF-α levels in serum and tissues (mean ± SE).

		Control (pg/ml)	Muscle Injury (pg/ml)	Healthy 5AA (pg/ml)	Healthy 10AA (pg/ml)	Injury 5AA (pg/ml)	Injury 10AA (pg/ml)
Serum	**IL-6**	0.047 ± 0.000[a]	0.055 ± 0.001[ab]	0.049 ± 0.002	0.047 ± 0.000	0.045 ± 0.000[b]	0.047 ± 0.000
	IL-10	0.272 ± 0.007	0.228 ± 0.017	0.248 ± 0.009	0.223 ± 0.021	0.198 ± 0.006	0.258 ± 0.022
	TNF-α	0.052 ± 0.001	0.050 ± 0.000	0.047 ± 0.000	0.050 ± 0.000	0.049 ± 0.000	0.048 ± 0.000
Muscle	**IL-6**	0.478 ± 0.073	0.676 ± 0.086	0.703 ± 0.027	0.511 ± 0.058	0.855 ± 0.102	0.894 ± 0.090
	IL-10	0.095 ± 0.010	0.066 ± 0.008	0.077 ± 0.007	0.068 ± 0.008	0.053 ± 0.003	0.056 ± 0.003
	TNF-α	1.576 ± 0.065	1.786 ± 0.076[c]	1.569 ± 0.023	1.576 ± 0.013	1.416 ± 0.008[c]	1.589 ± 0.104
Liver	**IL-6**	0.512 ± 0.019	0.518 ± 0.004	0.539 ± 0.038	0.525 ± 0.005	0.550 ± 0.017	0.507 ± 0.014
	IL-10	0.754 ± 0.023[d]	0.690 ± 0.009	0.833 ± 0.015[d]	0.814 ± 0.017	0.763 ± 0.023	0.731 ± 0.015
	TNF-α	0.633 ± 0.055[e]	0.723 ± 0.023	0.637 ± 0.051[f]	0.845 ± 0.035[ef]	0.754 ± 0.017	0.818 ± 0.024

Significant statistical differences between the groups were shown in letters (P<0.05).
a: Control - Muscle injury,
b: Muscle injury - Injury 5AA,
c: Muscle injury - Injury 5AA,
d: Control - Healthy 5AA,
e: Control - Healthy 10AA,
f: Healthy 5AA - Healthy 10AA.

Table 2. Collagen type I levels in muscle tissues (mean ± SD).

		Control (ng/ml)	Muscle Injury (ng/ml)	Healthy 5AA (ng/ml)	Healthy 10AA (ng/ml)	Injury 5AA (ng/ml)	Injury 10AA (ng/ml)
Muscle	**Collagen Type I**	0.100 ± 0.025	0.101 ± 0.018	0.093 ± 0.014	0.106 ± 0.025	0.095 ± 0.021	0.089 ± 0.013

Table 3. Mean muscle fiber diameter values of groups (mean ± SD).

Groups	Mean Muscle Fiber Diameter (µm)
Control (n = 11)	61.807 ± 3.881
Muscle Injury (n = 11)	58.783 ± 5.291
Healthy 5AA (n = 11)	58.893 ± 5.602
Healthy 10AA (n = 11)	57.344 ± 4.567
Injury 5AA (n = 11)	58.195 ± 3.734
Injury 10AA (n = 11)	59.171 ± 5.033

Discussion and Conclusion

In this study, we made an incisional wound model in the gastrocnemius muscle of rats and discuss the effects of dose-dependent AA administration on collagen synthesis and tissue cytokine levels.

In our study, no significant difference was found in weight gain between the groups. Similarly, in the animal study of Loizidis et al. (20) it was found that the administration of vitamin C did not have a significant effect on body weight gain, feed, and water intake. In our study, there was no adverse effect on the viability and comfort of rats, except to avoid standing on the injured leg during the first 24 hours after surgery.

It is well-known that cytokines are important regulators of the tissue repair process. Serum IL-6 levels were observed to be significantly lower in Injury 5AA compared with the muscle injury group according to our results. In the same group (Injury 5AA), the decrease in serum IL-10 level was detected but was not statistically significant. There was no significant difference in serum TNF-α levels between the groups. In a study that examined the effects of single-dose intraperitoneal vitamin C treatment (250 mg/kg) on serum cytokine levels in rats, levels of IL-6, IL-10, and TNF-α in serum were found to be lower in the vitamin C treated group (3). Different from our study, a single dose of AA was administered in the above-mentioned study. A human study concluded that vitamin C uptake (500 mg/day) did not have a significant effect on IL-6 and IL-10 concentrations in plasma and blood mononuclear cells in response to exercise (1). On the other hand, in one study, the effects of dose-dependent vitamin C uptake (500-1500 mg/day) on cytokine levels of runners after the ultramarathon were examined. Levels of IL-6 and IL-10 in plasma after the race were found to be significantly lower in the vit C-1500 group compared with the placebo and vit C-500 groups (22).

In this study, IL-6 levels in muscle tissue were not significantly different between the Injury 5AA, Injury 10AA, and muscle injury groups. TNF-α levels in muscle tissue were observed to be significantly lower in Injury 5AA compared with the muscle injury group. In a study that investigated the effects of AA on inflammatory markers in the case of multifidus muscle injury in rats, it was found that AA reduced the inflammatory response in muscle tissue. In that same study, expression of muscle tissue IL-6 and TNF-α was significantly inhibited in the

AA group on the 1st, 3rd, and 7th days after surgery (26). In another study, it was reported that AA administration (200 mg/kg) reduced TNF-α levels, myonecrosis, and inflammation in the diaphragm muscle (27). Different from our study, a single dose of AA was administered in both studies. This situation causes difficulties in interpreting the dose-dependent effects of AA on muscle tissue cytokine responses. In our study, the decrease in muscle tissue IL-10 levels in the Injury 5AA and Injury 10AA groups was not statistically significant. We were not able to find a study evaluating the effects of dose-dependent AA administration on muscle tissue IL-10 levels, searching the literature data. In one study, it was reported that vitamin C supplementation reduced IL-10 production by blood mononuclear cells (28).

In our study, liver tissue IL-6 levels were not significantly different between the groups. Liver tissue IL-10 levels were significantly higher in Healthy 5AA compared to the control group. In the Injury 5AA and Injury 10AA groups, the increase in liver tissue IL-10 levels was not statistically significant. Liver tissue TNF-α levels were observed to be significantly higher in the Healthy 10AA group compared to the control and Healthy 5AA groups according to our results. In the Injury 5AA and Injury 10AA groups, the increase in liver tissue TNF-α levels was not significant compared to the muscle injury group. In one study, it was reported that vitamin C supplementation under cyclic heat stress, significantly reduced rat liver tissue IL-6 and TNF-α expression (31). In another study, the effects of vitamin C administration on perfluorooctane sulfonate (PFOS)-induced liver steatosis in mice were examined. It was reported that TNF-α positive cells that increased PFOS exposure in the liver were reduced by vitamin C treatment (24).

In the literature, the effect of the topical AA in rats with an incisional wound model was examined in one study. It was reported that the topical application of AA accelerated the tissue repair process. In the same study, it was observed that no inflammatory symptoms were found in the AA-treated group on the 7th postoperative day. In histological evaluation, it was observed that collagen fibers in the papillary layer were thicker in the AA-treated group on the 14th postoperative day (19). In a study that investigated the effect of AA deficiency on skeletal muscle in senescence marker protein-30 knockout mice, it was found that the cross-sectional area of muscle was significantly smaller in the AA deficiency group compared with the AA-treated group (1.5 g/L) (25). In the animal study that examined the effect of vitamin C on muscle renewal in the case of cytotherapy, it was observed that vitamin C enhanced muscle tissue collagen type I expression (17). However, in our study, AA administration did not show a significant effect on collagen type I levels at the dose and time administered. In the study of Kim et al. (17) the effect of AA on collagen synthesis was examined at the level of gene expression. In our study, the measurement of collagen was performed with the ELISA method. This suggests that the effect of AA on collagen synthesis may be limited at the level of gene expression. On the other hand, the heterogeneity in AA supplementation protocols in terms of the route of administration, dose, frequency, and duration causes difficulties in interpreting the effects of AA.

In conclusion, our results showed that AA administration could affect cytokine responses in the case of skeletal muscle injury and be effective in alleviating the inflammatory state. The absence of a significant difference in muscle tissue collagen levels may either suggest that AA administration is not effective on collagen synthesis in the process of healing muscle injury or may be effective on a different level other than tissue level. However, to understand the clear relationship, examinations at the level of gene expression, at different doses, and periods will be useful. In our study, it is thought that AA administration after tissue injuries could be effective, but it is emphasized that further studies should be conducted to define the safe and effective dose range.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

SI, NY and PAA conceived and planned the experiments. SI, NY, BK, DB, SA, FTÇ and PAA carried out the experiments and contributed to sample preparation. BB carried out the statistical analysis. SI, NY, SA, FTÇ and PAA took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was approved by the Experimental Animals Local Ethics Committee at the Ankara University (Decision Number: 2019-9-96).

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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The effects of different choice feeding methods on laying hen performance, egg quality, and profitability

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ABSTRACT

This study was conducted to investigate the effects of using whole wheat in choice feeding on performance, egg quality, and income of laying hens. In the study, a total of 160-Hyline W-80 white commercial laying hens aged 28 weeks were divided into four treatment groups and fed for 84 days. The treatment groups were as follows: 1) Control (C, standard commercial laying hen feed), 2) C feed+choice feeding continuously with whole wheat in a separate cup (CW), 3) C feed+weekly intermittent choice feeding with whole wheat in a separate cup (WW), 4) C feed+choice feeding with continuous whole wheat+limestone together in a separate cup (WL). Choice feeding by using whole wheat methods (continuous, weekly intermittent, and with limestone) caused a significant decrease in the live weight change of laying hens ($P<0.01$), increased feed consumption, and feed conversion ratio compared to the control group ($P<0.05$). Total egg yield and egg weight were not affected by choice feeding with whole wheat methods ($P>0.05$). Choice feeding by using whole wheat caused a decrease in total eggshell weight, thickness, shell weight per unit area, and egg yolk color ($P<0.01$). However, it did not significantly affect the egg shell ratio, surface area, albumen height, and Haugh unit values of eggs ($P>0.05$). Also, total production costs, egg sales income, and net profit values were similar in all groups ($P>0.05$). In conclusion, the choice feeding by using whole wheat in laying hens did not affect the performance and economic parameters negatively; however, it caused reductions in some egg quality values.

Introduction

The egg poultry industry is an alternative branch of animal production in which an economical, non-substituted, and highly biologically valuable protein source is produced in a short period. The continuously increasing world population requires reducing the production costs (mainly feed) and increasing productivity for a profitable and sustainable production in the poultry industry due to its competitive structure. In recent years, natural disasters such as droughts, floods, hail, and tornados experienced as an effect of changing climatic conditions have adversely affected crop production, thus increasing livestock production costs. Considering the current conditions, scientists are making great efforts to prepare the lowest cost rations that can meet the changing nutritional needs of animals because the forecasted savings in feed cost have a tremendous economic impact on poultry enterprises. The increase in feed costs has brought about alternative feeding methods such as choice feeding in laying hens, as it is in all livestock sectors.

Nowadays, choice feeding studies in chickens have become increasingly important, particularly in the poultry industry, both for profitable production and for the public's concerns about animal welfare. Wheat is one of the most essential options for the whole grain feeding of hens. Since wheat can be produced directly on the farm, and feed processing, transportation, labor, and operating profits are not included in the costs, it creates a cheaper alternative feed source than a complete mixed feed (12). In addition, studies have shown that when feeding grains

are used as a whole, the size of the gizzard increases (20, 26), and there is more digestive fluid secretion, which increases the acidity in the digestive system (11, 13), and pathogen microorganisms are eliminated in the acidic environment. The damage caused by pathogen microorganisms is prevented and an increase in performance and yield characteristics can be seen due to the savings in the nutrients they use (25).

Feed selection in poultry is done by combining the metabolic effects of these feeds with one or more previously learned sensory characteristics. In addition, factors such as the smell, taste, form, and color of the feeds are also determinative in the feed selection of poultry (9, 10). Studies were conducted to determine the ability of poultry to create the most suitable combinations among feed sources when allowed to choose free feed (19, 20, 25, 26). By recognizing the feed selection characteristics of poultry, choice feeding can be applied to specific environmental (such as temperature, humidity) or physiological features (sex, yield, age, etc.), a flexible, practical, and economical feeding technique that can meet the individual needs of chickens has been created. This provides poultry nutritionists several advantages in practice, including reducing commercial food consumption, utilizing crop over-production, minimizing available opportunities for feed, and reducing manure production (9). Saikhlai et al. (28) reported that the inclusion of wheat between 0 and 25% of laying hen diets might not affect performance and egg quality traits, digestibility of dry matter, organic matter, apparent metabolizable energy, and also economic compared to corn diets. However, due to the presence of xylene as an antinutritional factor in wheat (6), dirty egg amounts may increase in laying hens (34). As an alternative, sequential feeding with whole wheat may not negatively affect egg production and feed efficiency and can be used as an alternative to conventional feeding of laying hens (8). On the other hand, whole wheat has low calcium (Ca, 0.05%) concentration compared to laying hen diets (more than 3.25 % Ca) (23). When the hens are choice fed with wheat, their Ca intake will be decreased, so deterioration in the quality of the shell and weakening of the bones may occur. For this reason, when selective feeding with cereals, the poultry should reach Ca sources as grit feeding, which may support bone and egg shell quality (1).

Therefore, this study aimed to determine the effects of whole wheat (continuous and weekly intermittent) and limestone given to laying hens as choice feeding, on performance, egg internal and external quality characteristics, and their economic reflections on the production process.

Materials and Methods

Animals and experimental design: This experiment was carried out in the Erciyes University Poultry Experimental Unit (ERUTAM) and approved by the local ethical committee before the experiment (approval date and number: 03.06.2020 and 20/087).

In the study, a total of 160 Hyline W-80 white commercial laying hens, 28 weeks old, were used in enriched cages (90 x 60 x 50 cm) for 84 days, excluding the 14-day adaptation period before the experiment. The hens were divided into four experimental groups, each consisting of 40 chickens, and distributed to 10 cages as replicate and four chickens were placed in each cage. Before the experiment, hens were weighed individually, and egg production and egg weight were recorded for 14-day intervals and ranked according to their body weight and egg traits to minimize differences among the groups. Feed and water were given to all groups *ad-libitum*. The daily lighting was arranged for 16 h light (05:00 am to 9:00 pm). The composition of the basal ration used in the study is given in Table 1.

Table 1. The composition of the basal ration used in the study.

Feed raw materials	**Control**
Corn	515.83
Sunflower meal (%36 HP)	180.40
Soybean meal (% 46 HP)	98.00
DDGS¥	41.90
Meat-bone meal	34.22
Rendering oil	22.42
Molasses	20.00
Limestone	79.31
Salt	2.50
Lysine	0.69
Methionine	0.73
Phytase	1.00
Toxin binder	0.50
Sodium bicarbonate	0.50
Vit.-Min. mixture*	2.00
Total	1000
Analyzed nutrient content, %	
Dry matter	90.60
Crude protein	18.10
Raw oil	4.60
Ash	13.10
Raw cellulose	5.30
Calcium	3.20
Total phosphorus	0.53
Calculated nutrient content, %	
Methionine	0.40
Lysine	0.80
Sodium	0.21
Metabolizable energy, MJ/kg	11.21

¥Dried distiller grain solubles. *Vitamin-mineral premix per kilogram of the diet, Vitamin A, 12,000 IU; Vitamin D3 2000 IU; Vitamin E, 30.0 mg; Vitamin K, 5.0 mg; Vitamin B1 (thiamine), 3.0 mg; Vitamin B2 (riboflavin), 6.0 mg; Vitamin B6, 5.0 mg; Vitamin B12, 0.03 mg; Niacin, 30.0 mg; Biotin, 0.1 mg; Calcium D-pantothenate, 12 mg; Folic acid, 1.0 mg, Choline chloride, 400 mg, Manganese, 80.0 mg; Iron, 35.0 mg; Zinc, 50.0 mg; Copper, 5.0 mg; Iodine 2.0 mg; Cobalt, 0.4 mg; Selenium, 0.15 mg assures. 2 Dry matter, crude protein and calculate nutrient composition of diets calculated according to NRC (1994) nutrient values.

Laying hens were fed in addition to the standard (basal) feed through the normal feeder of the chickens. Approximately 500 g of wheat was given in a separate metal cup (15 cm long x 12 cm high and 10 cm wide) continuously or with weekly changes (one-week basal feed, and one-week basal feed + wheat alternately). In another group, 100 g of granule limestone and 500 g of wheat in a separate metal cup, and basal feed, were added together with the metal cup *ad libitum*. The experimental groups were designed as follows: 1) Control (C, only commercial feed), 2) C feed + whole wheat with separate cups, 3) C feed + weekly interval whole wheat with separate cups, 4) C feed + whole wheat and limestone in the same feeder with separate cups.

Determination of performance traits: Body weights of hens were recorded at the beginning (initial) and end of the experiment. Body weight changes were calculated by considering the initial and final weights. Feed consumption was recorded every 14 days and divided by the number of hens (4) in a cage. Feed consumption was calculated by subtracting the weights of the remaining feeds from the feeds given based on subgroups. Average daily feed consumptions (FC) and feed conversion ratio (FCR) were determined for each 14 d (on day 14, 28, 42, 56, 70, and 84) however, data were given for each 28 d in three periods in the tables 2- 6). Egg mass and FCR were calculated according to the formula below;

Egg mass:egg yield (%) x egg weight (g)

FCR: feed consumption (g) in a period/egg mass (g)

There was no mortality throughout the experimental period.

Egg production was recorded daily at 15:00, and egg yield (% of egg production number/hen per cagex100) was calculated at 14-day intervals. Daily egg production was recorded and calculated at 28-day intervals for three periods.

Determination of egg quality characteristics: To determine the internal and external quality of eggs for three consecutive days at the end of each period (on days 14, 28, 42, 56, 70, and 84; however, data were given for each 28 d in the tables 2-6, eight eggs were randomly selected (80 eggs/group, a total of 320 eggs) from each subgroup. Egg weight, albumen height (Hmm), Roche yolk color fan, and Haugh unit (HU) values were determined by an automated egg analyzer (EggAnalyzer, Orka Food Technology, Israel).

The cracked egg shells were washed and dried for three days, then weighed with a precision balance (±0.1 g) to determine the shell weight (g). The shell ratio was determined by dividing the shell weight by the egg weight. The shell thickness was measured with a sharp digital gauge micrometer (±0.01 mm) from three points (sharp end, blunt end, and the equator) of the broken eggs; then, the shell thickness was determined by taking the arithmetic mean of these three measurements.

The egg shell surface area (ESA) was calculated according to the formula reported by Carter (3); Surface Area = 3.9782 × egg weight$^{0.7056}$. Per unit area, shell weight (USW, mg/cm^2) was calculated by dividing the absolute shell weight by the egg surface area.

Determination of protein and energy consumption for per kg egg production: Basal feed consumption is calculated every 14 days. The total protein and energy amount of the consumed feed were divided by the number of eggs mass-produced, and the protein and energy consumed per kg of egg was calculated.

Economic analysis: Economic analyses were made according to the following criteria;

- Egg sales income by weight (<62 gr = $ 0.069; ≥ 62 gr = $ 0.071) and
- Feed cost is in the total expenses; commercial chicken feed (0.37 $/kg), wheat (0.3 $/kg) limestone (0.037 $/kg) chicken cost ($ 2.2) and 15% (30) other expenses (labour, electricity, water etc.) are taken into account.

Total expenses (costs) are subtracted from total income in the net profit calculation. In economic analysis, it was accepted as 1 $ = 13.5 TL (converted in 2021).

Statistical analysis: The conformity of the data to the normal distribution and the homogeneity of the variances were checked using the Shapiro-Wilk and Levene tests, respectively. The significance of the difference between the beginning (initial) and final body weights within the group was evaluated with the Paired Sample T-test. The significance of the difference between the groups for all other performance traits, egg quality characteristics, protein and energy consumption for per kg egg production and economic characteristics was tested with One Way ANOVA. The statistical significance level was determined as $P<0.05$.

Results

The BW of hens at the beginning and at the end of the study, BW change, FC, and FCR values are given in Tables 2, 3 respectively.

There were no significant differences between the treatment groups regarding the beginning (initial) and final BW values ($P>0.05$). However, differences in BW changes of W, WW, and WL groups were significantly lower that of the control group ($P<0.01$). While the difference between beginning and final BW in the control group is not significant, within the treatment groups the difference was found to be significant ($P<0.001$; Table 2).

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Table 2. The effects of choice feeding with whole wheat continuous, weekly intermittent and with limestone on body weight values of laying hens.

Parameters (BW, g)	Groups ($\bar{X} \pm S_{\bar{X}}$)				P
	C	W	WW	WL	
Beginning	1643.72±19.80	1643.14±18.11	1640.02±16.25	1639.65 ±16.42	NS
Final	1646.56±17.55	1594.72±14.68	1604.23±17.05	1597.08±13.25	NS
Change	+2.85 ± 6.35^{a}	-48.41 ± 6.04^{b}	-35.77 ± 5.05^{b}	-42.55 ± 5.44^{b}	**
P (T-test)	NS	***	***	***	

C: Control. W: choice feeding with continuous whole wheat. WW: choice feeding with whole wheat weekly. WL: continuous choice feeding with whole wheat + limestone. P: probability. a.b: The differences between the averages shown with different superscripts on the same line are statistically significant. NS: non-significant. *: P<0.05. **:P<0.01. ***:P<0.001.

Table 3. The effects of choice feeding with whole wheat continuous, weekly intermittent and with limestone on FC, FCR, protein consumption, amount of protein consumed per kg of egg, metabolic energy consumption and amount of energy consumed per kg of egg of laying hens.

Parameters	Groups ($\bar{X} \pm S_{\bar{X}}$)				P
	C	W	WW	WL	
FC, g/day/hen					
1st period	113.73±2.44	113.06±4.32	113.56±2.60	117.39±1.79	NS
2nd period	124.84±2.70^{b}	123.81±2.60^{b}	133.52±3.54^{a}	130.26±1.01^{ab}	*
3rd period	109.38±2.90	114.91±1.67	113.75±1.69	117.74±2.18	NS
Total	115.98 ±1.80	117.26±2.12	120.28±1.91	121.80±1.25	NS
FCR, g feed/egg mass					
1st period	2.03±0.06	2.21±0.05	2.06±0.04	2.14±0.06	NS
2nd period	2.06±0.03	2.21±0.07	2.20±0.07	2.18±0.03	NS
3rd period	1.84±0.03^{b}	2.00±0.04^{a}	1.93±0.04^{ab}	2.02±0.03^{a}	**
Total	1.99±0.03^{c}	2.15±0.05^{a}	2.02±0.05^{bc}	2.12±0.03^{ab}	*
Protein consumption, g/day/hen					
1st period	19.33±0.42^{a}	17.08±0.64^{b}	18.15±0.40^{ab}	18.05±0.27^{ab}	*
2nd period	21.22±0.46^{a}	19.08±0.48^{b}	21.61±0.61^{a}	20.31±0.19^{ab}	**
3rd period	18.59±0.49	17.91±0.26	18.35±0.98	18.49±0.34	NS
Total	19.72±0.31^{a}	18.02±0.33^{b}	19.37±0.31^{a}	18.95±0.17^{a}	**
Amount of protein, consumed, per kg of egg					
1st period	345.06±10.92	334.51±7.41	328.93±7.21	329.49±8.26	NS
2nd period	349.99±4.51	340.40±10.14	356.85±11.48	340.06±5.40	NS
3rd period	312.79±4.68	311.94±5.72	312.28±7.11	317.23±4.27	NS
Total	335.68±4.24	328.00±5.45	339.12±7.95	328.85±3.86	NS
Metabolic energy consumption, g/day/hen					
1st period	312.76±6.72	321.60±12.40	318.10±7.36	332.34±5.28	NS
2nd period	343.32±7.42^{c}	350.31±7.04^{bc}	372.59±9.70^{a}	367.38±2.93^{ab}	*
3rd period	300.79±7.97^{b}	324.10±4.79^{a}	317.72±4.56^{ab}	331.43±6.22^{a}	**
Total	318.96±4.95^{b}	332.01±6.03^{ab}	336.14±5.34^{a}	343.71±3.76^{a}	*
Amount of energy, consumed, per kg of egg					
1st period	5581.77±176.69^{c}	6298.09±150.33^{a}	5764.12±121.00^{bc}	6069.03±168.65^{ab}	*
2nd period	5661.64±73.01^{b}	6263.24±213.82^{a}	6151.17±185.52^{a}	6151.50±94.69^{a}	*
3rd period	5059.78±75.77^{b}	5645.64±110.82^{a}	5405.79±113.65^{a}	5697.27±89.35^{a}	**
Total	5430.13±68.55^{b}	6044.84±118.65^{a}	5884.02±136.08^{a}	5965.99±89.89^{a}	**

C: Control. W: choice feeding with continuous whole wheat. WW: choice feeding with whole wheat weekly. WL: continuous choice feeding with whole wheat + limestone. P: probability. a.b: The differences between the averages shown with different superscripts on the same line are statistically significant. NS: non-significant. *: P<0.05. **:P<0.01.

Table 4. The effects of choice feeding with whole wheat continuous, weekly intermittent and with limestone on egg production, yield, weight and mass traits of laying hens.

Parameters	Groups ($\bar{X} \pm S_{\bar{X}}$)				P
	C	W	WW	WL	
Egg production, number					
1st period	52.25±1.15	49.60±1.75	52.15±0.76	52.80±0.73	NS
2nd period	54.00±1.12	52.15±2.00	55.20±0.25	55.05±0.38	NS
3rd period	53.60±0.55	53.15±0.68	54.05±0.54	53.90±0.29	NS
Total	53.28±0.0.87	51.63±1.31	53.80±0.38	53.92±0.23	NS
Egg yield, %					
1st period	93.30± 2.05	88.57 ± 3.13	93.13 ± 1.36	94.29 ± 1.31	NS
2nd period	96.43 ± 2.00	93.13 ± 3.57	98.57 ± 0.45	98.30 ± 0.68	NS
3rd period	95.72 ± 0.98	94.91 ± 1.22	96.52 ± 0.96	96.25 ± 0.51	NS
Total	95.15 ± 1.55	92.20 ± 2.35	96.07 ± 0.67	96.28 ± 0.40	NS
Egg weight, g					
1st period	60.35±0.35^{a}	57.80±0.54^{c}	59.31±0.51^{ab}	58.28±0.37 ^{bc}	**
2nd period	62.96±0.67^{a}	60.74±0.41 ^{b}	61.55±0.62^{ab}	60.82±0.29^{b}	*
3rd period	62.06±0.93	60.60±0.54	61.01±0.52	60.43±0.38	NS
Total	61.79±0.63	59.71±0.42	59.64±1.15	59.84±0.30	NS
Egg mass, g/day/hen					
1st period	56.31±1.27^{a}	51.19±1.90^{b}	55.19±0.55^{a}	54.96±0.94^{a}	*
2nd period	60.73±1.50	56.54±2.17	60.68±0.74	59.79±0.49	NS
3rd period	59.42±1.15	57.49±0.73	58.90±0.87	58.17±0.49	NS
Total	58.83±1.20	55.10±1.40	57.32±1.18	57.65±0.40	NS

C: Control. W: choice feeding with continuous whole wheat. WW: choice feeding with whole wheat weekly.
WL: continuous choice feeding with whole wheat + limestone. P: probability. a.b: The differences between the averages shown with different superscripts on the same line are statistically significant. NS: non-significant. *: P<0.05. **: P<0.01.

There were no differences between the treatment groups regarding FC values in the 1st, 3th, and total periods (P>0.05). However, in the 2nd period, the FC value of the WW group was higher than those of the C and W groups (P<0.05). In the 3rd period and total, the FCR value of the control group was lower than those of the W and WL groups (P<0.01), similar to that of the WW group (P>0.05). There were significant differences among the treatment groups regarding total protein (P<0.01) and metabolic energy consumption (P<0.05) and also the amount of energy per kg of egg (Table 3).

Egg production (number) and yield (%), egg weight, and egg mass values are presented in Table 4.

The differences between the treatment groups in terms of egg yields (number and %) were not significant (P>0.05). The egg weight of the W and WL groups was significantly lower than that of the control group in the 1st (P<0.01) and 2nd (P<0.05) periods. The egg mass value was significantly lower in the W group than in other groups in the 1st period (P<0.05; Table 4).

Egg internal and external quality parameters are given in Tables 5 and 6.

There were no differences amongst groups regarding albumen height and Haugh unit values (P>0.05). However, the yolk color values obtained in the W group were generally lower than the other groups (P<0.01; Table 5).

The egg shell ratio was lower in the W group than in other groups in the 1st and 2nd periods (P<0.01). Also, in the 1st and 2nd periods of the study, the eggshell weights of the W and WL groups were significantly lower than the control group (P<0.05). However, the difference was insignificant in the WW group (P>0.05). Eggshell thickness was significantly higher in the control group than in the other groups throughout the experiment (P<0.01). At the same time, the egg shell thickness in the W group was significantly lower than in the WW and WL groups in the 1st, 2nd, and total periods (P<0.01). ESSA values in the 1st and 2nd periods were significantly lower in the W and WL groups than in the control group (P<0.05). Eggshell unit weight was found to be lower in the W group in the 1st, 2nd, and total periods than in the other groups (P<0.01; Table 5).

The results of the economic analysis (egg income by weight, net income, and total expenses) amongst the study groups are given in Table 6.

In terms of total expenses, the W group was lower than other groups in the 2nd period (P<0.05). The lowest expense per laying hen was in the W group (0.205 $/hen). Egg sales income and net profit values were similar in all groups throughout the experiment (P>0.05). The lowest egg income per laying hen was in the W group (0.269 $/hens), but the net profit per laying hen was similar with control group (0.064 $/hens; Table 6).

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Table 5. Effect of choice feeding on egg internal (albumin height, yolk color, haugh unit) and external (shell ratio. shell weight. shell thickness, shell surface area, shell unit weight) quality characteristics of the study groups.

Parameters	Groups ($\bar{X} \pm S_{\bar{X}}$)				P
	C	W	WW	WL	
Albumen height, mm					
1st period	3.98±0.18	3.96±0.18	4.36±0.25	4.53±0.21	NS
2nd period	4.43±0.28	3.76±0.13	4.04±0.27	4.01±0.15	NS
3rd period	3.74±0.13	3.89±0.19	4.22±0.18	3.99±0.07	NS
Total	4.05±0.13	3.87±0.12	4.18±0.17	4.18±0.08	NS
Yolk color, Roche color fan value					
1st period	9.13±0.12^{a}	7.11±0.34^{c}	9.17±0.06^{a}	7.81±0.29^{b}	**
2nd period	9.03±0.11^{a}	7.15±0.17^{c}	8.73±0.11^{a}	7.70±0.18^{b}	**
3rd period	8.44±0.20^{a}	6.83±0.28^{b}	7.34±0.26^{b}	7.53±0.18^{b}	**
Total	8.86±0.07^{a}	7.03±0.21^{d}	8.38±0.14^{b}	7.68±0.18^{c}	**
Haugh unit					
1st period	53.75±1.90	54.53±2.64	58.99±2.96	61.43±2.71	NS
2nd period	57.63±4.00	51.20±2.00	52.52±3.64	54.70±1.93	NS
3rd period	52.35±1.43	54.77±2.24	57.69±1.98	55.99±1.19	NS
Total	54.58±1.64	53.50±1.64	56.04±1.92	57.38±0.81	NS
Shell ratio, %					
1st period	10.50±0.08^{a}	10.00±0.10^{b}	10.46±0.08^{a}	10.57±0.09^{a}	**
2nd period	10.03±0.08^{a}	9.69±0.06^{b}	10.08±0.07^{a}	10.10±0.05^{a}	**
3rd period	10.01±0.09	9.91±0.09	9.88±0.07	10.09±0.07	NS
Total	10.18±0.08	9.87±0.06	9.97±0.20	10.26±0.06	NS
Shell weight, g					
1st period	6.34±0.02^{a}	5.78±0.06^{c}	6.20±0.05^{ab}	6.16±0.06^{b}	**
2nd period	6.31±0.03^{a}	5.88±0.04^{c}	6.20±0.04^{ab}	6.15±0.04^{b}	**
3rd period	6.21±0.05	6.00±0.07	6.02±0.06	6.10±0.07	NS
Total	6.28±0.02^{a}	5.89±0.04^{c}	6.04±0.12^{bc}	6.13±0.05^{ab}	**
Shell thickness, mm					
1st period	35.56±0.34^{a}	32.30±0.39^{b}	35.76±0.51^{a}	35.70±0.56^{a}	**
2nd period	41.35±0.23^{a}	37.75±0.40^{c}	39.97±0.23^{b}	40.08±0.22^{b}	**
3rd period	41.35±0.27^{a}	39.45±0.34^{b}	40.03±0.26^{b}	41.03±0.30^{a}	**
Total	39.42±0.19^{a}	36.50±0.26^{c}	38.59±0.25^{b}	38.93±0.17^{ab}	**
Egg shell surface area (ESSA)					
1st period	71.80±0.30^{a}	69.64±0.46^{c}	70.92±0.43^{ab}	70.05±0.31^{bc}	**
2nd period	73.97±0.56^{a}	72.13±0.35^{b}	72.80±0.52^{ab}	72.19±0.25^{b}	*
3rd period	73.22±0.77	72.00±0.45	72.35±0.44	71.87±0.32	NS
Total	73.00±0.52	71.26±0.35	71.18±0.98	71.37±0.25	NS
Egg shell unit weight					
1st period	0.0884±0.00056^{a}	0.0829±0.00069^{b}	0.0875±0.00054^{a}	0.0879±0.00074^{a}	**
2nd period	0.0852±0.00039^{a}	0.0817±0.00047^{b}	0.0851±0.00041^{a}	0.0850±0.00045^{a}	**
3rd period	0.0848±0.00039	0.0833±0.00073	0.0833±0.00060	0.0848±0.00066	NS
Total	0.0862±0.00042^{a}	0.0825±0.00043^{b}	0.0848±0.00076^{a}	0.0860±0.00058^{a}	**

C: Control. W: choice feeding with continuous whole wheat. WW: choice feeding with whole wheat weekly. WL: continuous choice feeding with whole wheat + limestone. P: probability. a.b: The differences between the averages shown with different superscripts on the same line are statistically significant. NS: non-significant. *: P<0.05. **: P<0.01.

Table 6. Economic reflection of choice feeding in laying hens.

Parameters	Groups ($\bar{X} \pm S_{\bar{X}}$)				P
	C	W	WW	WL	
Total cost, $					
1st period	2.79±0.06	2.61±0.08	2.70±0.06	2.74±0.05	NS
2nd period	3.06±0.06^{ab}	2.88±0.06^{b}	3.18±0.09^{a}	3.05±0.02^{ab}	*
3rd period	2.69±0.07	2.70±0.04	2.71±0.04	2.77±0.05	NS
Total	8.54±0.13	8.19±0.13	8.59±0.12	8.56±0.07	NS
Average	2.85±0.04	2.73±0.04	2.86±0.04	2.85±0.02	NS
Per hen	0.214±0.003	0.205±0.003	0.215±0.003	0.214±0.002	NS
Egg sales income, $					
1st period	3.58±0.09	3.43±0.13	3.66±0.07	3.64±0.06	NS
2nd period	3.80±0.08	3.63±0.14	3.88±0.03	3.82±0.03	NS
3rd period	3.74±0.05	3.70±0.05	3.77±0.05	3.74±0.02	NS
Total	11.12±0.19	10.76±0.28	11.31±0.11	11.20±0.05	NS
Average	3.71±0.06	3.59±0.09	3.77±0.04	3.73±0.02	NS
Per hen	0.278±0.005	0.269±0.007	0.283±0.003	0.280±0.001	NS
Net Profit, $					
1st period	0.79±0.11	0.82±0.09	0.96±0.09	0.90±0.07	NS
2nd period	0.74±0.03	0.75±0.10	0.70±0.08	0.77±0.05	NS
3rd period	1.05±0.05	1.00±0.06	1.06±0.06	0.97±0.04	NS
Total	2.58±0.13	2.57±0.20	2.72±0.11	2.64±0.11	NS
Average	0.86±0.04	0.86±0.07	0.91±0.04	0.88±0.04	NS
Per hen	0.064±0.003	0.064±0.005	0.068±0.003	0.066±0.003	NS

C: Control. W: choice feeding with continuous whole wheat. WW: choice feeding with whole wheat weekly. WL: continuous choice feeding with whole wheat + limestone. P: probability. a.b: The differences between the averages shown with different superscripts on the same line are statistically significant. NS: non-significant. *: P<0.05. **: P<0.01. 1 US$=13.5 TL.

Discussion and Conclusion

One of the main costs of livestock production in laying hens is feed. If the difference between the daily feed expense and the next day's egg income is positive, laying hen farms can continue to produce. Although more studies have been carried out in broilers (26) and other poultry (20) to continue without impairing performance and product quality in poultry, the number of choice feedings based on grains in laying hens is less. Especially in laying hens, due to the low Ca level in grains, free-choice feeding studies have been concerned about the deterioration of egg shell quality and, therefore, the decrease in the number of eggs that can be marketed. Both choice feedings with continuously whole wheat and limestone (given as grit against the possible Ca deficiency) and, at the same time, the effects of weekly choice feeding with whole wheat were examined.

In the current study, the initial and final BW values at the trial were not significantly affected by the treatments, but the BW change increased in the control group and decreased in the choice feeding groups. Body-weight gain or loss is related to the difference between the energy taken into the body and the energy lost or given. Under normal conditions, small increases in body weight occur in commercial flocks fed on a standard diet. Although small body weight gains occurred in this study, choice wheat consumption with different methods caused a loss in body weight. Other researchers found similar results (14, 31, 33) when they gave wheat selection. The BW of laying hens decreased.

In contrast, some reports showed that choice feeding with wheat did not change the BW of laying hens (15) and quails (22). This may show the ration balance slightly deteriorated with the addition of wheat. This situation manifested itself with the increase in feed consumption and the decrease in FCR in the selectively fed groups. The FC values obtained from the control group were generally lower than the choice feed offering groups. Bennett and Classen (2), reported that feeding with whole barley increased the feed intake and body weight gain in laying hens, in contrast to current findings (14-16). Also, Robinson (27) showed that combined use of wheat and limestone decreased feed intake in hens.

Although more studies have been carried out in broilers (7, 18, 26) and quail (19) and Türkiye chicks (20) to continue without impairing performance and product quality in poultry, the number of selective feedings based on grains in laying hens is limited. Especially in laying hens, due to the low Ca level in grains, free-choice feeding studies have been concerned about the deterioration of egg

shell quality and therefore the decrease in the number of eggs that can be marketed. In this study, both choice feeding was applied and limestone was given as grit against the possible Ca deficiency. At the same time, the effects of choice feed presentation in weekly changes were examined. Feed efficiency was lower in whole wheat selection groups, but group C used feeds more effectively than the choice feeding treatment groups. Similar results were obtained by Traineau et al. (32) and Mirzaie et al. (21). However, Cho et al. (5) and Kerman (17) reported that whole wheat feeding did not affect FCR in laying hens. Ciftci et al. (4) showed that triticale and additional enzymes did not affect FC and FCR in laying hens.

In the current experiment, continuous/weekly choice feeding with whole wheat and limestone addition did not affect egg yield, mass, or weight (except periods 1 and 2). These results showed that hens adapted to choice feeding of wheat in two programs, and they were given together with limestone as a calcium source. Similar results were reported by Karunajeewa (16), Cho et al. (5), Kerman (17) and Jordan et al. (15) that egg yield was not significant amongst the groups; however, egg weight increased (16, 27) and decreased (14, 27) in laying hens. Bennett et al. (2) reported that choice feeding with barley and access to insoluble grit did not affect production parameters in hens.

There were no significant differences among the treatment groups in HU and albumen height values in the present study. However, there was a substantial difference in terms of egg yolk color. One of the critical issues to be considered in whole wheat choice feeding practices is the changes in the egg yolk color (16). Although corn is rich in carotenoids, however, other common grains such as barley and wheat have a low concentration of carotenoids (24), and may cause a decrease in egg yolk color. Saikhlai et al. (28) reported a lighter yolk color between 15, 20, and 25% in wheat additions in laying hen diets.

Eggshell weight, thickness, ratio, shell surface area, and unit shell weight were decreased in the group (W) in which whole grain wheat was given continuously compared to the control and other treatment groups. Weekly administration of wheat and wheat+grit limestone did not improve eggshell properties compared to the control. Still, it was found to be slightly superior to group W. The effect of the low Ca content of wheat was reflected in the weight and thickness of the eggshell. As it is well known, Ca, P, and vitamin D are essential traits for bioavailability in the body, and a deficiency or excess of one of them reduces the level of utilization of the other two. Therefore, giving grit limestone along with wheat could not improve the thinning of the eggshell. A similar result was reported by Faruk et al. (8), and sequential feeding with wheat causes a decrease in eggshell weight in laying hens. However, Karunajeewa (16) issued with whole wheat feeding and Sakomura et al. (29) exclusive wheat+limestone feeding did not change eggshell weight and thickness.

In conclusion, choice feeding with wheat (continuous and weekly) and limestone in laying hens did not negatively affect some performance and economic parameters (FC, cost, income, and net profit). However, some quality parameters (yolk color, shell weight, and thickness) were regressed. With choice feeding, which does not have a negative economic impact, both animal welfare is ensured and animals are protected from some metabolic diseases. In addition, by means of choice feeding, transport, grinding, and feed mixing costs are decreased. Thus, it is thought that the profitability of the enterprises may increase due to a decrease in metabolic diseases of poultry. Therefore, in the future the use of choice feeding is considered important in terms of both economic and animal welfare.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

MK, contributed to data collection and writing. GG, designed the study material and carried out analyzes. BRY, contributed to data collection and literature review. SS, designed the research, contributed to reviewing and editing the manuscript. YK, carried out the literature reviewing process and writing the manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was approved by the Erciyes University Animal Experiments Local Ethics Committee (approval date and the number: 03.06.2020 and 20/087).

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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Seasonal variation in the nutrient composition of mussels (*Mytilus galloprovincialis*) from farms in Boka Kotorska Bay, Southern Adriatic Sea

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ABSTRACT

The aim of this study was to investigated the seasonal variation in the nutrient composition of Mytilus galloprovincialis, cultivated in Boka Kotorska Bay, Montenegro, during all seasons in one year. Biometric parameters, meat yield, condition index, proximate composition, minerals, and the lipid and fatty acid compositions of mussels were analyzed. The most significant factors determining these parameters were temperature, food amount and gametogenesis The biometric parameters showed no significant differences during the sampling period. In the spring, meat yields and mussel condition index increased at substantial levels. Low values od meat yield and condition index during cold months have resulted from food shortage and reproductive cycle, when mussels use carbohydrates and protein reserves. The highest amount of protein was detected in mussels harvested in August (10.76%), while the highest amount of lipids was recorded in the winter months (2.11%). Docosahexaenoic acid and eicosapentaenoic acid were the most abundant PUFA. The concentration of metals found in mussels from the study area is within the range of mean values reported in the literature. Our results indicate that the best period for mussels harvesting was during the spring and summer (April and August), opposite the winter months when the mussels were not favorable for harvesting.

Introduction

Mussels are a highly nutritious foodstuff, rich sources of digestible proteins, vitamins, and minerals. The mussel lipid contents are rather low, the polyunsaturated fatty acids (n-3 PUFAs), eicosapentaenoic acid (EPA, C20:5 n-3), and docosahexaenoic acid (DHA, C22:6 n-3) composition are crucial. Mussels feed on the phytoplankton from which they acquire proteins, carbohydrates, lipids and other components for their biomass (10). Also, different types of metals were detected in their tissue, such as calcium, zinc, copper, iron, iodine, manganese, however they can be contaminated with heavy metals which are dangerous to human health (18).

Factors influencing mussel biochemical composition, meat content and condition index are water temperature, plankton composition and gametogenesis cycle, which change during the season (12). Phytoplankton growth and microalgal blooms rates increase with increasing water temperature in spring and summer and reduce during the cold months (autumn and winter) (19). In addition, the abundance of food resources (e.g., diatoms, dinoflagellates) leads to an increase in mussels biomass (15).

The contribution of the aquaculture in the Montenegrin national economy is insignificant, but there is significant potential for its development because suitable oceanographic, physical, chemical and biological conditions in the Boka Kotorska Bay (8). The natural environment with sheltered bays and estuaries give this area an ideal geoformation.

The objective of this study was to analyze the biometric and biochemical characteristics of mussels during one year. Based on the obtained results, we can propose an ideal time for mussel harvesting. On this location, the study for the first time considers such a large number of analyzes, therefore it constitutes encourages a model for local entrepreneurs and could be helpful for developing the mussel as a functional food.

Materials and Methods

Mussel sampling and environmental conditions of seawater: In the southern Adriatic Sea, at the border between Montenegro and Croatia there is a Boka Kotorska Bay, one of the largest bays of this part of the coast (87.3 km^2 of maritime zone). Bay consists of four smaller sub-bays: the Bay of Kotor, the Bay of Risan, the Bay of Tivat, and the Bay of Herceg Novi. Three large farms located in this Bay: Orahovac, Lipci and Kukuljina, were selected as sampling sites.

Mussel samples for this study were collected from three harvesting areas during four seasons (April, August, October 2018 and February 2019). Specific properties of this bay are caused by variable weather conditions, like abundant, rainfall during the autumn and winter and a lot of arid days during the summer. More than 2 kg of mussels were collected at each sampling site and were transported to the laboratory under refrigeration (+4°C) immediately. The biggest 25-30 mussels were separated for condition index, meat yield determination and biometric measurements. The rest of the mussels were washed, opened, and the flesh scraped out of the shells. At the same time, seawater environmental parameters were measured (below 1 m surface). Basic physical-chemical water parameters (temperature, salinity) were measured every season in situ using the multiparameter instrument (MultiLine 4, WTW, Germany). This research was conducted under commercial conditions.

Biometric parameters, condition index and meat yield*: Mytilus galloprovincialis* collected were measured for their biometrical parameters - length (maximum measure along the anterior-posterior axis), height (maximum dorsoventral axis) and width (maximum lateral axis). Mussels were measured using a 0.05 mm precision caliper (21). After opening the adductor muscle with a scalpel, the total weight of mussels, wet meat and shell weight were measured.

Using the following we calculate the condition index and meat yield of mussels. Condition Index (%): (dry meat weight (g) / dry shell weight (g)) × 100 (23).

Meat Yield (%): (wet meat weight (g)/total weight (g)) × 100 (22).

For condition index determination, to obtain dry weight, mussels (soft tissues and shells) were dried at 60°C for 48 h.

Proximate composition and mineral analysis: For proximate chemical composition studies, three pooled samples of mussels from each sampling site were minced in a processor for food (IKAR M 20 universal mill, IKA 1603601; IKA, Germany) and, until further analyzed, kept in dry conditions. Biochemical analyses on homogenized samples of mussels tissues were carried out in triplicate.

For chemical composition (moisture, ash, proteins and lipids) mussels samples were analyzed according to AOAC (3). In pre-measured porcelain trays, the moisture and ash content were established gravimetrically at the 80°C for 24 h. Then the dried mussel was ashed at 450°C for four h in a muffle furnace and weighed to the nearest 0.001 g. The total protein content was determined using the Kjeldahl technique (N × 6.25). In a digestor (BÜCHI B-435, Labortechnik AG, Flawil, Switzerland) mussel tissue was digested and then distilled using a Rotavapor R-210 (BÜCHI Labortechnik AG, Flawil, Switzerland) and automatic volumetric titration (T-50 automatic titrator, Mettler Toledo, United States).

The total fat content was determined gravimetrically, using the Soxhlet technique, by virtue of ether-mediated lipid extraction. In a muffle furnace (550°C/16h) the amount of inorganic material was measured by incinerating the samples to ash. Fatty acid profiles were determined by capillary gas chromatography using GC Shimadzu 2010 (Kyoto, Japan) gas chromatograph with flame ionization detector and capillary HP-88 column (100 m x 0.25 mm x 0.20 µm, J&W Scientific, USA). Working conditions were as previously described (23). Comparison of retention times to authentic standards were used for fatty acids identification. After digestion of the samples, the concentration of micro and macro elements were determined by inductively coupled plasma-mass spectrometry (ICP-MS). During ICP-MS, simultaneously with the samples, a working solution of internal standard was introduced into the system. The internal standard included low, middle and high mass elements. Based on the recorded values (i.e. according to the percentage of intensity reduction or increase) of the internal standard, the software performed an automatic correction of the obtained concentration of elements in the minced mussel tissue digests. Quality control was carried out by analyzing the certified reference material NIST 1577c.

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Statistical analysis: Results were presented by descriptive statistical parameters mean±standard deviation. Significance was fixed at level P<0.05 in all cases. At all sampling times, analyses were carried out in duplicate. For all parameters conducted to test significant seasonal fluctuations, the analysis of variance (One-Way ANOVA) was using (14). All obtained results are presented in tables and figures.

Results

Environmental parameters (temperature, salinity) in the water samples from three different stations in the Boka Kotorska Bay were monitored in April, August, October 2018, and February 2019. During the seasons, the lowest seawater temperature and water salinity recorded in February (11.6°C; 14.9‰) and the highest values recorded in August, when the seawater temperature was 27.6°C and salinity was 33.9‰. The seasonal distribution of mean temperature and salinity were shown in Figure 1.

The biometric of mussel results in all seasons are shown in Table 1. Width shell showed significant variation (P<0.05) reaching minimum values in April (20.72±1.02 mm) and maximum values in October (24.31±0.83 mm). In spring, both meat yield (MY) and conditions index (CI) values began to grow significantly, and this high values continued all summer. CI values varying between 7.73+1.04% in February, and 18.83+0.55% in August and MY was maximum (31.07+0.76%) in April and minimum (22.63+0.45%) in October (Figure 2).

Table 1. Biometric measurement (length, width, height) of *Mytilus galloprovincialis* collected from Boka Kotorska Bay.

	n	April	August	October	February
Length (mm)	25	51.99±1.51[a]	60.66±6.85[a]	56.42±2.84[a]	53.32±1.80[a]
Width (mm)	25	20.75±1.02[a]	23.07±1.35[ab]	24.31±0.83[b]	21.01±0.95[a]
Height (mm)	25	11.25±0.49[a]	13.88±3.14[a]	11.23±0.31[a]	12.15±0.60[a]

[a,b,c] Values within a row with different superscripts differ significantly at P<0.05.
n= number od samples

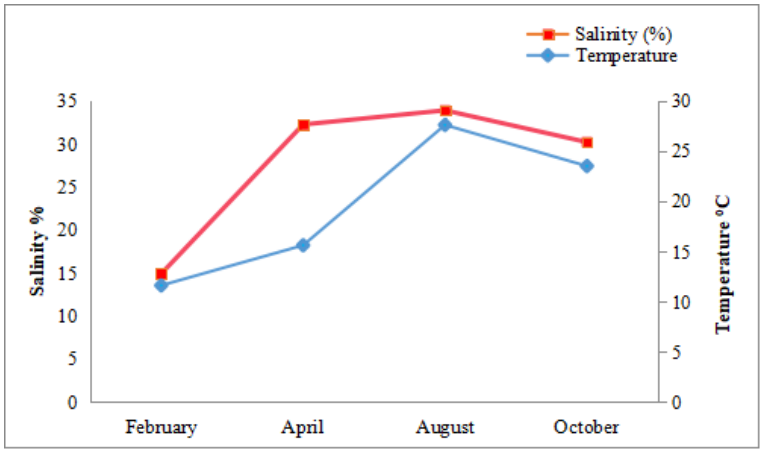

Figure 1. Seasonal distribution of mean temperature and salinity.

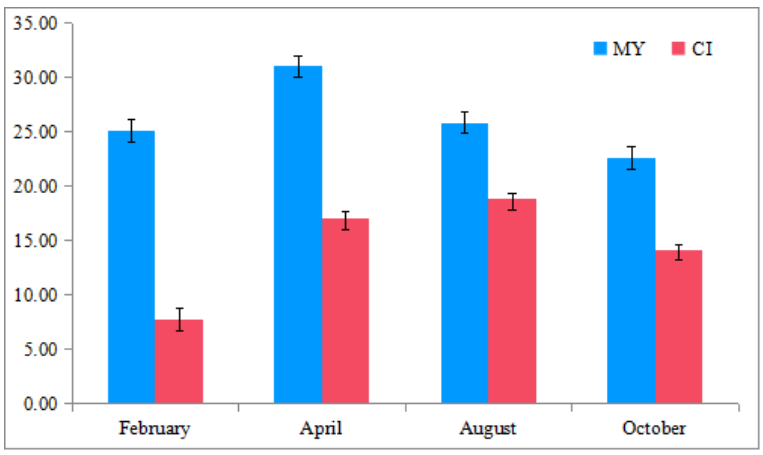

Figure 2. Seasonal variations in percentage condition index and meat yield of mussels. CI, condition index; MY, meat yield.

Table 2. Total fatty acid composition (saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids) and ratio ω-3/ω-6 fatty acids for *Mytilus galloprovincialis* samples from different locations in Bokakotorska Bay during four season.

	April	August	October	February
C14:0	2.39 ± 0.43	3.76 ± 0.62	2.53 ± 0.09	1.66 ± 0.55
C15:0	0.50 ± 0.03	0.88 ± 0.05	0.79 ± 0.04	0.62 ± 0.07
C16:0	23.69 ± 0.82	25.86 ± 0.82	23.26 ± 0.02	22.12 ± 1.22
C17:0	0.68 ± 0.08	2.43 ± 0.08	1.91 ± 2.07	1.17 ± 0.21
C18:0	3.42 ± 0.20	3.94 ± 0.11	4.11 ± 0.12	3.11 ± 0.07
ΣSFA	**30.68 ± 0.75**	**36.87 ± 1.02**	**32.6 ± 2.07**	**28.68 ± 0.84**
C16:1	4.33 ± 1.02	2.72 ± 0.26	3.51 ± 0.12	5.11 ± 1.29
C18:1 cis-9	3.21 ± 0.80	3.42 ± 0.12	3.44 ± 0.16	2.53 ± 0.39
C20:1	3.55 ± 0.04	5.39 ± 0.29	5.98 ± 0.04	3.16 ± 1.00
ΣMUFA	**11.09 ± 1.05**	**11.53 ± 0.64**	**12.93 ± 1.01**	**10.8 ± 1.23**
C18:2 n-6	2.98 ± 0.22	2.12 ± 0.18	3.47 ± 1.45	1.26 ± 0.28
C18:3 n-3	1.41 ± 0.32	1.13 ± 0.12	1.41 ± 0.12	1.33 ± 0.26
C20:2 n-6	4.41 ± 0.27	5.22 ± 0.08	4.81 ± 0.21	3.98 ± 0.31
C20:3 n-3	1.42 ± 0.18	1.84 ± 0.21	1.98 ± 0.30	1.32 ± 0.10
C20:3 n-6	1.14 ± 0.02	0.87 ± 0.03	0.98 ± 0.12	0.68 ± 0.12
C20:5 n-3 (EPA)	11.31 ± 0.71	9.23 ± 0.31	7.99 ± 0.34	13.05 ± 3.21
C22:5 n-3	0.55 ± 0.04	1.46 ± 0.11	1.08 ± 0.21	0.97 ± 0.55
C22:6 n-3 (DHA)	12.26 ± 0.65	16.58 ± 0.21	12.41 ± 1.11	12.23 ± 1.44
ΣPUFA	**38.48 ± 1.29**	**38.45 ± 0.74**	**34.13 ± 0.59**	**34.92 ± 1.66**
Total n-3 PUFA	26.95 ± 0.58	30.13 ± 0.42	24.87 ± 0.61	28.9 ± 0.95
Total n-6 PUFA	8.53 ± 0.72	8.21 ± 0.87	9.26 ± 0.75	5.92 ± 0.60
n-3/n-6	3.16 ± 0.03	3.67 ± 0.02	2.68 ± 0.03	4.88 ± 0.19

All samples were analyzed in duplicate (n = 2) from a pooled sample (20 numbers) and expressed as mean ± standard deviation.

Table 3. The mean concentrations of metals (mg/kg dry weight) in the sampled mussels.

	April	August	October	February
Ca	1401 ± 506^{a}	3714 ± 206^{b}	2370 ± 710^{c}	7085 ± 445^{d}
K	1876 ± 109.8^{a}	2216 ± 140.9^{b}	2037 ± 83.91^{c}	1824 ± 207.8^{d}
Ni	4.1 ± 0.03^{a}	7.9 ± 0.35^{b}	5.1 ± 0.15^{c}	7.2 ± 0.11^{d}
Cr	2.6 ± 0.06^{a}	1.3 ± 0.04^{b}	2.4 ± 0.06^{a}	5.3 ± 0.12^{c}
Fe	120.7 ± 15.27^{a}	267.2 ± 1.28^{b}	381.8 ± 7.96^{c}	341.7 ± 8.52^{d}
Zn	148.4 ± 2.11^{a}	189.1 ± 1.84^{b}	224.8 ± 2.09^{c}	246.8 ± 2.88^{d}
Mn	57.4 ± 2.43^{a}	15.8 ± 0.29^{b}	62.6 ± 1.60^{c}	77.5 ± 2.78^{d}
Pb	0.22 ± 0.02^{a}	0.27 ± 0.06^{b}	0.28 ± 0.06^{bcd}	0.30 ± 0.03^{d}
Cd	0.13 ± 0.04^{a}	0.14 ± 0.02^{a}	0.12 ± 0.02^{a}	0.19 ± 0.03^{b}
Hg	0.2 ± 0.001^{a}	0.3 ± 0.004^{a}	0.3 ± 0.01^{a}	0.4 ± 0.001^{a}

All samples were analyzed in duplicate (n = 2) and expressed as mean ± standard deviation.

The fluctuations of total lipid (0.72–2.12%), ash (2.56-3.59%), and protein (8.42–10.76%) contents of mussels on a dry basis during the experimental period are shown in Figure 3. Quantitative analyses of proteins, responsible, for energy storage in animals, revealed elevated levels in spring and summer and lower levels during autumn and winter. The percentage of lipids ranged from 0.72 ± 0.18% in October to 2.11 ± 0.69% in the winter months, when it reached its highest value. The fatty acid profile of the mussels in Boka Kotorska Bay is shown in Table 2, where a total of 16 FAs were identified. Polyunsaturated fatty acids (34.13-38.48%) predominated oversaturated (28.68-36.87%) and monounsaturated fatty acids (10.8-12.93%) throughout the year. Amongst all PUFAs, a high proportion of ω-3 long-chain PUFAs, especially EPA and DHA, is apparent. Within the group of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs), palmitic (C16:0) and palmitoleic (C16:1n-7) acids were the most abundant.

The mean concentrations of the investigated metals (Ca, K, Ni, Cr, Fe, Zn, Mn, Pb, Cd and Hg) in mussel *M. galloprovincialis* samples are given in Table 3. The

concentrations of the these metals in the mussel along the Montenegrin coastline decreased in the following order: Ca > K > Fe > Zn > Mn > Ni > Cr > Pb > Cd > Hg for all investigated samples. As can be seen from Table 3, the greatest mean metal concentrations were observed in samples from the autumn and winter seasons.

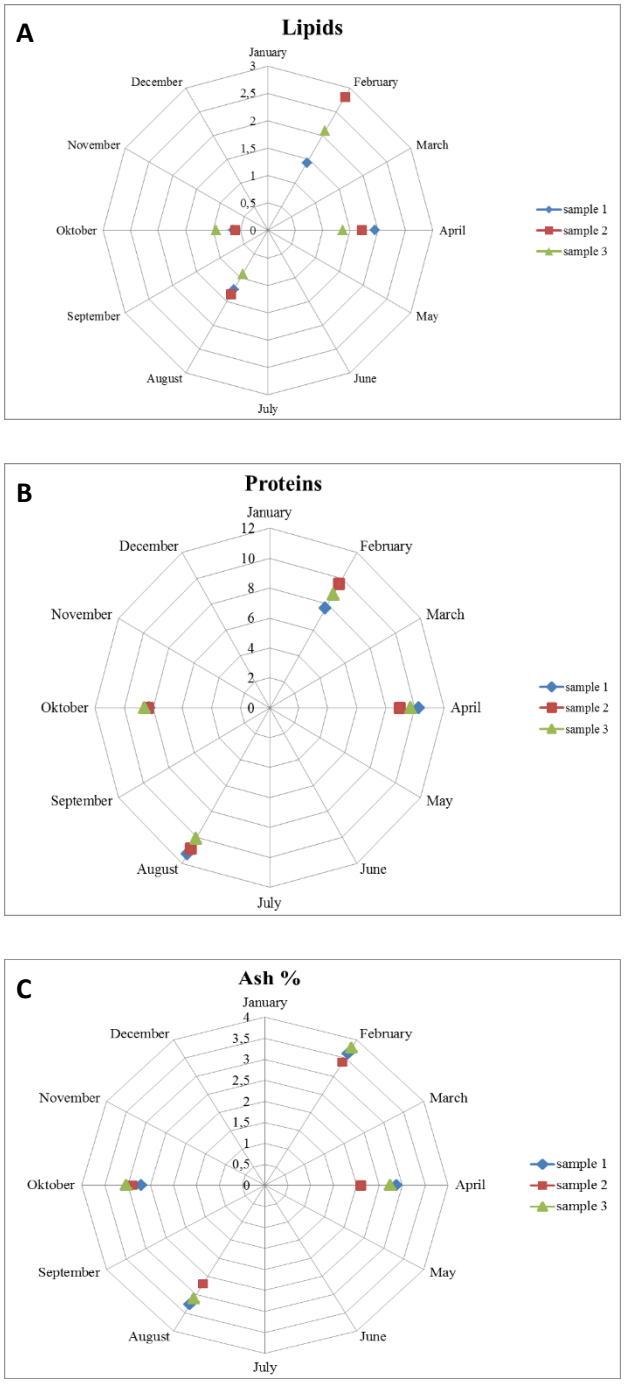

Figure 3. Polar diagram - seasonal variation a) lipids; b) proteins; c) ash

Discussion and Conclusion

The results suggest an influence of environmental parameters such as water temperature, food availability, and the reproductive cycle of the mussels on their biochemical composition. Boka Kotorska Bay has very pronounced seasonal water temperature fluctuations. Perennial research shows minimum temperatures in January and February and peak temperatures in July and August (28). Our study reported the mean minimum temperature in February (11.6°C), and the maximum temperature was recorded in August (27.6°C). The salinity of the Adriatic Sea (38.30‰) is higher than the western Mediterranean (37‰) and but slightly lower than the eastern Mediterranean (39‰) (28). In our examination the salinity was constant throughout the year (30-33‰), except in February when it was 14.9‰, resulting from higher amounts of precipitation.

In 2004, Ojea et al. found some variations in the weight, length, width, height and proximal composition of mussels that occur in response to changes in environmental conditions, especially in food availability (20). While parameters such as the decrease of sunlight or the cooling of the sea surface reduce phytoplankton biomass production during autumn and winter, they have the opposite effect in spring and summer, resulting in microalgal blooms (19). When there is enough food, mussels use the energy to grow their tissues and develop the gonads. It takes about 18 months to reach a commercial size of at least 50 mm, because during the winter months, they generally stagnate and lose weight, and after that they grow again. Shell growth can be continuous during the year as it is formed mainly from dissolved calcium present in seawater, still meat weight appears to be seasonal, with gross growth concentrated in specific seasons (2). In our study, the biometric parameters showed no significant differences during the sampling period.

The biochemical compositions of mussels are characterized by phases of accumulation and depletion of food reserves in the spawning season. Shortage of food typically occurs between late September and early December. Then low values of biochemical resources may be expected (17).

The commercial quality and physiological state of bivalve molluscs are adequately described by the condition index (CI), and also the meat yield (MY), parameters of economic relevance reflecting the ecophysiological conditions and the health of *Mytilus galloprovincialis* (7). The fluctuations in condition index and meat weight have important implications for the cultivation and harvesting strategy. For optimum exploitation the harvesting season should be timed according to the peak period for the condition index. In our study, in spring, both meat yield (MY) and conditions

index (CI) began to grow significantly. A variety of extrinsic and intrinsic factors, like salinity and temperature of the water, the gametogenic cycle of animals, and food availability, affect these environmental parameters (22). A positive correlation between CI and biochemical constituents of bivalves has been reported in different mollusc species (23). Low meat yield values and condition index values during winter have been attributed to the utilisation of carbohydrates (i.e., glycogen) reserves and the depletion of protein contents due to of food shortage and gametogenesis.

The variations in protein content appeared to be due to the seasonal differential availability of food (microalgae). The protein content varied from 8.42-10.76%; a maximum of 10.76% was recorded in samples collected in August. In general, a rapid tissue restoration occurred in spring and mussels remained in good condition during summer when protein was maximal and decreased through winter. During winter the lipid reserves in the developing eggs leads to higher lipid content in mussels (5).

Considering the economic value of biochemical components from marine mussels, fatty acids (FAs), especially PUFAs, are regarded as the single most crucial nutritional indicator dictating the quality of *Mytilus galloprovincialis*. The prevalence of PUFAs, amounting to about 34 - 38% of total fatty acids and predominated over the SFAs and MUFAs. Similar profiles have been reported previously for *M. galloprovincialis* grown in the northwest of Spain (9), the Adriatic Sea (29) and two different Italian sites (23). The main factor explaining these results might be the availability of phytoplankton, the bivalve's major source for n-3 PUFAs, particularly linoleic (C18:2n-6) and linolenic (C18:n-3) acids, known to be synthesized by diatoms and dinoflagellates (2). Therefore, the quantitative and qualitative availability of food influences the composition of fatty acids (11). The proper balance of dietary n-3/n-6 PUFAs is integral to prevent chronic and cardiovascular diseases. It is potentially significant because the ratios of essential fatty acids in the tissues are determined mainly by their proportions in the diet (6). In present study n-3/n-6 ratios were 2.7–4.9 which is paralleled previous studies.

Mussel fatty acid profiles usually contain about ~30–40% SFA (1), a level found in the present study. The predominant SFA was recorded to be 16:0 (22–25% of total fatty acids), with a maximum recorded in August. It is as expected because SFAs are used for energy storage, and therefore, their concentration increases during periods of enhanced feeding activity (13).

The metal level in mussel tissues represents integrated response to bioavailable metal in seawater and the food available to them. Because they are filter feeders, metals in their interior can also be found in higher concentrations in seawater (24). In the mussel samples K, Mg, and Ca concentrations were higher than the remaining tested elements. That is understandable since these macronutrients have many vital roles in the organisms (27). The high content of Ca and Mg in mussels is their requirement for the shell formation while the mussel growth (4).

Based on the measured concentrations of metals in mussel sampled in four different seasons, it can be observed that generally the highest average concentrations were measured in the winter and autumn months. This result agrees with that of other authors (26, 16) and is explained by the higher solubility of heavy metals and minerals due to increased rainfall and fresh water inflow. It can also be explained by the decrease in the weight of mussels during this time of year, less food available, while the metal concentration remains the same. A significant seasonal variability was observed for the tissue concentrations of thesome trace metals. Chromium, iron, calcium and zinc exhibited higher levels in winter and autumn, and lower in spring or summer months (Table 3).

The present study provided a detailed biochemical profile of the *Mytilus galloprovincialis* collected from Boka Kotorska Bay for the first time from this part of the Southern Adriatic Sea. The CI, MY and biochemical parameters proved suitable conditions for mussel cultivation in Boka Kotorska Bay. The most significant factors determining these parameters were temperature, food amount, and gametogenesis. In addition the metal concentrations found in the mussels are within the range of the mean values reported in the literature. Therefore, we can conclude that ideal periods for mussels harvesting were April and August. Spawning periods following these months and winter (between December and February) were not suitable for harvesting.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Author Contributions

NG, VDj and MD conceived and planned the experiments. NG, IZB, NČ and SB carried out the experiments. NG, MD planned and carried out the simulations. NG, VD and

IZB contributed to sample preparation. NG, NK and SDj contributed to the interpretation of the results. NG took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

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Vasoactive use in early goal-directed therapy in dogs with severe sepsis and septic shock

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ABSTRACT

The goal of this study was to see how to evaluate the changes in macrovascularand microvascular parameters in survivors (Sv) and non-survivors (non-Sv) dogs with severe sepsis and septic shock (SEVS & SEPS) in response to goal-directed hemodynamic optimization at the intensive care unit (ICU), and to evaluate norepinephrine (NE) and dobutamine (DT) ICU applications, and their effectiveness for predicting death. Thirty-five dogs with SEVS & SEPS were used. NE was given to 10 hypotensive dogs, despite receiving a single bolus of fluid therapy, at a constant infusion rate of 1.5 µg/kg/min for 2 h. The rate of NE infusion was doubled (3.0 µg/kg/min) if the clinical response was insufficient after 2 hours. DT was administered to 5 dogs with left ventricular systolic dysfunction (LVSD) (LVS'<7.5 cm/s) at a constant infusion rate of 5 µg/kg/min for 2 h. The Sv and non-Sv groups had no major differences in macrovascular and microvascular characteristics, PW-TDI septal mitral annulus systolic (S') and early diastolic (E') velocities, or DT applications. The only difference was the use of NE applications. non-Sv received a greater amount of NE, while Sv received a smaller amount of NE. In contrast, more Sv received a greater amount of DT applications. Total mortality rate was 25.7%. In conclusion, the effects of DT and NE in dogs with SEVS & SEPS are limited. To provide evidence-based guidelines for dogs with SEVS & SEPS, more research is needed.

Introduction

In both veterinary and human medicine, sepsis generates a high rate of morbidity and mortality (38). When the immune system overreacts to an infection, organ failure results. In dogs, sepsis-induced myocardial dysfunction (SiMD) is caused by canine parvovirus infection (CPVI) (22).

The basic pathologic developments resulting in SEVS & SEPS are absolute or relative dehydration, LVSD and left ventricular diastolic dysfunction (LVDD) (24), and right ventricular (RV) dysfunction, Chan and Klinger (12) marked peripheral vasodilation and vasoplegia. Even following the fluid balance correction, microcirculatory abnormalities may be constant and lead to the maldistribution of cardiac output (CO) (21) multiple organ dysfunction syndromes (MODS), and death (13). The incidence levels of SEVS & SEPS is unreported in veterinary medicine. SEVS & SEPS is observed in five percent to nineteen percent of human ICU admissions (1). The mortality rate in dogs and cats with SEVS & SEPS has been reported to range from 20% to 68% (37).

Early goal-directed therapy (EGDT) protocols have been developed to normalise irregular measurable indices of tissue perfusion and oxygenation (32). Macrovascular parameters such as SBP and MAP, together with microvascular parameters such as oxygen saturation (SpO2), lactate and base deficit (BE), which are used to reflect tissue perfusion and organ dysfunction, are often used to monitor patients with SEVS & SEPS because of their good prognostic value (11).

Vasoactive agents have both vasopressor and inotropic effects, however, vasopressor actions increase

blood pressure, whereas inotropic actions increase CO in EGDT (20). NE is a sympathetic catecholamine with mixed alpha and beta adrenergic effects. Its primary site of activity is the alpha 1 receptor (35). NE should be used in all hypotensive septic patients following adequate fluid replacement therapy (2, 14). In septic patients with hypotension unresponsive to other vasopressors, Jhanji et al. (23) discovered that NE was an effective rescue medication. DT has inotropic and vasodilatory properties, potentially improving oxygen delivery and tissue perfusion (13). Therefore, current guidelines recommend the use of DT for septic patients with low CO after appropriate fluid replacement therapy (5, 14). However, the optimal treatment of hypotension with NE and low CO with DT in volume-replete patients is debatable (38). Despite its extensive use, veterinary research on the effects of DT in LVSD and NE in hypotensive dogs with SEVS & SEPS is sparse (11). We expected that improvements in EGDT would be linked to higher survival rates in dogs with SEVS & SEPS. In veterinary medicine, the value of these macrovascular and microvascular monitoring metrics, as well as the effect of NE and DT in dogs with SEVS & SEPS, has not been widely examined. Therefore, the first purpose of this study was to evaluate the changes in the monitored parameters (SBP and MAP, lactate, SpO2, BE) in canine patients with SEVS & SEPS in response to goal-directed hemodynamic optimization. The second purpose was to evaluate NE and DT applications in ICU and their effectiveness in predicting death.

Materials and Methods

Ethics committee approval for this study was obtained from Local Ethics Committee of the Near East University (permit number: 2019/03). From January 2019 to September 2020 the records of dogs that were admitted to Near East University's Animal Hospital were evaluated.

Animals: The study included 35 dogs which are 16 female and 19 male sexes and different mix breeds who were 6 months old and suffering from CPVI with SEVS & SEPS. Clinical symptoms (vomiting and/or bloody diarrhoea) and test findings (leukopenia) were consistent with CPVI in all dogs with SEVS & SEPS. None of the dogs had been inoculated with a commercial parvovirus vaccine, and all SNAP CPV antigen tests (IDEXX, SNAPshot Dx) were positive. SBP, MAP, lactate, SpO2, BE and HR, LVSD, LVDD, and cardiac troponin I (cTnI) were measured at admission, as well as after one bolus fluid therapy, low dosage vasoactive medication, double dose vasoactive medication, and recurrent double dose vasoactive medication for dogs with SEVS & SEPS. The detection of systemic inflammatory response syndrome (SIRS) and SEVS & SEPS were the inclusion criteria in the dogs with SEVS & SEPS. Any dogs who had previously undergone other therapies and had congenital heart disease or inadequate echocardiographic images/measurements were eliminated.

Definitions of sepsis: To diagnose sepsis, researchers used SIRS and a positive SNAP CPV antigen test (IDEXX, SNAPshot Dx). Case definitions for SIRS in adults (25) and children (18), as well as established reference intervals for dogs (38) were used to determine whether the dogs had SIRS if they had two or more of the following abnormalities: leukopenia (5000 cells/L), hypothermia or hyperthermia (reference interval; 37.5–39.3 °C), tachycardia (>120 beats per minute), or tachypnea (>35 breaths per minute). Severe sepsis is characterized as sepsis accompanied by organ failure, hypoperfusion, or hypotension. Septic shock was described as a case of severe sepsis that did not respond to a single bolus of IV fluid. Hypotension was defined as SBP 90 mmHg and MBP 65 mmHg (34).

Blood pressure measurement: An oscillometric approach was used to assess systolic blood pressure and MAP indirectly (Compact 7, Medical Econet, Oberhausen, Germany). After the patient had had time to acclimate to their circumstances, BP readings were taken in a quiet, secluded area. The cuff was 40 percent of the circumference of the limb. The dogs were confined to a lateral recumbency position. The initial reading was eliminated, and the average of the next five readings was calculated (11).

Echocardiographic evaluation: An echocardiograph was used to perform transthoracic echocardiography (TTE) in the ICU (GE LOGIQ e R7 CONSOLE). All dogs with SEVS & SEPS had comprehensive PW-TDI echocardiographic exams (apical 4-chamber view). All measures were taken from three cardiac cycles in dogs with sinus rhythm, and mean values were determined. Heart rate (HR) was assessed using a base-apex or lead II electrocardiogram at the same time as echocardiographic procedures (40). Two investigators (ICU staff) performed all echocardiographic measurements, and one investigator (non-certified cardiologists) analysed the videotape recorded examinations. The systolic (S') and early diastolic (E') velocities of the PW-TDI septal mitral annulus were determined (8). When the S' was less than 7.5 cm/s, LVSD was diagnosed. When the E' was less than 8 cm/s, LVDD was determined (15).

Pulse oximetry: The clamp probe of a pulse oximeter (Compact 7, Medical Econet, Germany) was placed on the dog's buccal mucosa to detect tissue oxygenation. Pulse oximetry was used to determine the buccal mucosa's SpO2.

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Blood gas analysis and cTnI: Peripheral venous blood samples were collected for blood gas analysis to determine BE and lactate concentrations (GEM Premier Plus). Commercial ELISA tests (MyBioSource, USA) were used to measure cTnI.

Treatment Protocol: We used a standardized EGDT procedure that included fluid therapy, vasoactive medicine, antimicrobial therapy, blood products, anticoagulants, venous thromboembolism prophylaxis, stress ulcer prophylaxis, and nourishment after taking blood samples and measurements. Lactate, glucose, SBP and MBP, SpO2, BE, and ECG recordings were used to guide shock treatment in dogs with SEVS & SEPS, according to current understanding of EGDT protocols for SEVS & SEPS (31).

For the initial 30 minutes of treatment, intravenous fluid delivery was started with a 0.9 % NaCl solution at 30 ml/kg BW. SBP, MAP, and S' were assessed after one bolus of fluid therapy, and NE and DT administration were used, respectively. The effect on clinical indicators (e.g., HR, respiration rate, mucous membrane colour, and pulse quality) was then examined after multiple (up to four) boluses of 10–20 ml/kg were given over 10–15 minutes. Following that, 0.9 percent NaCl was given at a rate of 20 ml/kg/day as fluid maintenance therapy. Intravascular volume, hypoglycaemia, and continuing fluid losses were all monitored in the dogs. If hypoglycaemia was observed, dextrose (5%) was added to the intravenous fluids. A colloid solution (hydroxyethyl starch 6 percent, 10 ml/kg/h, i.v) was administered to patients with LVDD to prevent the establishment of positive fluid balance because of the administration of massive volumes of crystalloid solution.

Ceftriaxone (Novosef®, Sanofi İlaç San. ve Tic. A.Ş., Türkiye, 30 mg/kg BW, i.v, every 8 h), enrofloxacin (Enrocure®, Türkiye, 5 mg/kg BW, i.m, every 12 h), metronidazole (Flagyl®, Aventis Pharma, Türkiye, 10 mg/kg BW, i.vevery 12 h), and meloxicam (Bavet Meloxicam®, Bavet İlaç San., Türkiye, 0.1 mg/kg BW, i.v, 24 h) were used as wide-spectrum antibiotics and anti-inflammatory treatments, respectively.

A nasal oxygen mask was used to administer oxygen (100 ml/kg, BW/min) to dogs with a SpO2 of less than 90%. Metoclopramide was given if needed for vomiting or nausea. Pantoprazole (Protaz®, HTA, Türkiye, 1 mg/kg BW, i.v, every 24 h), was applied daily for stress ulcer prophylaxis. Potassium was supplemented if K+ levels were below 3.5 mEq/l.

To provide external warmth, hypothermic dogs were placed under an infrared heat lamp. Dalteparin (FRAGMIN®, Pfizer, Belgium) was given as a venous thromboembolism prophylaxis dose of 100 IU/kg s.c every 8 to 12 hours. In dogs with a haematocrit of less than 20%, fresh complete blood was given at a dosage of 20 ml/kg (33).

Vasoactive therapy: Despite receiving a single bolus of fluid treatment, hypotensive dogs were given vasopressor therapy by delivering NE (1.5 µg/kg/min in 0.9 percent NaCl solution) without a loading dose. If an acceptable clinical reply was not obtained after 2 hours, the dosage of NE administration in 0.9 percent NaCl solution was increased to 3.0 µg/kg/min (22, 41). DT was administered to dogs with LVS'<7.5 cm/s at a constant infusion for 2 h of 5 µg/kg/min in 0.9% NaCl solution. In cases which do not result in an adequate clinical response in 2 h a doubling of the DT infusion (10 µg/kg/min) was administered (40, 41).

Small amounts of food were introduced once feeding did not majorly exacerbate the levels of vomiting. We administered parenteral nutrition if prolonged anorexia occurred. For this reason, dogs received a 10 ml/kg i.v infusion of a solution (Duphalyte® solution, Zoetis, London, United Kingdom) each day. Before infusion, intravenous fluids were warmed to 38°C in a hot water bath.

Patient follow-up: The dogs were observed for 28 days to see if they died. According to their treatment response, the dogs were classified into two groups: survivors (Sv) and non-survivors (non-Sv). Dogs alive at discharge were considered Sv, and dogs that died were considered non-Sv.

Statistical analysis: Statistical software was used to analyse the data (SPSS 25.00 for windows). The Shapiro-Wilk test was done to see if the variables had a normal distribution. The independent samples t-test was used to examine the parametric data, and the results were provided as mean standard deviation (SD). The Mann Whitney U test was used to examine non-parametric data, and the median (min/max) was provided. Fisher Exact test was used to examine categorical variables. For NE and DT - treated pups, a Kaplan–Meier analysis and log-rank tests were utilized to assess survival probability. $P<0.05$ was used to determine statistical significance.

Results

Animals: Age, body weight, and sex differences were not significantly the distinction between Sv and non-Sv groups. The differences in macrovascular (SBP and MAP) and microvascular parameters (lactate, SpO2, BE), S' and E', and DT applications were also not significantly distinction between the two groups (Tables 1 and 2). The only the distinction between the Sv and non-Sv groups was the use of NE applications (Table 2).

Table 1. Base excess (mean ± standard deviation), lactate (mean ± standard deviation), SpO2 (%) (mean ± standard deviation), SBP and MAP (mean ± standard deviation), norepinephrine application, and Dobutamine application compared between survivors (n = 25) and non-survivors (n = 10) among the dogs treated for SS/SS.

Parameters	Non-Survivor	Survivor	P value
SBP (mmHg)	115.18±33.69	110.62±29.66	0.705
MAP (mmHg)	81.18±28.72	82.54±21.60	0.891
SpO_2 (%)	64 (19-91)	64.50 (20-99)	0.563
Lactate (mmol/L)	1.60 (0.70-31.00)	3.37 (0.80-26.00)	0.124
BE (mmol/L)	-5.60 (-17.90-1.90)	-6.35 (-17.90-12.80)	0.713
Norepinephrine (n)	**8 (72%)**	**2 (8%)**	**0.000**
Dobutamine (n)	1 (%9)	4 (%16)	0.491

SpO_2: oxygen saturation, SBP: systolic blood pressure, MBP: mean blood pressure, BE: base excess

Table 2. Heart rate (mean ± standard deviation), echocardiographic parameters (mean ± standard deviation) and cTnI (mid) compared between survivors (n = 25) and non-survivors (n = 10) among dogs treated for SS/SS.

Parameters	Non-Survivor	Survivor	P value
HR (bpm)	185.18±30.19	189.83±29.42	0.675
cTnI(pg/ml)	251 (57-920)	414 (57-1000)	0.211
S' (cm/s)	8.65±2.35	9.54±2.88	0.357
E' (cm/s)	6.99±2.31	5.41±1.36	0.054

HR: heart rate, cTnI: cardiac troponin I, S': septal mitral annulus systolic, E': early diastolic

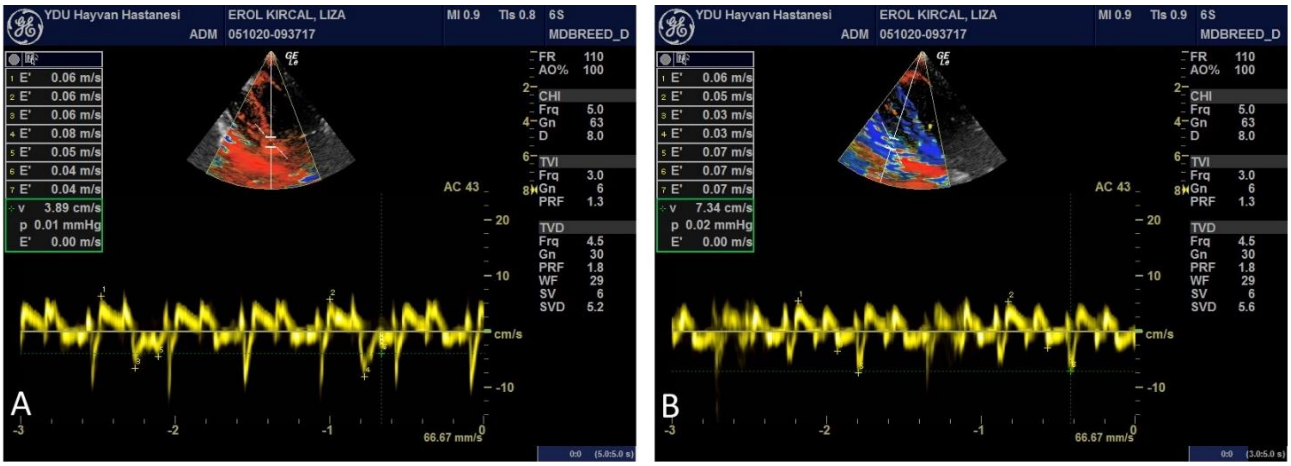

Figure 1. Left apical 4 chamber view in a dog for measurement of PW – Tissue Doppler Imagining mitral annulus systolic (Sm), early (Em) and late (Am) diastolic pick velocities. The Sm is less than 10cm/s and Em is less than 8 cm/s (A). No change was observed in the echo data and they died despite the administration of norepinephrine (B).

Systolic and diastolic dysfunctions: At the time of admission, 26 of the SEVS & SEPS dogs (74 percent) at least one type of myocardial dysfunction was present. LVSD (LVS' 7.5 cm/s) and LVDD (E' 8 cm/s) were both present in 7 (20%) of the participants, while both forms of dysfunction were present in 5 (14%), while 19 (60%) patients had both types of dysfunction. Isolated LVDD (E' <8cm/s) (Table 2) was more common type of dysfunction (16/35 46%). Neither LVSD nor LVDD were found in 9 dogs with SEVS & SEPS. A total of eight dogs with LVDD and one dog with LVSD died.

Response to vasoactive treatment: The NE group. At the time of admission, 12 of 35 dogs with SEVS & SEPS had hypotension. One SEVS & SEPS hypotensive dog had normal blood pressure after one bolus of fluid treatment. One hypotensive dog with SEVS & SEPS died during one bolus of fluid therapy. 10 of which still had low BP after one bolus fluid therapy (septic shock). 2 of which, were in the survivor group, responded to treatment with NE at the dose of 1.5 µg/kg/min. Five hypotensive dogs with SEVS & SEPS, which were in the non-Sv group, not responded to treatment with NE at the dose of 3.0 µg/kg/min (Figure 1).

The rest of 3 dogs with SEVS & SEPS, which were in the non-Sv group, not responded to treatment with repeated NE at the dose of 3.0 µg/kg/min. After one bolus of fluid therapy, the average rate of Sv for the NE group was 91 percent, according to Kaplan-Meier analysis. The average rate of the Sv was 82% after 1.5 µg/kg/min. The average rate of the Sv was 35% after 3.0 µg/kg/min. The average rate of the Sv was 0% after repeated 3.0 µg/kg/ (P<0.05) (Figure 2).

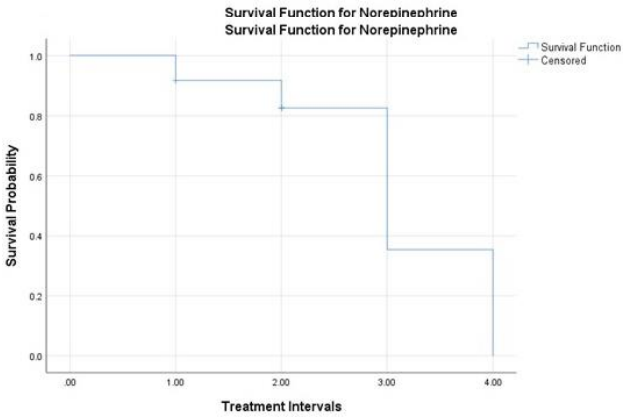

Figure 2. Kaplan-Meier's analysis showed that the average rate of the Sv was 91% after one bolus of fluid therapy for the NE group. The average rate of the Sv was 82% after 1.5 µg/kg/min. The average rate of the Sv was 35% after 3.0 µg/kg/min. The average rate of the Sv was 0% after repeated 3.0 µg/kg/ (p<0.05).
Description of treatment intervals for Norepinephrine
0: Hypotensive
1: One bolus fluid
2: Administration of norepinephrine 1.5 µg/kg/min in 2 h
3: Administration of norepinephrine 3.0 µg/kg/min, CRI in 24h
4: Administration of repeated norepinephrine 3.0 µg/kg/min, CRI in 24h

More non-Sv received a greater amount of NE, while more Sv received a smaller amount of NE.3 of 12 (25%) Sv received either no (one bolus fluid) or a single NE (1.5 µg/kg/min), while 8 of 12 (67%) non-Sv received at least 1 NE (P<0.000). The only dogs that received more than 1 NE consisted of 7 non-Sv.

DT group, 7 of 35 dogs with SEVS & SEPS had systolic dysfunction (S' 7.5 cm/s) at admission. Two of which responded to the one bolus fluid therapy and had normal systolic function (S' ≥ 7.5 cm/s). 5 of which still had S' < 7.5 cm/s after one bolus fluid therapy. All the dogs did not respond to the treatment with DT at the dose of 5µg/kg/min. 1 dog died despite the treatment with DT at the dose of 10 µg/kg/min. 1 dog responded to the treatment with DT at the dose of 10 µg/kg/min (Figure 3). 3 dogs did not respond to the treatment with DT at the dose of 10 µg/kg/min. The rest of 3 dogs with SEVS & SEPS, which were in the survivor group, responded to treatment with repeated DT at the dose of 10 µg/kg/min. The average rate of Sv for the DT group was 100 percent following one bolus of fluid treatment, according to Kaplan-Meier analysis. The average rate of the Sv was 100% after 5 µg/kg/min. The average rate of the Sv was 80% after 10 µg/kg/min. The average rate of the Sv was 100% after repeated 10 µg/kg/min (P<0.05) (Figure 4).

In contrast, more Sv received a greater amount of DT applications. While 1 (20%) non-Sv received 2 DT (5 µg/kg/min in 2 h + 10 µg/kg/min in 24 h), the 4 Sv dogs received 3 DT (5 µg/kg/min in 2 h, 10 µg/kg/min in 24 h, repeated 10 µg/kg/min in 24 h).

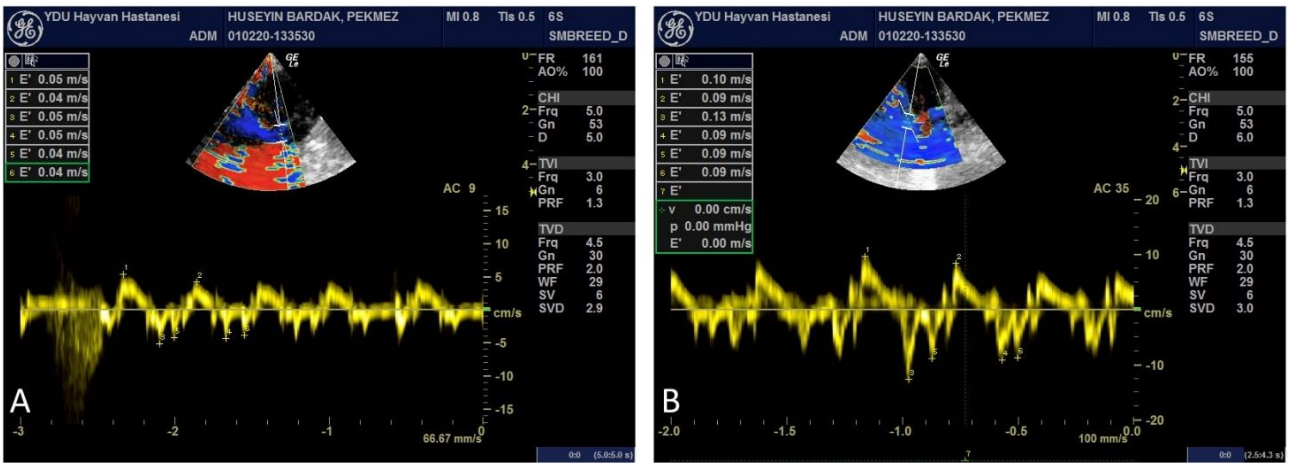

Figure 3. Left apical 4 chamber view in a dog for measurement of PW – (A). Tissue Doppler Imagining mitral annulus systolic (Sm), early (Em) and late (Am) diastolic pick velocities. (B) Normal systolic function was observed (S' ≥ 7.5 cm/s) responded to the treatment with dobutamine.

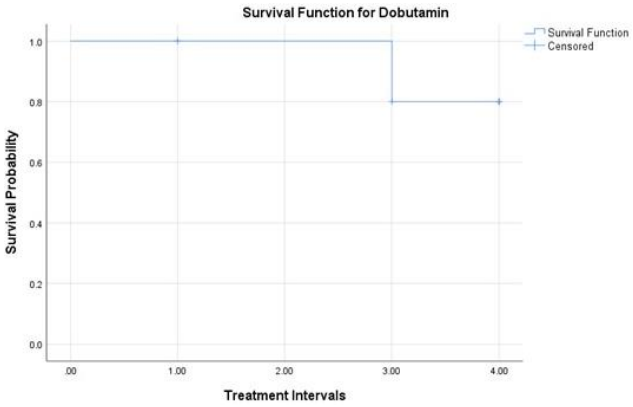

Figure 4. Kaplan-Meier's analysis showed that the average rate of the Sv was 100% after one bolus of fluid therapy for the DT group. The average rate of the Sv was 100% after 5 µg/kg/min. The average rate of the Sv was 80% after 10 µg/kg/min. The average rate of the Sv was 100% after repeated 10 µg/kg/min (P<0.05).
Description of treatment intervals for Dobutamine
0: Hypotensive
1: One bolus fluid
2: Administration of dobutamine 5 µg/kg/min, CRI in 2h
3: Administration of dobutamine 10 µg/kg/min in 24 h
4: Administration of repeated dobutamine 10 µg/kg/min in 24 h.

Mortality rate: Thirty-five dogs matched the criteria for inclusion, with 26 (74%) of those with SEVS & SEPS surviving and being discharged from the ICU (Sv). Nine individuals died, for a total mortality rate of 25.7 percent (non-Sv). The canines with LVDD (E'8 cm/s) (8 cases) and those who had NE applications (8 cases) had the lowest survival rates.

Discussion and Conclusion

Sepsis is a complex clinical syndrome, and can be complicated by tissue hypoperfusion. Septic shock is a life-threatening condition that arises when sepsis causes life-threatening hypotension (25). Septic shock death rates in dogs and cats have been found to range from 20% to 68 percent (37). LVSD and LVDD both developed in humans with SEVS & SEPS, according to studies (9). LVSD was present at admission in 7 of 35 (20%) dogs with SEVS & SEPS in our research. Two of them responded to the one bolus fluid therapy and had a normal systolic function. 1 dog died despite the treatment with DT. The rest of 4 dogs with SEVS & SEPS, which were in the Sv group, responded to treatment with DT. The patients with LVSD (7 cases) had a better survival outcome. Very high prevalence rates (60–84%) of LVDD with increased mortality have also been found in human with sepsis (24). M. E. Ince et al. (22) found that the E', an LVDD index, had the best sensitivity and specificity to distinguish Sv and non-Sv dogs, with values of 100 percent (95 percent CI: 55.2–100) and 100 percent (95 percent CI: 78.9–100), respectively, at an ideal cut-off point of 6.50. Therefore, LVDD was a good independent outcome predictor. Although there was no statistical difference (P <0.054) for E' between Sv and non-Sv dogs with SEVS & SEPS in this study, isolated LVDD (Table 2) was more common (16/35 46%), and the patients with LVDD (8 cases) had the worse survival outcome. The mitral annulus E' can be used with PW-TDI to properly quantify LV relaxation (40). E' does not alter much in response to diverse loading situations, according to several research (42). The lateral E'<10 and septal E'<8 cm/s have been observed to be highly indicative of LVDD and increased LA pressures (LAP) (17). By inhibiting LV dilation, LVDD may prevent stroke volume augmentation in response to fluid load. Lung congestion may be exacerbated by LVDD. Pulmonary hypertension and RV dysfunction might develop as a result of non-cardiogenic pulmonary oedema.

The EGDT protocols have been developed to normalise irregular measurable indices of tissue perfusion and oxygenation (32). There are two broad groups of monitoring parameters: macrovascular and microvascular. Macrovascular parameters, which are also referred to as upstream parameters, deal with systemic measures of cardiopulmonary status, such as SBP and MAP, central venous pressure, and urine output. Microvascular parameters, which are also referred to as the downstream parameters, are related to tissue oxygenation and include lactate and lactate clearance, ScvO2, and BE (30). Therefore, monitoring both macrocirculation and microcirculation parameters provide a broader picture which is required to enable informed clinical decisions (11). Our macrovascular (SBP and MAP) and microvascular (lactate, SpO2, and BE) measures did not differ significantly between the Sv and non-Sv groups (Table 1). This means that SBP and MAP, lactate concentration, SpO2, and BE were not correlated with the severity of dogs with SEVS & SEPS. These parameters can be useful, but flawed as a goal for the treatment of shock (4).

Hypotension, on the other hand, was present as a macrovascular measure in 12 of 35 dogs with SEVS & SEPS at admission. 8 of them did not respond to fluid resuscitation and NE applications. All of them were in the non-Sv group. A MAP of less than 60 to 65 mm Hg is considered hypotension (38). Restoration of systemic blood pressure to a MAP between 65-70 mm Hg is a good initial goal in EGDT (2). Hypotension is a common consequence in those suffering from septic shock, and it can be caused by hypovolemia, low CO, or improper vasodilation (11). Between Sv and non-Sv groups, there was no difference in SBP and MAP (Table 1). Hypotension could be explained in our investigation by the emergence of distributive shock. In sepsis or SIRS, the release of inflammatory mediators causes distributed shock. Septic shock is a type of shock that is classified as

a subtype of distributive shock (22). Different processes, including as hypovolemia, vasoplegia, and septic cardiomyopathy, are frequently found simultaneously or separately in this complex process.

Vasopressors raise circulatory system tone and, as a result, MAP (26, 37). If patients do not respond to fluid resuscitation, vasopressor medications are used as the next step in treating hypotension (14). As suggested by the sepsis surviving campaign (SSC), In a patient with septic shock, the initial vasopressor that should be given is NE (31). NE and its vasoconstricting effects move blood from the general circulation to increase the preload. This is essential in the early stages of septic shock because it can result in a positive fluid balance (29). In the early stages of septic shock, a MAP of >65 mmHg is the resuscitation aim to enhance the perfusion of important organs such as the brain and kidney (23, 31). NE has been used in dogs with endotoxic shock, (5) septic shock, (27) tamponade-induced stagnant hypoxia, (44) and haemorrhagic shock (16). Despite one bolus of fluid treatment, NE was given to the 10 hypotensive dogs in our trial. Dogs being treated with NE generally had no increase in their blood pressure, with 2 of 10 dogs achieving normotension. Persistent hypotension in dogs and cats has previously been associated with a poor outcome (36). All of the dogs that were perceived to be fluid tolerant in this study received one bolus of fluid therapy consisting of crystalloids before vasopressor initiation. Blood pressure measurements were consistently low in the non-Sv group despite fluid resuscitation. As in humans, it is difficult to determine at times if an adequate fluid challenge has been administered before starting vasopressors, (6) but there is growing evidence that a positive fluid balance is linked to an increase in mortality (10). Whether the non-Sv dogs died due to positive fluid balance or hypotension is unknown in this study.

In humans, unresolved hypotension and inadequate tissue perfusion are recognized as precursors to MODS and death and are important and tangible targets for intervention (3, 39). Evidence-based guidelines advocate the use of vasopressors in hypotensive humans who are adequately volume resuscitated and are therefore considered to have refractory hypotension, (31) but evidence-based guidelines do not exist for dogs surrounding the optimal use of vasopressors. We used NE as a vasopressor in dogs with SEVS & SEPS. The only difference between Sv and non-Sv was the use of NE (Table 1). 10 dogs with SEVS & SEPS were started on NE, 8 of which did not respond to NE applications and all of them were in the non-Sv group. This could be the result of distributive shock, vasopressor failure, hypotension, MODS, and LVDD or a combination of all these reasons. In this study, the patients received NE applications (8 cases) that had a worse survival outcome.

In dogs with SEVS & SEPS, the use of multiple vasopressors is linked to a poor prognosis. It has been found that dogs with septic peritonitis who were hypotensive due to surgery and dogs who received more than one vasopressor were less likely to survive (7). This implies that dogs with SEVS & SEPS requiring vasopressor therapy had higher levels of mortality. In addition to the maladaptive inflammatory responses that occur during sepsis and their impact on cardiovascular tone, (19) there may be a reduced reactivity due to long-term use of catecholamine medications and consequent down regulation of α-adrenergic receptors in the arterial smooth muscle (43). In our study, a proportionally higher number of non-Sv received a greater amount of NE, whilst more survivors received a smaller amount of NE. Kaplan-Meier's analysis supported this conclusion. The survivorship rate was 91% after one bolus of fluid therapy for the NE group. This rate was 82% after 1.5 µg/kg/min in 0.9% NaCl solution, CRI in 2h and 35% after 3.0 µg/kg/min in 0.9% NaCl solution, CRI in 24h; with 0% after repeated 3.0 µg/kg/min in 0.9% NaCl solution, CRI in 24h ($P<0.05$) (Figure 2).

Increased oxygen delivery may have enhanced tissue perfusion with inotropic treatment (14, 19). DT is used as part of standard care in clinical trials of EGDT (28). In dogs, the use of DT has been infrequently reported. In dogs with tamponade-induced stationary hypoxia, it enhanced oxygen availability to the tissues (44). In a dog model of endotoxic shock, Bakker and Vincent (5) reported that DT had a favourable effect on oxygen transport and consumption. In our study, although it was not significantly different for DT use between Sv and non-Sv, a larger number Sv received a greater amount of DT applications. Kaplan-Meier's analysis support this conclusion. The average rate of the survivor was 100% after one bolus of fluid therapy for the DT group. The average rate of the survivor was 100% after 5 µg/kg/min. The average rate of the survivor was 80% after 10 µg/kg/min. The average rate of the survivor was 100% after repeated 10 µg/kg/min ($P<0.05$) (Figure 2). We may suggest that DT can be used in cases of LVSD at the dose of 10 µg/kg/min.

In our research, 26 of 35 dogs with SEVS & SEPS survived and were released from the ICU (Sv). Nine patients died, resulting in a 25.7 percent total mortality rate (non-Sv). This relatively low mortality rate could be the result of the use of EGDT protocols in our study.

There is one more flaw in our research. The study's sample size was limited, but it was comparable to early echocardiographic studies in septic individuals. The specific rationale behind vasoactive decisions was to only use NE and DT, which may have affected the overall outcome.

In dogs with SEVS & SEPS, tissue perfusion is inadequate. Therefore, oxygen and nutrient delivery is impaired. EGDT is a protocol for the monitoring and management of hemodynamic in dogs with SEVS & SEPS. In dogs with SEVS & SEPS, therapy with NE and DT can be started if fluid administration fails to restore appropriate arterial pressure and organ perfusion. NE and DT can be used for patients with persistent hypotension and LVSD, respectively. In dogs with SEVS & SEPS, the ultimate goals of EGDT procedures are to increase effective tissue perfusion and regulate cellular metabolism. DT and NE in dogs with SEVS & SEPS can be used to improve outcomes. The use of NE applications was the only the distinction between the Sv and non-Sv groups. The dogs with an LVDD and the dogs received NE applications had the worse survival outcome. Veterinary medicine's evidence foundation for the use of DT and NE in dogs with SEVS & SEPS is inadequate, despite their frequent use. In order to produce evidence-based guidelines for dogs, more study is required.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

KT, conceptualization, methodology, writing-review & editing. AN, HS, ME and MEI, writing-review & editing.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

Ethics committee approval for this study was obtained from Local Ethics Committee of the Near East University (permit number: 2019/03).

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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The effects of physical forms of corn and forage source on growth performance, blood parameters and ruminal fermentation of Holstein calves

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ABSTRACT

This investigation's purpose was the evaluation the effects of physical forms of corn and forage sources on growth performance, blood parameters, and ruminal fermentation of Holstein calves. Forty-eight Holstein calves (38.2 ± 1.2 kg of BW) were used in a completely randomized design with 4 treatments and 12 replications for 65 days. The treatments were: 1) Basal diet + mashed form corn + wheat straw (MCWS), 2) Basal diet + flacked form corn + wheat straw (FCWS), 3) Basal diet + mashed form corn+ Sugarcane bagasse (MCSB) and 4) Basal diet + flacked form corn + sugarcane bagasse (FCSB). Final body weight and daily weight gain were affected significantly by the forage (F) factor whereas no effect from C (corn physical forms) and C×F interaction were observed. Skeletal growth parameters were not affected by C, F, and F×C factors. The trial factors did not affect triglyceride, urea and BHBA levels. Glucose level and urea were affected by C and F factors. At 30 and 60 days, calves fed on FCWS had the highest glucose. Also, calves fed the FCSB diet presented higher urea at 30 and 60 days. Acetic acid was affected significantly by C, F, and C×F interaction. Calves fed the MCWS diet had the maximum concentration of acetic acid. At 30 days, propionic acid was affected by C, F, and C×F interaction. Valeric acid was affected by C and F effect at 30 days. While for valeric acid no differences were observed at 60 days.

Introduction

The physical and metabolic development of the rumen facilitates the shift from the pre-ruminant phase to the adult ruminant (9, 21). Pre-weaning growth is a difficult progression that is influenced by nutritional factors, nutritional plans, and feed administration (4). Differences in grain fermentability (20, 23), grain processing (29), forage level (25), and forage kind or source (7, 23) can affect growth performance in calves. Feed processing and its physical shape affect the palatability and feed intake in suckling calves (17, 36), and consequently, the physical and metabolic growth of the rumen seems to be essential (4). Also, the processing of cereal grains along with methods used in feed manufacturing influences the ruminal and intestinal digestibility of feed and starch (14) and performance (12, 20) of both young and adult cattle.

Providing a ground starter feed along with forage is another way to inhibit the abnormal growth of the ruminal epithelium without the use of a textured or pelleted starter feed, which ultimately reduces the production cost for the dairy farming system (6). Some reports support the use of forage sources in improving the growth performance of suckling calves (7, 21). Studies have revealed that feeding forage in pre-weaning calves improves feed intake, daily weight gain, and ruminal fermentation (8, 24). Stimulation of the rumen muscle layer (23, 35) stimulates rumination

(13) and reduces behavioral problems (31). Probably in contradiction the results of forage feeding in suckling calves may be related to foraging type and quality, starch source, feed processing, and physical form of starter. The hypothesis of this study was whether the use of forage can reduce the negative effect of corn shape in dairy calves or are ineffective. Therefore, this study project was designed and implemented to explain the effect of corn grain treatment with different types of fiber sources on growth performance, blood parameters, and ruminal fermentation of Holstein calves.

Materials and Methods

Forty-eight neonatal Holstein calves (mean birth weight 38 ± 3 kg; age 1–3 days; on July and August 2018) were carefully chosen from the Peghah dairy herd to evaluate the influence of corn grain processing with different fiber sources on growth performance, blood parameters, and ruminal fermentation. Trial treatments were: 1) Basal diet + mashed form corn + wheat straw (MCWS), 2) Basal diet + flacked form corn + wheat straw (FCWS), 3) Basal diet + mashed form corn+ sugarcane bagasse (MCSB) and 4) Basal diet + flacked form corn + sugarcane bagasse (FCSB). In the first 24 hours next birth, calves were detached from their maternal cows and allotted to the experimental groups randomly from day 4 of life until the end of the experiment (days 70 of life). Calves were assigned randomly among the groups based on their age and birth weight to get similar average weight and age among the treatments. Calves were drunk 4 L of colostrum twice in the first 8 hours of birth, the rest of the colostrum was fed in 2 days according to the 10% of body weight. Milk whole for calves to intake was provided twice a day at 8:00 and 18:00 by plastic bucket and controlled to completely consuming by calves. On day 4, water and starter were offered adlibitum to the calves. Dried alfalfa was offered to the calves from day 20 of life as 10 percent of the starter diet. Investigational diets and their chemical composition are presented in Table 1.

Sampling and analysis

Chemical composition: Before chemical analysis, feed samples for crude protein, ether extract, ash (1), neutral detergent fiber, and acid detergent fiber (37) were air-dried at 60 °C and ground with a 1 mm sieve.

Table 1. The components of trial diets (starter feed) and their chemical composition (Dry matter %).

Items	Mashed corn		Flacked corn	
	WS	SB	WS	SB
Mashed form corn	62.4	62.4	-	-
Flacked form corn	-	-	62	62
Soybean meal	27.6	27.6	28	28
Wheat straw	5	-	5	-
Sugarcane bagasse	-	5	-	5
Pre-mixed				
Salt	0.5	0.5	0.5	0.5
Minerals[1]	1	1	1	1
Sodium bicarbonate	1.5	1.5	1.5	1.5
Vitamin[2]	1	1	1	1
Bentonite	1	1	1	1
Chemical mixture (% of DM)				
Dry matter (% of as fed)	92.2	92	92.2	92
NEm (Mcal/kg)	2.20	2.21	2.20	2.21
NEg (Mcal/kg)	1.67	1.67	1.68	1.68
Crude protein	19.8	20	19.8	20
Ether extract	2.26	2.27	2.26	2.28
Neutral detergent fiber	11.2	11.5	11.2	11.5
Acid detergent fibre	6.31	6.33	6.32	6.34
Calcium	0.62	0.68	0.63	0.67
Phosphorus	0.50	0.55	0.51	0.54

WS=wheat straw, SB= sugarcane bagasse.
NEm = net energy for maintenance; NEg = net energy for growth.
[1]Mineral premix provided per kilogram of diet: Cu, 3300 mg/kg; Fe, 100 mg; Zn, 16 500 mg/kg; Mn, 9000 mg; I, 120 mg/kg; Co, 90 mg/kg; Se, 90 mg/kg.
[2]Vitamin premix provided per kilogram of diet: vitamin A, 200 000 IU; vitamin B, 300 000 IU; vitamin E, 10 000 IU; vitamin K, 2 mg; antioxidant 1000 mg/kg.

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Growth performance: Throughout the experiment, the calves were weighed once every two weeks in the morning individually without prior deprivation of feed and water, and weight change was calculated by subtraction method. During the experiment, feed intake was measured daily by the difference between feed offered and feed refused.

Skeleton growth: To quantity skeletal growth, the parameters of the longitudinal line from the shoulder to rump as body length, the longitudinal line from the base of the rear feet to hook bones as the height of the withers, the longitudinal line from the base of the front legs to the withers for the height of the withers and the circumference of the chest as heart girth were measured at weaning time (56 days) and end of study (70 days) according to Nemati et al. (25).

Blood parameters: Blood samples were taken from the jugular vein, on days 30 and 65 of experiment 3 to 4 h after morning feeding, into two separate tubes (one with heparin and the other with no anticoagulant). Plasma and serum were collected by centrifuging the blood samples at 2000×g for 15 min at 4°C. The samples were kept at −80°C until analysis. The obtained plasma samples were analysed for glucose, triglyceride, urea, and beta-hydroxybutyric acid (BHBA) colorimetrically using commercial kits (imported by Pars Azmoon Co, Tehran, Iran from Hitachi 917, Modular P, Tokyo, Japan, and Randox, Antrim, UK).

No severe disease incidence occurred during the experiment. No mortality was observed among the experimental groups.

Ruminal fermentation: Rumen fluid sampling was performed on day 35 and at the end of the study period (70 days), at 3 hours after feeding in the morning by stomach tube technique. Rumen pH was measured immediately using a hand-held pH meter (HI 8314 membrane pH meter, Hanna Instruments, Villaf+ranca, Italy). For acidification of the rumen fluid, an aliquot (4 mL) was used with 1 mL of 25% meta-phosphoric acid and kept (−20 °C) until analysis for volatile fatty acids (VFA) by gas chromatography (0.25 × 0.32, 0.3 mm i.d. fused silica capillary, model no. CP- 9002 Vulcanusweg 259 a.m., Chrompack, Delft, the Netherlands) (18).

Statistical analysis: Statistical analyses were conducted using PROC MIXED (34) with the individual calf as the experimental. Given that growth performance was over time as repeated data was analyzed by relationship:

$$Y_{ij} = \mu + A_i + B_j + (A \times B)_{ij} + \beta(X_i - \bar{X}) + \varepsilon_{ij}$$

Where Y_{ij} is the dependent variable; μ is the overall mean, A_i is the effect of corn physical form (mashed vs. flacked), B_j is the effect of forage (hay vs. sugarcane bagasse), $(A\times B)_{ij}$ is the interaction between corn physical form and forage, $\beta(X_i-\bar{X})$ is the covariate variable (used only for BW with initial BW as a covariate) and εijk is the overall error term.

Results

Results related to the effects of corn grain processing with different fiber sources on the growth performance of Holstein calves are shown in Table 2. The source and type of forage (F) affected the growth performance of the calves ($P<0.05$). Whereas no effect from the C effect and an interaction effect between F and C groups were observed. Calves consuming the MCSB diet had a higher final body weight than those consuming FCWS and FCSB ($P<0.05$). The highest feed intake (the second months and the total period of the experimental) compared with other groups belonged to calves fed MCSB diet ($P<0.05$). Calves consuming the MCSB diet had a higher average daily gain during the first months and total period than those consuming FCWS and FCSB ($P<0.05$). So, this difference might be the result of the forage type whereas no effect from C factor was observed. An interaction was observed between the physical form of corn and the forage source for FCR during the second month ($P<0.05$). FCR was numerically improved for calves FCSB-fed diets than that other group in the second month, but this effect was not significant ($P>0.05$).

Results related to the effects of corn grain processing with different fiber sources on the performance skeleton of Holstein calves are shown in Table 3. The parameters of body length, hip and withers height, and heart circumference were no differences between treatments at weaning (d 56) or the final trial (d 65).

Results related to the effects of corn grain processing with different fiber sources on blood parameters of Holstein calves are shown in Table 4. Trial factors did not affect the concentration of triglyceride, urea, and beta-hydroxybutyric acid (BHBA). But the concentration of glucose was affected by F factors for 30 days. So, Calves fed on FCWS had the highest glucose, and calves fed on MCSB had the minimum value.

The results of volatile fatty acids (acetate, propionate, butyrate, and valerate) are presented in Table 5. Acetic acid and butyric acid were affected significantly by C and F treatment ($P<0.05$). Furthermore, there was an important effect ($P<0.05$) of C×F interaction on acetate of concentration at one and second months ($P<0.05$). But the concentration of butyrate was not affected by the C×F interaction effect. During the first month, propionic acid was affected by C, F, and C×F interaction effect ($P<0.05$). While, in the second month, the C factor and C×F interaction did not affect the concentration of propionate. At 30 days, the concentration of valeric acid was affected by C and F effect. But, no differences were observed in valeric acid among at d 65 of the study.

Table 2. Effects of physical forms of corn (mashed and flaked) and forage source (sugarcane bagasse and wheat straw) on growth performance of dairy calves (*n* = 12 calves per treatment).

	Mashed corn		Flacked corn			P-value		
	WS	SB	WS	SB	SEM	C	F	C×F
Bodyweight (kg)								
Birth	38.18	38.06	38.22	38.19	0.69	0.90	0.90	0.94
Final	83.16^{ab}	87.47^{a}	80.29^{b}	81.68^{b}	1.63	0.15	0.005	0.55
Feed intake (g/d)								
1-30 day	262	284	236	223	23.06	0.85	0.06	0.45
31-60 day	1304^{b}	1533^{a}	1243^{b}	1190^{b}	57.36	0.13	0.001	0.01
1-65 day	769^{b}	870^{a}	717^{b}	713^{b}	23.64	0.06	0.001	0.03
Daily weight gain (g/d)								
1-30 day	307.74^{ab}	359.21^{a}	281.25^{b}	303.30^{ab}	20.74	0.08	0.05	0.47
31-60 day	759.63	845.78	738.21	761.23	36.44	0.32	0.06	0.70
1-65 day	656.5^{ab}	705.25^{a}	601.41^{b}	621.45^{b}	21.33	0.11	0.002	0.50
Feed conversion ratio								
1-30 day	0.89	0.80	0.88	0.75	0.082	0.19	0.72	0.81
31-60 day	1.65	1.84	1.73	1.56	0.084	0.90	0.25	0.03
1-65 day	1.17	1.24	1.22	1.14	0.049	0.91	0.68	0.15

^{abc}Means within a row with different superscripts differ significantly, P<0.05.
WS=wheat straw, SB= sugarcane bagasse, C= physical forms of corn, F= forage source, C×F= the interactions between physical forms of corn and forage source.

Table 3. Effects of physical forms of corn (mashed and flaked) and forage source (sugarcane bagasse and wheat straw) on body measurements of suckling calves (*n* = 12 calves per treatment).

	Mashed corn		Flacked corn			P-value		
	WS	SB	WS	SB	SEM	C	F	C×F
Body length (cm)								
Birth (1 d)	45.08	46.00	45.75	47.00	1.25	0.87	0.15	0.69
At weaning (56 d)	54.25	55.66	57.08	56.33	3.55	0.06	0.60	0.17
Final (65 d)	60.08	59.66	61.41	60.36	3.47	0.30	0.43	0.73
Withers height (cm)								
Birth (1 d)	80.00	80.91	80.58	79.33	6.50	0.89	0.92	0.78
At weaning (56 d)	88.16	89.17	90.29	88.84	1.48	0.54	0.73	0. 07
Final (65 d)	91.16	92.58	93.58	92.50	1.85	0.09	0.76	0.06
Heart girth (cm)								
Birth (1 d)	79.41	80.66	80.50	80.33	1.47	0.47	0.81	0.12
At weaning (56 d)	95.26	96.83	97.13	96.58	4.43	0.33	0.99	0.30
Final (65 d)	100.50	101.04	102.32	101.33	7.22	0.17	0.60	0.26
Hip height (cm)								
Birth (1 d)	81.00	82.41	80.25	80.91	1.88	0.13	0.05	0.76
At weaning (56 d)	91.19	91.25	93.16	91.16	1.94	0.15	0.61	0.10
Final (65 d)	94.40	94.91	96.24	94.44	1.98	0.14	0.33	0.06

^{abc}Means within a row with different superscripts differ significantly, P<0.05.
WS=wheat straw, SB= sugarcane bagasse. C= physical forms of corn, F= forage source, C×F= the interactions between physical forms of corn and forage source.

Table 4. Effects of physical forms of corn (mashed and flaked) and forage source (sugarcane bagasse and wheat straw) on blood parameters of dairy calves (*n* = 12 calves per treatment).

	Mashed corn		Flacked corn			P-value		
	WS	SB	WS	SB	SEM	C	F	C×F
Glucose (µg/dL)								
30 day	97.50^{b}	107.24^{ab}	113.25^{a}	106.25^{ab}	5.80	0.11	0.02	0.54
65 day	73.25	70.25	80.75	76.25	3.50	0.009	0.57	0.07
Triglyceride (µg/dL)								
30 day	19.50	18.25	19.17	18.89	2.51	0.76	0.95	0.87
65 day	26.50	29.05	26.00	28.50	2.38	0.74	0.13	0.98
Urea (g/dL)								
30 day	22.14	21.57	20.00	23.82	1.70	0.004	0.002	0.18
65 day	25.00	26.23	27.37	28.25	1.65	0.38	0.001	0.69
BHBA (µmol/L)								
30 day	0.127	0.132	0.125	0.123	0.03	0.45	0.73	0.53
65 day	0.202	0.212	0.197	0.202	0.01	0.59	0.59	0.85

^{abc}Means within a row with different superscripts differ significantly, P<0.05.
WS=wheat straw, SB= sugarcane bagasse, C= physical forms of corn, F= forage source, C×F= the interactions between physical forms of corn and forage source.

Table 5. Effects of physical forms of corn (mashed and flaked) and forage source (sugarcane bagasse and wheat straw) on the individual volatile fatty acids of the rumen fluid of dairy calves (*n* = 12 calves per treatment).

	Mashed corn		Flacked corn			P-value		
	WS	SB	WS	SB	SEM	C	F	C×F
Acetic acid								
30 day	62.32^{a}	50.26^{b}	50.16^{b}	49.10^{b}	0.82	0.001	0.001	0.001
60 day	75.12^{a}	60.97^{b}	59.42^{b}	58.20^{b}	1.11	0.001	0.001	0.004
Propionic acid								
30 day	22.75^{c}	36.63^{b}	35.30^{b}	41.54^{a}	1.29	0.001	0.001	0.018
60 day	23.21^{b}	34.19^{ab}	27.62^{ab}	38.75^{a}	3.27	0.290	0.009	0.986
Butyric acid								
30 day	10.88^{b}	13.52^{ab}	13.09^{ab}	14.64^{a}	0.76	0.059	0.024	0.491
60 day	8.12^{c}	12.49^{b}	15.20^{a}	16.22^{a}	0.81	0.054	0.095	0.258
Valeric acid								
30 day	1.59^{b}	1.81^{a}	1.37^{c}	1.86^{a}	0.35	0.008	0.015	0.740
60 day	1.21^{b}	0.81^{c}	1.50^{a}	1.01^{b}	0.21	0.291	0.073	0.823
pH								
30 day	5.4	5.3	5.2	5.2	0.6	0.54	0.88	0.89
60 day	5.5	5.3	5.3	5.2	0.5	0.55	0.65	0.82

^{abc}Means within a row with different superscripts differ significantly, P<0.05.
WS=wheat straw, SB= sugarcane bagasse, C= physical forms of corn, F= forage source, C×F= the interactions between physical forms of corn and forage source.

Discussion and Conclusion

In the present study, the relatively low consumption of starter-containing flacked form corn can be related to the texture and physicality of the feed and the low digestibility of feed, reducing the rate of feed passage and increasing the mean retention time (MRT) of feed in the rumen. Rumen volume is one of the factors limiting the voluntary consumption of dry matter. In the ruminal wall, there are sensitive receptors that are stimulated against the stretching and expansion of the ruminoreticulum wall. In this way, they reduce the fermentation and digestion of feed in the rumen, which will eventually lead to a reduction in feed intake (22). In the current study, the forage factor affected body weight and daily weight gain of suckling calves. So, Calves fed the MCSB diet had better growth performance. Thus, the C effect was not significant. The inclusion of MCSB in the diet compared to other nutritional groups caused an increase in feed

intake, as measured in the present study, and this issue in the studies of Bach et al. (3) and Porter et al. (33) approved. Imani et al. (16) Stated that adding forage increases feed intake, but this increase depends on the type of forage, the amount of forage, and the feeding method. So, the use of sugarcane bagasse compared to straw increased feed consumption (Table 2). It has been reported that the use of wheat straw increases the shelf life of feed in the rumen and spends more time on digestion and absorption. In contrast, the rate of ruminal emptying reduced due to the reduction of passage rate and feed consumption decreases (24). According to Khan et al. (21), the physical shape of the starting feed and the type of processing can make changes in the consumption of calf feed. Azimzade et al. (2) reported that the average body weight of calves fed cracked corn and steam-flaked corn was significantly higher than those fed whole corn and ground corn treatments at weaning and days 84. Franklin et al. (10) stated calves that consume pellet starter feed intake are higher compared with the ground starter. The researchers observed a decrease in feed intake in calves receiving flacked from corn and an increase in feed intake in calves receiving mashed versus textured (23, 27). However, Pazoki et al. (29) reported no differences in feed intake of suckling calves when comparing a ground starter feed with a textured starter feed. According to reports, the processing of grain and ingredients of starter feed can alter the rumen fermentation pattern and the nutrients digestibility (15, 28), thereby affecting starter intake and growth performance in dairy calves (29, 30). Regarding the effects of forage on the performance of claves, Imani et al. (16) stated that the consumption of starter feed intake in the ground or pelleted starter-fed calves is improved by supplementation with forage. Also, Mirzaei et al. (23) have observed increased ADG in calves provided with forage, either during the pre-weaning stage. But, these researchers did not observe any interactions between the physical forms of starter and forage provision on feed intake of calves during the pre-weaning periods. Castells et al. (7) described that the including of wheat straw in the diet of calves improved daily weight gain compared with alfalfa. In our study, the increased daily weight gains and feed intake of calves observed with the inclusion of SB in CM compared with WS supplementation were likely due to the sufficient supply of effective fibers to stimulate chewing activity and salivation, resulting in more rumen environment stability.

In the present work, experimental treatments did not affect the skeletal growth (length, hip and withers height, and heart circumference) of suckling calves. Terre et al. (36) reported that structural body measures (hip height, hearth circumference, and body size) were unaffected by texturized starter feed with or without straw, a pelleted starter feed plus straw offered to calves. Ghassemi-Nejad et al. (11) reported that length of body, withers and hip height, and heart girth of calves fed textured starter was not significant effect compared with those fed mashed. Nilieh et al. (26) showed that heart girth, body length, and hip height were not affected by the physical form of the starter diet or its interaction with alfalfa hay. However, Omidi-Mirzaei et al. (27) reported that body length, height and width hip, and heart circumference were greater for calves texturized offered feed starter feed than those fed coarsely mashed starter diets. Beiranvand et al. (6) described that body length, hip height, or hip-width of calves were not different by fed 10% hay. In the study of Mirzaei et al. (23), the body size of calves fed corn silage was higher than that of calves fed non-forage fiber. According to them, filling the gut can also be a confounding factor in determining the effects of forage on the body size measurement of dairy calves. Also, Kazemi-Bonchenari et al. (19) indicated body size of calves is not affected when diets contained no forage.

In the present study, the effects of corn grain processing with different fiber sources on triglyceride, urea, and BHBA content were not significant. But calves receiving FCWS diet had the highest and the MCWS diet had the lowest glucose at 30 days. Azimzadeh et al. (2) investigated the effect of corn processing (cracked corn and steam-flaked corn) on the blood of Holstein calves and reported that glucose, triglyceride, and beta-hydroxybutyric acid (BHBA) concentration were not affected by the experimental treatment. The physical form of the starter diet, forage inclusion, or their interaction according to the research of Nilieh et al. (26) could not change the levels of blood parameters such as glucose, beta-hydroxybutyric acid (BHBA), albumin, and total protein. However, in the present study, blood urea levels increased with the forage factor. This is our finding of blood urea levels according to a study by Phuong et al. (32) found that by including alfalfa in the diet of weaned calves, who observed an increase in blood urea, which was associated with low nitrogen efficiency with increased dietary fiber content.

In the present study, calves receiving MCWS diet had higher acetic acid concentrations compared to other treatments (Table 5). At 60 days, propionic acid and Butyrate acid concentrations were highest in FCSB treatment and lowest in MCWS treatment. The valeric acid concentration was affected by the forage factor. Thus, MCSB treatment had the lowest value compared to other treatments at 60 days. Research by Mirzaei et al. (23) has shown that the total amount of VFA rumen does not accurately indicate the amount of fermentation because their clearance rate is higher than their presence, but the ratio between individual VFA is probably an indicator of the type and process of fermentation. Beharka et al. (5) reported that calves fed with ground starter had no significant effect on the ratio of acetate to propionate compared to starter unground. Castells et al. (7) stated the

highest total rumen VFA values in calves fed pelleted starter feed over the total trial period. Although the molar proportion of propionate was higher for pelleted starter feed but was no difference between treatments in the molar proportion of acetate, butyrate, and the acetate to propionate ratio. Nilieh et al. (26) stated that the higher SCFA value in the ruminal fluid of pelleted starter fed compared with coarsely mashed starter fed calves, as well as lesser ruminal pH in these animals, representing that pelleted starter form, has perhaps faster break down compared to the coarsely mashed starter. According to Castells et al. (7), acetate levels in the ruminal fluid of calves consuming forage supplements (alfalfa hay or oat hay) were higher than those of calves supplemented with non-forage fiber. Mash form of the starter as well as the wheat straw positively influenced acetate concentration in the ruminal fluid. Mirzaei et al. (23) found that mashed starter feed produced more acetate in comparison with the texturized starter in dairy calves. Results reported herein for ruminal acetate proportions are by Terré et al. (36) who reported higher acetate pro-portions on forage inclusion (alfalfa hay or chopped oat hay) in the diet. Our results showed that feeding the coarsely mashed starter with wheat straw increased the molar proportion of acetate and decreased the molar proportion of butyrate in the ruminal fluid before. Forage may displace concentrate intake and shift rumen fermentation in favor of acetate rather than butyrate production and, thus, delay rumen papillary development (38).

In conclusion, mashed corn feed compared with flacked corn was able to increase feed intake and daily weight gain, and final body weight, especially with the type of forage from the sugarcane bagasse. Therefore, providing sugarcane bagasse into mashed corn feed affects the calf's performance. Whereas, providing sugarcane bagasse with flacked corn improved the concentration of propionate and butyrate in the ruminal fluid. Based on the results, feeding corn grain in mash form with sugarcane bagasse resulted in better growth performance and it could be suggested for suckling calves compared to flacked corn and wheat straw.

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Conflict of interest

The authors declare that they have no competing interests.

Author Contributions

SJ, JS and GRG conceived and planned the experiments. SJ and JS carried out the experiments. HA and RS planned and carried out the simulations. SJ, JS and GRG contributed to sample preparation. HA, RS and JS contributed to the interpretation of the results. SJ and SS took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

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Gastrointestinal helminths of stray dogs in Erzurum province: Prevalence and risk to public health

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ABSTRACT

Dogs play an important role in public health due to their close contact with humans. This study aimed to investigate the presence and prevalence of gastrointestinal helminths in stray dogs in Erzurum province. Gastrointestinal helminth eggs were detected in 190 out of 446 (42.6%) dog fecal samples collected in Erzurum. More specifically, taeniid eggs (17.71%, 79/446), *Toxocara canis* (16.82%, 75/446), *Toxascaris leonina* (12.78%, 57/446), *Linguatula* spp. (4.48%, 20/446), *Alaria* spp. (2.91%, 13/446), hookworms (2.69%, 12/446), spiny-headed worms (1.12%, 5/446), and *Trichuris* spp. (0.22%, 1/446) eggs were identified. These findings indicate zoonotic parasites to be common in stray dogs in Erzurum. The public health risk associated with the presence of these zoonotic parasites can be reduced through the regular deworming of stray dogs, the prevention of environmental contamination with dog feces, and the adequate enforcement of sanitation protocols.

Stray dogs play a critical role in the epidemiology of several parasitic infections known to pose a risk to human health due to their adaptation to human habitation (21). For instance, people face the danger of infection when gardens and public areas are contaminated with the feces of dogs infected with zoonotic agents (7). The zoonotic transmission can occur through direct contact, water consumption and/or contaminated food with dog feces, or direct contact with contaminated soil. Another significant risk factor for infection is pica, especially in children (12).

The most prevalent gastrointestinal (GI) helminths found in dogs are *Taenia* spp., *Dipylidium* spp., *Echinococcus* spp., *Toxocara* sp., *Toxascaris* sp., *Ancylostoma* spp., *Uncinaria* spp., *Capillaria* spp., and *Trichuris* spp. (9). Among these helminths, *Toxocara* sp., *Echinococcus* spp., and *Ancylostoma* spp. are particularly significant in both under-developed and developing countries due to the limited use of antiparasitic drugs, poor socio-economic conditions, and lack of education (16).

The present study aimed to determine the presence and prevalence of GI helminths, including zoonotic species, in stray dogs in Erzurum province. To gather the required data, the animal shelter run by Erzurum Metropolitan Municipality was visited periodically between October 2015 and February 2016. Sterilization, vaccination, and antiparasitic drug (praziquantel) administration are routinely performed for every dog brought to this shelter. The sampling schedule was planned by the antiparasitic drug administration protocol followed by the shelter's management. The antiparasitic drugs were individually administered to the dogs, and the fecal samples were collected 24 hours after the administration.

A total of 446 (female [n = 237], male [n = 209]; 0–6 months age group [n = 15], 6–12 months age group

[n = 39], >1 year age group [n = 392]) dogs' fecal samples were collected. The samples were picked up in individually numbered plastic bags. The bags were taken to the laboratory on the same day that the samples were collected. The fecal samples were stored at -80°C for seven days to eliminate the zoonotic agents' infectivity.

The fecal samples were examined to detect the presence of adult parasites and cestode proglottids. The observed helminth eggs were further investigated using a light microscope (Nikon Eclipse Ci, Japan) according to the Fulleborn flotation and Benedek sedimentation methods. Saturated saline solution (specific gravity: 1.45 g/mL) was used in the flotation method. The helminth eggs were identified based on established morphological criteria (22).

All the statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 20 software (SPSS Inc., Chicago, IL, USA). The chi-squared (χ^2) test was used to calculate the possible correlations between the dogs' age and sex and the presence of the different parasite species. A difference was considered statistically significant at $P<0.05$.

The microscopic examination revealed the presence of GI helminth eggs in 190 out of 446 (42.6%) fecal samples, including trematodes (2.91%), cestodes (21.97%), nematodes (32.5%), spiny-headed worms (1.12%) and pentastomids (4.48%). Moreover, the most prevalent helminth eggs were taeniid cestodes (17.71%, 79/446), followed by *Toxocara canis* (16.82%, 75/446), *Toxascaris leonina* (12.78%, 57/446), *Linguatula* spp. (4.48%, 20/446), *Alaria* spp. (2.91%, 13/446), hookworms (2.69%, 12/446), spiny-headed worms (1.12%, 5/446), and *Trichuris* spp. (0.22%, 1/446) (Table 1). In addition, adult *Mesocestoides* spp. (3.14%, 14/446), *Taenia* spp. (2.69%, 12/446), *Dipylidium caninum* (1.12%, 5/446), *Alaria* spp. (0.22%, 1/446), *T. canis* (0.22%, 1/446), and *T. leonina* (0.22%, 1/446) were identified macroscopically. The prevalence of *T. canis* was also determined by age (Table 2), which revealed that the 0–6 months age group was associated with a significantly higher prevalence than the 6–12 months and >1 age groups ($P<0.05$).

Table 1. Occurrence of gastrointestinal helminths in stray dogs.

	Trematode		**Cestode**		**Nematode**			**Others**			**Total**
	***Alaria* spp.**	**Taenidae**	***Dipylidium* spp.**	***Mesocestoides* spp.**	***T. canis***	***T. leonina***	**Hookworm**	***Trichuris* spp.**	**Spiny-headed worm**	***Linguatula* spp.**	
np/n (%)	13/446 (2.91)	79/446 (17.71)	5/446 (1.12)	14/446 (3.14)	75/446 (16.82)	57/446 (12.78)	12/446 (2.69)	1/446 (0.22)	5/446 (1.12)	20/446 (4.48)	190/446 (42.6)**

n: number of examined samples; np: number of positive samples; %: prevalence; **some animals suffered from mixed infections.

Table 2. Distribution of gastrointestinal helminth infections according to age and sex in stray dogs.

	Age			**Total**	**Sex**		**Total**
Helminth species	**0-6 Months (n = 15) np/%**	**6-12 Months (n = 39) np/%**	**>1 Ages (n = 392) np/%**	**n = 446 np/%**	**Female (n = 237) np/%**	**Male (n = 209) np/%**	**n = 446 np/%**
Alaria spp.	-	-	13/3.3	13/2.9	7/3	6/2.9	13/2.9
Taeniidae	-	8/20.5	71/18.1	79/17.7	45/19	34/16.3	79/17.7
T. canis	8/53.3*	14/35.9	53/13.5	75/16.8	43/18.1	32/15.3	75/16.8
T. leonina	-	3/7.7	54/13.8	57/12.8	26/11	31/14.8	57/12.8
Hookworm	-	-	12/3.1	12/2.7	5/2.1	7/3.3	12/2.7
Trichuris spp.	-	-	1/0.3	1/0.2	1/0.4	-	1/0.2
Spiny-headed worm	-	-	5/1.3	5/1.1	2/0.8	3/1.4	5/1.1
Linguatula spp.	-	-	20/5.1	20/4.5	9/3.8	11/5.3	20/4.5
Total	8/53.3	25/64.1	229/58.4	262/58.74**	138/58.23	124/59.33	262/58.74**

n: number of examined samples; np: number of positive samples; %: prevalence;
* $P<0.05$; **some animals suffered from mixed infections.

Due to their close relationship with humans, dogs serve as important reservoirs of many zoonotic agents of relevance to public health (11). In the present study; *Alaria* spp., *Dipylidium* spp., *Mesocestoide*s spp., taeniid cestodes, *T. canis*, hookworms, *Trichuris* spp., *Linguatula* spp., and spiny-headed worms were all observed.

Taeniasis is recognized as a problem regarding both veterinary medicine and human health because some *Taenia* species exhibit zoonotic potential. These species can affect human health due to being agents of cystic and alveolar echinococcosis (*Echinococcus* spp.), coenurosis (*T. multiceps* and *T. serialis*), and cysticercosis (*T. crassiceps*) (10). Erzurum is considered a highly endemic area for both cystic and alveolar echinococcosis. Studies conducted in the province have shown that cystic and alveolar echinococcosis are prevalent in humans (14), while cystic echinococcosis is prevalent in livestock (5). Moreover, coenurosis has been detected in cattle, which rarely serve as intermediate hosts (3), emphasizing the presence of this agent in the province. In prior studies conducted in Türkiye, taeniid eggs were detected in 2.8%–46.28% of fecal samples (8, 27). In the present study, taeniid eggs were detected in 17.71% of samples, indicating a prevalence higher than that previously observed in Erzurum (2.9%) (6). It is thought that the administration of antiparasitic drugs in this study and disuse in the previous study may have led to the difference in the prevalence rates in the study area. Although several studies have investigated the prevalence of canine dipylidiasis in Türkiye (0.89%–65%) (19, 24), this study represents the first report from Erzurum (1.12%). Additionally, in Türkiye, the prevalence of *Mesocestoides* spp. has been reported to vary between 1.66% and 12.5% (4, 26), although this is the first study to examine the presence of *Mesocestoides* spp. (3.14%) in stray dogs in Erzurum.

Toxocara canis is a soil-associated nematode that is recognized as the most common intestinal parasite in dogs and wild canids (15). Furthermore, it is known to cause visceral larva migrans and ocular larva migrans in humans. The prevalence of *T. canis* has been determined to be between 4.2% and 47.8% in Türkiye (13, 27). In a previous study conducted in Erzurum (2), *T. canis* eggs were detected in 35.29% of dog fecal samples and 64.28% of park soil samples. In the present study, *T. canis* was detected in 16.82% of the fecal samples. The prevalence of *T. canis* infections is highest in puppies and young dogs aged less than six months old (15). In this study, the *T. canis* positivity was statistically significant in the 0–6 months age group ($P<0.05$). It is thought that the decrease in the infection rate between the two studies is related to the ages of the sampled dogs. Most of the sampled dogs (431/446) were older than 6 months in this study.

Similar to *Toxocara* spp., canine hookworms (*Ancylostoma* spp. and *Uncinaria* spp.) are soil-associated nematodes that are transmitted via contact with contaminated soil. The prevalence of canine hookworms has been reported to range between 1.1% and 80% in Türkiye (24, 27). In this study, hookworms were detected in 2.69% of the fecal samples, which is by the finding of a previous study conducted in Erzurum (2.3%) (6). Moreover, the prevalence of *Trichuris* spp. in Türkiye has been reported to be between 0.6% and 4.02% (6, 26). In the present study, *Trichuris* spp. eggs were detected in 0.22% of the fecal samples, which is also in line with the finding of the study previously conducted in Erzurum (0.6%) (6).

Adult *Alaria* spp. can cause inflammation in the bowel or general intoxication in the final hosts (17). The larval stage (mesocercariae) of *Alaria* is a causative agent of infection in both humans and animals. The prevalence of *Alaria* spp. was determined to range from 1.9%–5% in dogs in Türkiye (21, 25). In the present study, it was found to be 1.12%, which is consistent with the finding of the earlier study conducted in Erzurum (2.9%) (6). The prevalence of *Linguatula* spp. in dogs in Türkiye was reported between 0.6% and 53.3% (19, 23). In this study, the prevalence of *Linguatula* spp. was observed to be 4.48%. A previous study involving dogs in Erzurum reported that *Linguatula* spp.'s prevalence was 8.33% with necropsy (1). The difference between the two Erzurum-based studies is considered likely due to methodological differences. There are no other studies concerning the presence of spiny-headed worms (acanthocephalans) in stray dogs in Türkiye aside from the present investigation (2.91%).

In conclusion, this study determined the presence and prevalence of zoonotic GI helminths in stray dogs in Erzurum province. The results suggest that stray dogs play a critical role in human health. Indeed, dogs represent an effective contact between urban and rural life when it comes to the transmission of zoonotic disease agents. The identified presence of zoonotic GI helminths in dogs highlights the importance of prevention and control studies in the province. In addition, the humans living in Erzurum should be informed about the importance of regularly deworming dogs, ensuring the proper management of the process, preventing environmental contamination with dog feces, and ensuring the appropriate enforcement of sanitation protocols.

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Conflicts of Interest

The authors declared that there is no conflict of interest.

Author Contributions

RK, MA, İB, EG, HA, and VD conceived and planned the experiments. RK and MA carried out the experiments. RK, MA, and VD contributed to sample preparation. RK, MA, İB, EG, HA, and VD contributed to the interpretation of the results. RK took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was approved by Atatürk University Animal Experiments Local Ethics Committee (Approval no: 2015/27).

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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Maggot debridement therapy in an infected wounded dog: A case report

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ABSTRACT

Losing its importance over time with the discovery of antibiotics, maggot debridement therapy (MDT) to treat non-healing wounds tends to increase in recent years. However, its use in veterinary medicine is rather limited than human medicine. The material of this case report consisted of a 5-month-old Doberman dog bandaged for a fracture of the left hind toe. An anamnesis was taken that the bandage on the leg area was wet and remained on the wound for five days, and due to these, the foot turned into a macerated and gangrenous structure. Then, the gangrenous foot was amputated, and an infected wound that did not respond to antibiotic therapy occurred in the amputation area. The study was planned to investigate the efficacy of MDT on a post-operative infected wound. For this purpose, the sterile first and second instar larvae of *Lucilia sericata* were used. The first MDT administration was performed six days after the amputation. The second application was repeated after 24 hours, and the other two applications were repeated 48 hours intervals. After the first application, the amount of discharge gradually decreased in the wound, and rapid healing was determined. The wound was successfully treated by the wound healing-promoting characteristics of sterile *L. sericata* larvae. It is thought that this study will contribute to the spread of MDT applications, which are applied at a limited level for the treatment of non-healing chronic infected wounds in the field of veterinary medicine.

The calliphorid fly *Lucilia sericata* is one of the primary etiological agents of traumatic myiasis cases in animals and humans (4, 6). However, it is also known that the larvae of this fly make a significant contribution to wound healing by removing the necrotic tissues from the non-healing wounds. The use of *L. sericata* larvae for this purpose is known as biotherapy, biosurgery, maggot debridement therapy, or larval treatment (10, 12). Although widely used in human medicine in the treatment of chronic wounds, the use of MDT in veterinary medicine is limited. However, veterinary practices have reported that different types of wounds have been treated with MDT in animals in recent years (3, 8, 9, 13).

The literature review determined that MDT was applied in a post-operative infected wound covering the inguinal and abdominal regions of a cat in Türkiye (13). Apart from that study, no study regarding the application of MDT in animals has been found in Türkiye. This case report was prepared to give information regarding the efficacy of MDT in the treatment of a post-operative infected wound of a dog, which did not respond to the antibiotic regimen.

This case study aimed to evaluate the effectiveness of MDT in a post-operative infected wound of a dog. A 5-month-old Doberman breed dog constituted the material of this case study. The dog left hind leg was bandaged due to a finger fracture. An anamnesis was taken that the bandaged area got undesirably wet and remained this way for a long time. As a result, the wet leg turned into macerated tissue, and gas gangrene occurred (Figure 1). The left hind leg was amputated from the upper 1/3 of the femur due to gangrene. Due to the ongoing post-operative infection after the amputation, the wound did not respond to standard wound treatment and antibiotic regimens.

After the operation, wound treatment was performed for five days, but no successful results were obtained. Therefore, the case became a situation where euthanasia was considered.

In order to prevent euthanasia, MDT was considered as a treatment option. Six days after the amputation, sterile I. and II. instar larvae of *L. sericata* were obtained from the Maggot Production Laboratory of Selçuk University Faculty of Veterinary Medicine. After determining the size of the wound, 8-10 larvae were administered to 1 cm^2 of the wound (Figure 2). Systemic antibiotic ceftriaxone (Unacefin® 1 g IM Flankon, Yavuz İlaç San. Tic. A.Ş, Istanbul, Türkiye) and enrofloxacin (Baytril-K® 5 50 mL, injectable solution Bayer) applications were continued during MDT applications. After a bidirectional sticking plaster surrounded the wound edges, the wound was covered with sterile gauze to prevent the larvae from escaping the wound area. The gauze was left on the wound for 24 hours.

Figure 1. Status of the leg before amputation.

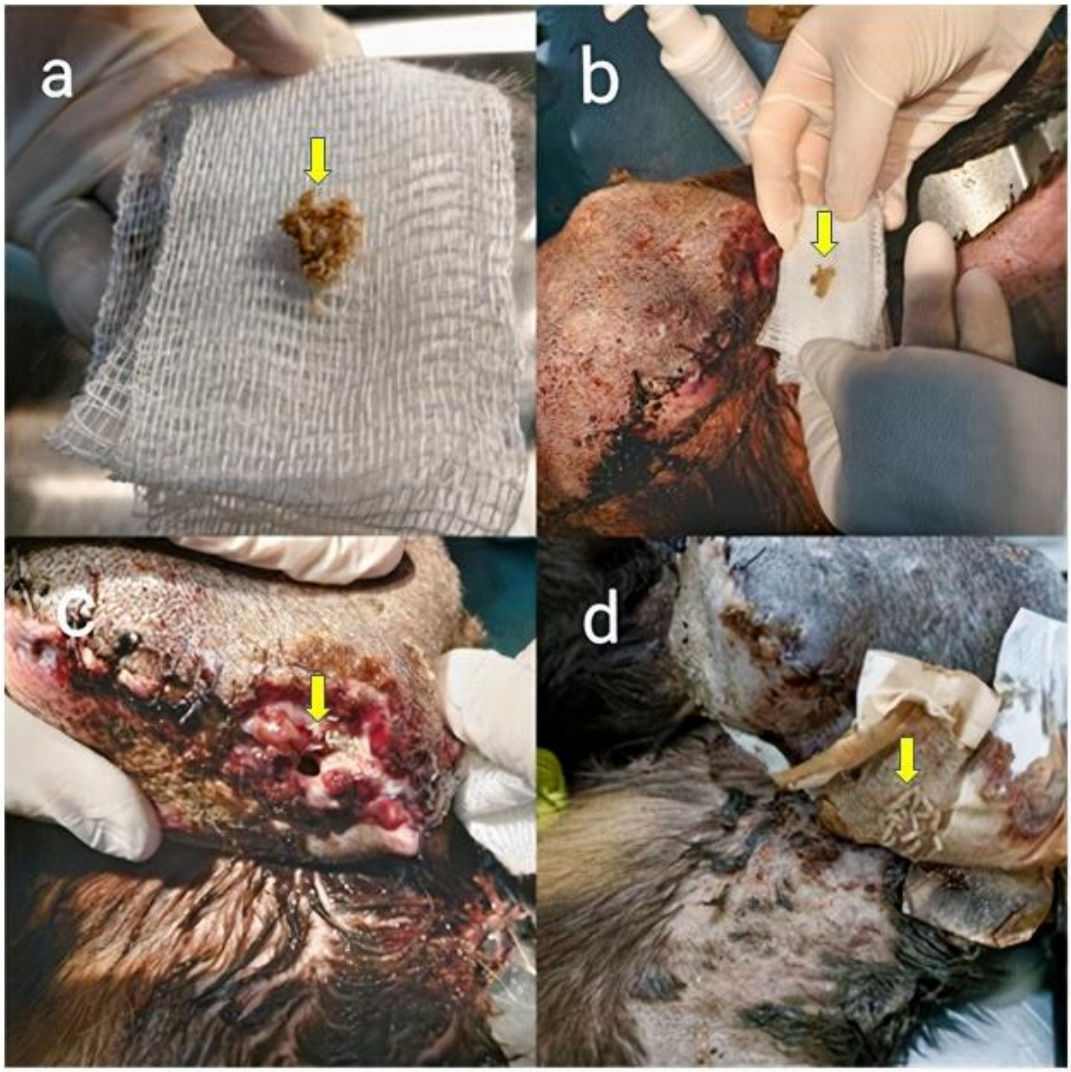

Figure 2. Application of sterile *Lucilia sericata* larvae to the infected wound (**a.** Sterile larvae, **b.** Application of the larvae to the wound site, **c.** The appearance of the larvae on the wound, **d.** Appearance of larva after 24 hours application).

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After 24 hours, the gauze opened, and the larvae that had passed into the III. larval stages were removed from the wound. The wound was carefully checked for larvae likely to remain in the wound. A decrease in the smell of the wound and discoloration in the discharge were detected. Before each larva application, the wound area was irrigated with isotonic solution (0.9% serum physiological). Two days later, the practice was repeated, and the maggots were left on the wound for 48 hours. Visible improvement was detected in the wound, which was rechecked. The application was repeated two more times at 2-days intervals, and the MDT was discontinued after a total of four applications (Figure 3). At the end of the applications, the larvae significantly promoted wound healing, and then treatment was continued with topical antibiotics.

Figure 3. Wound status before (**a**) and after (**b**) MDT application.

Maggot debridement therapy is the use of sterile *L. sericata* larvae in chronic, infected, and non-healing wounds. The larvae contribute to wound healing by four main mechanisms: debridement, disinfection, initiation of granulation, and improvement of blood flow in the wound area (5). The application of this treatment method, which is successfully applied in human medicine, is not at the desired level in veterinary medicine. In this study, an infected wound formed after amputation that did not respond to antibiotic treatment was successfully treated with maggot therapy in a 5-month-old Doberman.

The field of veterinary medicine has a limited number of studies on the use of MDT. Maggot treatment has been more widely used on horses than pet animals. It has been reported that MDT is successfully used to treat contaminated and septic navicular bursitis in horses than other methods, and most of the horses have reached their previous health thanks to MDT (2). In addition, MDT can be effectively used to treat many types of wounds, including foot/leg wounds and horses' wounds such as deep cuts, abscesses, and abdominal wounds (2). Lepage et al. (8) reported that 41 equines with wounds in different parts of their body were treated with MDT, and considerable results concerning wound healing were obtained in less than one week in 38 horses. Apart from these, various wounds such as panniculitis, soft tissue abscess, laceration of the limbs, dehiscence of the *linea alba*, fistulous withers, some musculoskeletal wounds, and laminitis in equines were treated by MDT (1, 8, 9, 11). Kocisova et al. (7) reported that MDT could be used to treat foot root and foot scald in sheep. Maggot debridement therapy is also reported as an effective treatment option for small animals (12). Gunshot wounds, pressure ulcers, necrotic tumors, and multiple bite wounds were successfully treated in dogs and cats (12). The literature search revealed the scarcity of MDT applications in the veterinary field in Türkiye. The first MDT application in Türkiye was performed by Uslu et al. (13) in 2021. A cat with a non-healing post-operative infected abdominal and inguinal wound was successfully treated in that study (13). Apart from that, no study has been encountered regarding the application of MDT, and the current study is the second study on the use of MDT in the veterinary field in Türkiye.

In this study, wound healing was promoted using sterile *L. sericata* larvae in a dog with an infected wound, which did not respond to antibiotic treatment. Sherman et al. (12) stated that MDT provides debridement of wounds of companion animals, which is expected to prevent euthanasia and amputation, and these animals regain their health. Thus, the treatment procedure for non-healing chronically infected or gangrenous wounds should be determined as soon as possible. In conclusion, MDT can be used before amputation and may prevent amputation in some animals, depending on the character of the wound. At this point, animal owners and veterinarians should be informed more regarding MDT. The current study and similar studies concerning the use of MDT have indicated that MDT will also be one of the alternative treatment options used for non-healing wounds in the future.

This case study showed that the dog with the non-healing infected wound could be successfully treated with MDT. It should be noted that MDT is an important

alternative treatment option used in veterinary medicine to treat such wounds. It should not be forgotten that MDT is an effective and safe method to promote wound healing. Although MDT is an effective and cost-efficient option used in the treatment of non-healing wounds, it has some undesirable conditions such as the difficulties in obtaining sterile larvae and the tickling sensation caused by the larvae at the application site. However, the development of resistance to antibiotics and expensive surgical applications indicate that the need for MDT will increase in the debridement of chronically infected non-healing wounds in the future.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

UU and OC conceived and planned the study. AE and HKA carried out the experiments. UU and OC took the lead in writing the manuscript. All authors have read and agreed to the submitted version of the manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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This study does not present any ethical concerns.

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Open surgical correction combined with an u-shaped external splint for pectus excavatum in a Scottish fold cat

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ABSTRACT

A 4 month-old, 1.9 kg, female, Scottish fold cat was brought to Kırıkkale University Veterinary Faculty Research and Practice Hospital with complaints of progressive dyspnea and exercise intolerance. Dorsal deformation in the caudal part of the sternum and displacement of the heart into the left hemithorax were determined in the clinical and radiographic examinations. In the measurements made on the radiographic images taken before the operation, the frontosagittal index (FSI) and vertebral index (VI) values were measured as 3.5 and 5.1, respectively. An external splint made of PVC material is fixed to the thorax with suture material passed around the sternebrae, in order to correct the sternal malposition in intraoperative treatment. The patient was followed for 8 weeks after the operation. The external splint was removed five weeks after the operation, and the FSI and VI values were measured as 1.4 and 11.3 on the radiographs, respectively. In the postoperative period, it was observed that the patient's problems such as dyspnea and exercise intolerance disappeared. In animals with bone development yet to be completed, it has been determined that external splint application gives successful results.

Pectus excavatum (PE) is the most common congenital deformity of the anterior chest wall. Dorsal deviation occurs in the caudal part of the sternum and associated costal cartilage or it can be seen abnormal growth on several ribs and sternum. As a result, the chest narrows and a collapsed appearance is formed. PE is sometimes referred to as Cobbler's chest, Sunken chest, Funnel chest, Shoemaker chest or a Dent in the chest (1, 15). Costo-sternal deformity causes the rib cage to dorsoventrally narrow. Due to the restriction in ventilation and cardiac compression, narrowing occurs especially in the caudal direction (6, 13). Cardiac and lung functions deteriorate due to decreased intrathoracic volume and restriction of diastolic filling in PE cases (10).

Although most of the cases are congenital, abnormalities are also observed during puberty. Large negative intrapleural pressures can cause the collapse of the sternum and intercostal cartilage in humans. There is information in the literature that conditions that cause upper airway obstruction at a young age lead to abnormal respiratory gradients and that PE may develop as a result. An example of this is the "swimmer syndrome", which causes collapse of the sternum as a result of newborn dogs unable to walk in a proper manner and constantly lying in the sternal position (16). Another information about PE formation is the formation of a concave abnormality in the caudal sternebrae as a result of the short and hypoplastic diaphragm inhibiting the development of the sternum and caudal ribs (18). In postmortem examinations in a cat with PE, it was determined that the primary abnormality included the ventral part of the diaphragm (17).

In the case presentation, the treatment of PE in a 4-month-old cat with external splint application is described.

A 4-month-old, 1.9 kg, female intact, Scottish Fold cat with the complaint of respiratory distress was brought to Kırıkkale University Veterinary Faculty Research and Practice Hospital. The first findings observed in the clinical examination were tachypnea, exercise intolerance, and sternum deformity that was noticeable on inspection. It was determined that a dorsal deviation of the last 4

sternebrae and sternum on the laterolateral radiography, and heart shifted to the left of the median line on ventrodorsal radiography. (Figures 2a and 2b). For determining the severity of the deformity, measurements were made according to the frontosagittal index (FSI) and vertebral index (VI), which were defined in previous studies (2). The FSI value was determined to be 3.53 (reference range 0.7-1.3) and the VI value was determined to be 5.1 (reference range 16.2-18.8) in these measurements (Table 1). According to the measurements made, it was decided that the clinical score of the cat was 'severe' and that an operation should be performed.

Upon premedication with butorphanol (0.01 ml/kg, IV) (Butomidor; Richterpharma, Austria), propofol (3 mg/kg, IV) (Propofol 2% Fresenius; Fresenius Kabi, Austria) was administered for anesthesia induction. After the intubation of the patient, anesthesia was maintained with isoflurane (Isoflurane USP, Adeka, Türkiye). Fluid therapy (Lactate Ringer 10ml/kg/hour) (Ringesol, Vilsan, Türkiye) was administered during the intraoperative period. Cefazolin sodium (25 mg/kg) (Cefazol, Mustafa Nevzat, Türkiye) was administered intravenously 20 minutes before the operation and during the intraoperative period.

Table 1. Grading of the clinical severity of PE using Frontosagittal and Vertebral indices.

Clinical Score	FSI	VI
Normal	0.7-1.3	12.6-18.8
Mild	2	>9
Moderate	2-3	6-9
Severe	>3	<6

FSI: Frontosagittal index. VI: Vertebral index.

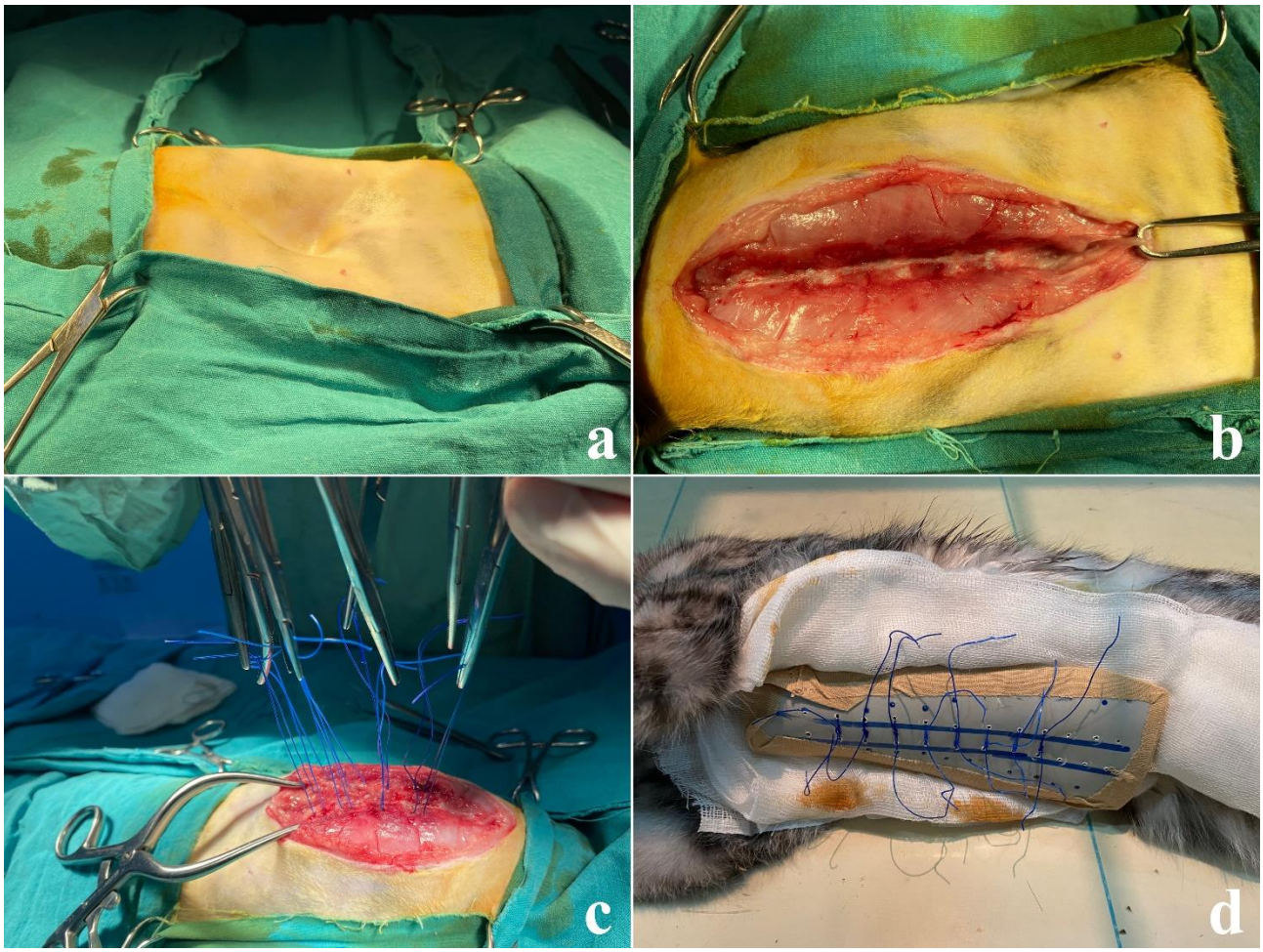

Figure 1. Preoperative appearance of a cat with PE showing dorsal deviation of the caudal sternum (a). Separation of deep pectoral muscles from the sternum (b). Circumsternal sutures are retracted ventrally to straighten the sternal deviation (c). Stabilization of external U-shaped splint. (d).

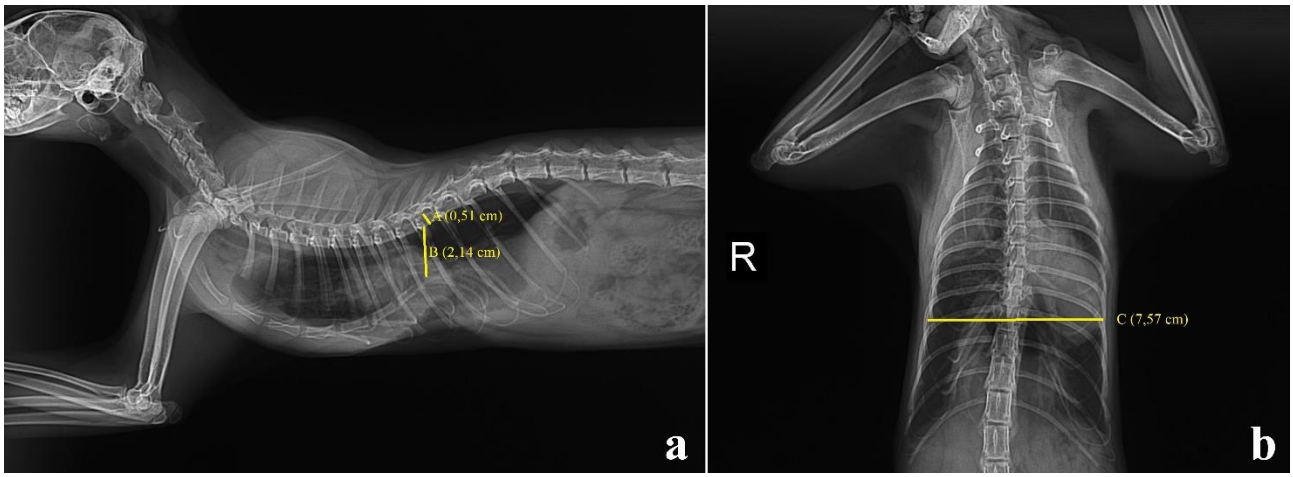

Figure 2. a: Measurement of the vertebral body for calculation of vertebral indices in laterolateral radiograph. The diameter of the vertebral body overlying the deformity and the distance of the same vertebra to the sternum added (A+B). The ratio of the result to the diameter of the vertebra determines the vertebral indices. ((A+B)/A). b: Measurement of thoracic width at the 10th thoracic vertebra on a ventrodorsal radiograph for calculation of frontosagittal index (C).

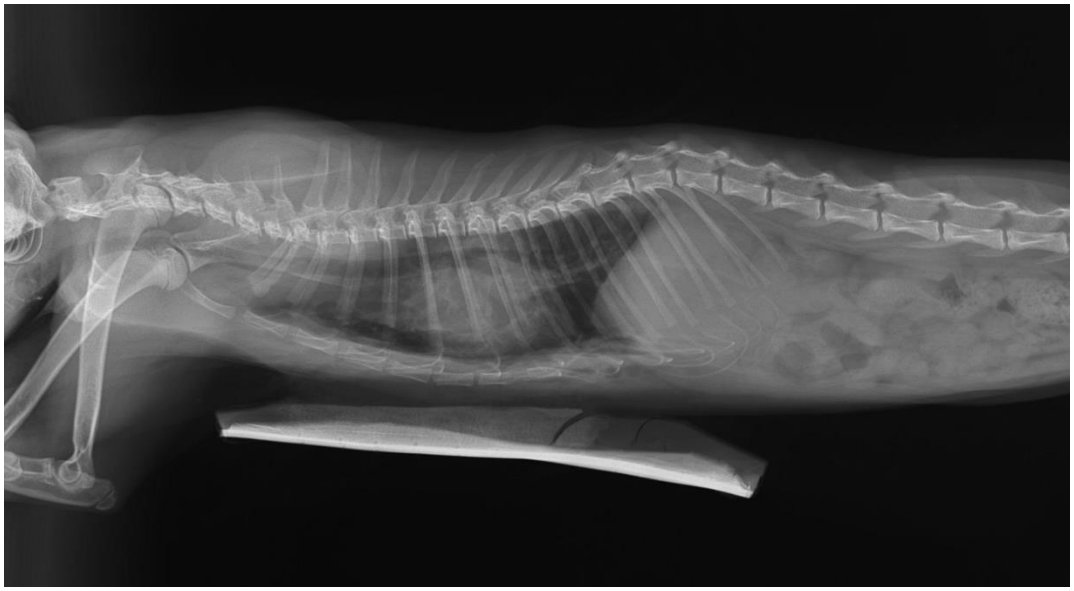

Figure 3. Laterolateral radiographic image of the thorax on first postoperative day.

The operation area was shaved and the patient positioned in the dorsal recumbency. The operation area was disinfected and prepared for the operation (Figure 1a). A skin incision was made in the midline of the thorax, extending from the second sternebra to the processus xiphoideus. The deep pectoral muscles were separated from the place where they were attached to the sternum with the help of a periosteal elevator (Figure 1b). With this method, the sutures were placed more easily and carefully, as the sternal deviation was made more prominent. As the suture material, monofilament polypropylene (USP 1) was preferred. As the deviation in the sternum was very deep, the first of the circumsternal sutures were placed on the processus xiphoideus. The placed sutures were fixed with hemostatics and pulled upwards (Figure 1c). Thereby, safe suture areas were created for other suture materials to be placed. After placing all the sutures, the skin was closed and the free ends of the suture materials were passed through the prepared U-shaped PVC support material. Surgical pads were put under the PVC support to as not to create an abrasion wound on the skin. Subsequently, the free ends of the suture materials were tie on the PVC and the external splint was fixed to the thorax (Figure 1d and 3).

Amoxicillin-clavulanic acid (Synulox; Zoetis, Finland) was used for 7 days postoperatively and meloxicam (Maxicam; Sanovel, Türkiye) was used for 4 days, and the patient was called for controls every week.

In accordance with the information obtained from the owner of the patient, it was learned that the cat's breathing improved and its activity increased 5 days after the operation. A bandage was changed during the controls, the pads between the skin and the external splint were removed, the area was cleaned as much as possible, and control radiographs were taken. On the radiographs taken in the fourth postoperative week, pulmonary edema was detected and furosemide (2 mg/kg) (Lasix, Sanofi, France) and dexamethasone (0.5 mg/kg) (Vetakort, Vetaş, Türkiye) were used for this purpose for 4 days. This complication is thought to result from reexpansion. The external splint was removed at the end of the fifth week. When the external splint was removed, no abrasion wound was observed on the skin and pulmonary edema had resolved. One week after removing the splint, clinical examinations of the patient were performed again and control radiographs were taken. FSI and VI scoring was performed on the radiographic images taken (Figure 4). In accordance with the results, it was determined that the patient's thorax index turned from severe to near normal (FSI: 1.4 and VI: 11.35).

A pectus anomaly is a chest wall deformity in which several ribs and the sternum are abnormally enlarged and consequently produce a convex (carinatum) or concave (excavatum) appearance on the ventral aspect of the chest wall. This anomaly is frequently seen in animals, particularly in cats and dogs (5, 8). Even though the incidence of PE in cats is not known clearly, it has been reported that it is more common in Bengal cats than domestic shorthair cats (4). It is reported that the presence of PE in Burmese cats is associated with flat-chested kitten syndrome (19). It is reported that the most common clinical symptoms in PE cases are exercise intolerance, tachypnea, growth retardation, or growth arrest (3). It is reported that the PE case can be easily felt on palpation of the thorax (2). The main clinical findings in this case were exercise intolerance and dyspnea. It was also noted by a previous studies (14, 21). In the weeks following the diagnosis of PE, progressive respiratory distress was detected.

Although the diagnosis of PE can be easily determined by palpation of the defect in the sternum, the clinical severity of the disease can be visualized by deformation of the sternum determined by orthogonal radiographs and decreased thoracic volume. FSI and VI assessments are designed for scoring purposes. Together with these, the displacement of the heart's position to the left or right according to radiographic findings is also shown to be among the remarkable findings in PE cases (2, 21). In this study, the PE was diagnosed at first by palpation and then by radiographs. Consistent with previous studies (2), dorsal deviation of the last 4 sternebrae in lateral radiographic findings and the displacement of the heart to the left hemithorax in the ventrodorsal radiograph was noted. The severity of PE in this case was graded according to the FSI and VI values (3.53 and 5.1, respectively) defined in previous studies. In our study, the assessment of FSI and VI was, as stated by Risselada et al. (14) and Charlesworth (2), made by considering the vertebra where the distance between the highest point of sternal deviation and the vertebra (min_{TH}) was minimum, and in accordance with that, the min_{TH} was determined at the 9th vertebra level. Because the T10 vertebral level was far from the point where the sternum deviation is maximum and therefore it was thought that such a measurement method is not suitable for the correct determination of the thorax index.

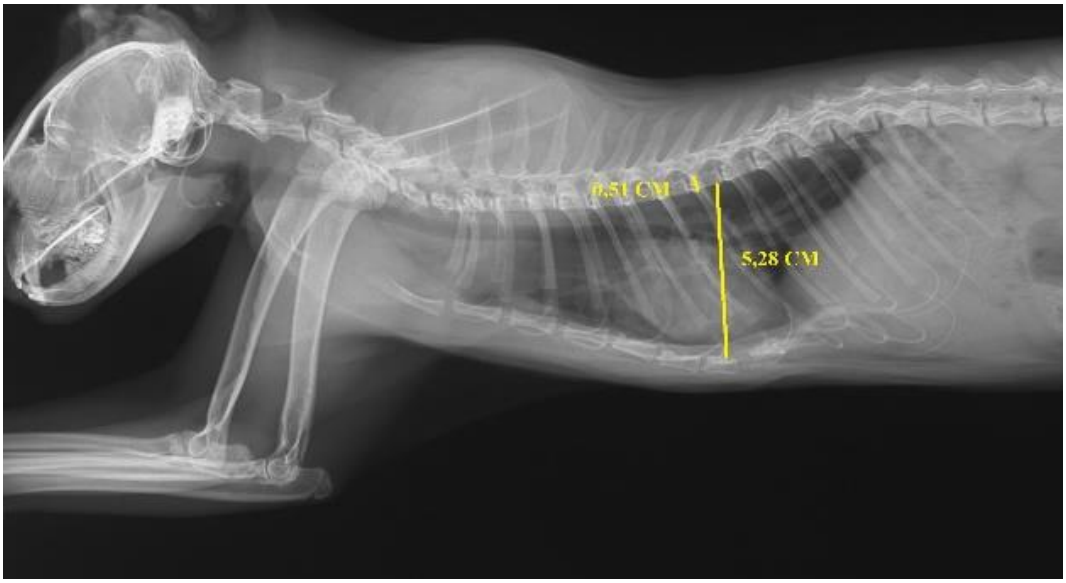

Figure 4. Lateral radiographic view of the thorax and thorax index values at 6th weeks postoperatively.

It was stated that the operation can be performed in cats and dogs with PE by evaluating according to clinical symptoms. It was reported that the diagnosis of patients with mild PE is often overlooked when they do not show clinical symptoms, and the operation will be contraindicated for these patients (10, 15). It was reported that this situation should be corrected by performing an operation in animals with severe PE, and for this purpose, external splint, internal splint (plate and rod application), or longitudinal sternebral pining combined with external splinting provides successful outcomes. It was reported that especially external splint application is indicated in young animals whose sternebra is still flexible (14, 15, 21). It was stated that the effectiveness of external splint application in adult and old animals is weak (11, 15). It was reported that the most common complications are internal thoracic vascular damage, heart or lung perforation, iatrogenic pneumothorax, and infection or dermatitis in the splinted area (2, 16). Because of the reason that the cat was 4 months old and the PE degree was severe according to the thorax index, it was thought that external splint application would be appropriate by performing the operation in the study conducted. In the external splint application, the suture placement was done very carefully because the sternum deviation was close to the heart. Even though no intraoperative complication was observed, the presence of reexpansion pulmonary edema was noted in the postoperative period. Postoperative FSI and VI values (1.4 and 11.35) were found to be rather close to the reference range.

In various studies (2, 5, 12, 16), the use of external splint for the treatment of PE in young animals has given successful results. In this study, PE was successfully corrected using an external splint in a 4-month-old cat. Our study also supports these results.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

Diagnose, surgical plans and procedure, writing-reviewing, editing and postoperative controls and evaluations are made by BK and MB.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

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