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Contents

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Comparative Evaluation of Colostrum Quality in Karya Ewes and Yearling Lambs Using the Brix Refractometer

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ABSTRACT

The aim of this study was to comparatively evaluate colostrum quality in ewes and yearling lambs using a digital Brix refractometer. A total of 18 Karya sheep, including 9 yearling lambs and 9 ewes, were used in the study. Colostrum/milk samples were collected from the sheep immediately after birth and at 12 and 24 hours, and 2, 3, 4, 7 and 15 days after birth. The Brix percentages of the fresh colostrum/milk samples were measured with a digital Brix refractometer. The colostrum Brix percentages of the ewes and yearling lambs and the change in the colostrum/milk Brix percentages up to 15 days after birth were compared by repeated measure of analysis of variances. The mean colostrum Brix percentages of the ewes immediately after birth, and at 12 and 24 hours and, 2 and 3 days after birth were determined as 32.8 ± 1.7%, 22.6 ± 1.2%, 17.2 ± 1.0%, 15.4 ± 0.8%, and 14.2 ± 0.3%, respectively. The mean colostrum Brix percentages of the yearling lambs immediately after birth, and at 12 and 24 hours, and 2 and 3 days after birth were determined as 33.3±1.8%, 21.0±1.9%, 17.2 ± 1.4%, 15.6 ± 0.6%, and 15.0 ± 0.4%, respectively. No differences were detected between the colostrum and milk Brix percentages of the ewes and yearling lambs. Colostrum Brix percentages decreased significantly from birth to 24 hours postpartum. Colostrum/milk Brix percentages remained at similar values from 24 hours to 15 days postpartum. In conclusion, colostrum quality did not differ between Karya ewes and yearling lambs and colostrum/milk Brix percentages decreased steadily from 24 hours to 15 days after lambing.

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INTRODUCTION

Colostrum, which is the first secretion of the udder tissue after birth, contains antibodies, fat, proteins, vitamins, and many other biological molecules such as hormones, growth factors and cytokines, which are necessary for the health, growth and development of lambs (Agenbag et al., 2021; Uysal and Yörük, 2022; Farooq et al., 2024). The syndesmochorial placental structure of ewes prevents immunoglobulins from crossing the placental barrier. Therefore, lambs are born agammaglobulinemic and get the antibodies they need through the consumption of colostrum (Agenbag et al., 2021). Failure of transfer of passive immunity (FTPI) develops in lambs that do not receive enough immunoglobulins through colostrum consumption. Serum IgG concentrations do not yet have a universally accepted threshold for lambs in determining adequate passive immunity transfer. Even so, considering the mortality and growth performances of lambs, several blood IgG thresholds indicating FTPI or different passive immunity strata have been proposed such as <1500 mg/dL (Alves et al., 2015), <600 mg/dL (Gökçe and Atakişi, 2019), <600 mg/dL, 600-1000 mg/dL, >1000 mg/dL (Gökçe et al., 2022) and <1000mg/dL, 1000-2000 mg/dL, >2000 mg/dL (Gökçe et al., 2013).

The radial immuno diffusion (RID) test is the gold standard method for determining the IgG concentrations of biological fluids. This laboratory method is both expensive and time-consuming, and requires special equipment to display the results of the analysis. A simple and practical method used to determine the quality of colostrum is refractometry. The Brix refractometer enables the simultaneous and rapid estimation of the total solid concentration of colostrum (Todaro et al., 2023). The Brix refractometer has been validated to estimate the IgG concentration of bovine colostrum (Buczinski and Vandeweerd, 2016). It has also been described as a useful and reliable tool for determining the quality of ovine colostrum (Santiago et al., 2020; Swinbourne et al., 2022; Todaro et al., 2023; Sarıca and Aydoğdu, 2024). The Brix refractometer has been previously used in studies on the investigation of factors affecting colostrum quality in sheep (Torres-Rovira et al., 2017; Uysal et al., 2024).

Many factors such as birth season, feeding conditions, gestational age, dry period length, parity, lamb birth weight, twinning and breed have an effect on colostrum quality in sheep (Torres-Rovira et al., 2017; Campion et al., 2019; Uysal et al., 2024; Sarıca and Aydoğdu, 2024). It has been reported that 22% of sheep cannot produce colostrum with an IgG concentration above 50 g/L (Dwyer et al., 2016). Based on previous research on the colostrum needs of newborn lambs, a lamb requires 200 ml of colostrum per kg of birth weight under mild weather conditions during the first 18 hours of life, and 50% more under rainy and windy conditions. To increase the viability of lambs, 25% of this colostrum needs to be consumed at birth (Banchero et al., 2015). Otherwise, it has been reported that higher passive immunity levels are achieved with bottle feeding compared to natural suckling, such that morbidity and mortality significantly decrease in bottle-fed lambs (Eğdir and Öcal, 2023). Therefore, to ensure adequate passive immunity transfer to newborn lambs, it is necessary to implement an efficient colostrum management and monitor the quality of colostrum (Uysal and Yörük, 2022). So, for this purpose, the quality of the colostrum of ewes is determined, and those of high quality are frozen to be later fed to lambs of ewes that produce poor-quality or not enough colostrum.

The rearing of the Karya sheep is common in western Anatolia (Karaca et al., 2018). While sheep breed is known to have a significant effect on colostrum quality, there is only limited literature information available on colostrum quality in the Karya breed (Eğdir and Öcal, 2023). The aim of this study was to compare the quality of the colostrum of Karya ewes and yearling lambs with the aid of the digital Brix refractometer and to determine any change in the colostrum/milk Brix percentages during the first 15 days postpartum.

MATERIAL and METHOD

Milking is not subject to local ethics committee approval in accordance with the provisions of "Regulation on Working Procedures and Principles of Ethics Committee of Experiments on Animal (Official Gazette No: 28914, 15 February 2014)". This study was conducted on a sheep farm located in the Serinhisar (Kizilhisar) district of the Denizli province in 2021. The sheep were fed with roughage consisting of wheat straw and oats, and concentrated feed (a mixture of barley and wheat) of about 500 g per animal per day. Clean and fresh water was provided ad libitum.

In this study, the data of a total of 18 Karya sheep, including yearling lambs (n=9) and ewes (n=9), were used. The parity number of the ewes ranged from 2 to 5. Five-ml colostrum/milk samples were collected from the sheep immediately after birth and at 12 hours, 24 hours, and 2, 3, 4, 7 and 15 days after birth into Falcon tubes. The quality of the colostrum was determined by analyzing fresh colostrum samples with a digital Brix refractometer (Atago PAL-1, Tokyo, Japan). Both the collection of the colostrum/milk samples and the Brix refractometer analyses were carried out by the farm owner under the supervision of the researcher. In this study, the secretion obtained from the udder of the sheep until the 3rd day after lambing was classified and used as colostrum, and the secretion obtained on the 4th, 7th and 15th days after lambing was classified and used as milk.

Whether the study data met the normality assumptions was determined according to the Shapiro-Wilk test. The postpartum colostrum/milk Brix percentages of the groups were compared using repeated measures of analysis of variance. The results are given in mean±SEM (standard error of mean). The statistical significance level was set at $p<0.05$. Statistical analyses were performed using SPSS version 24.

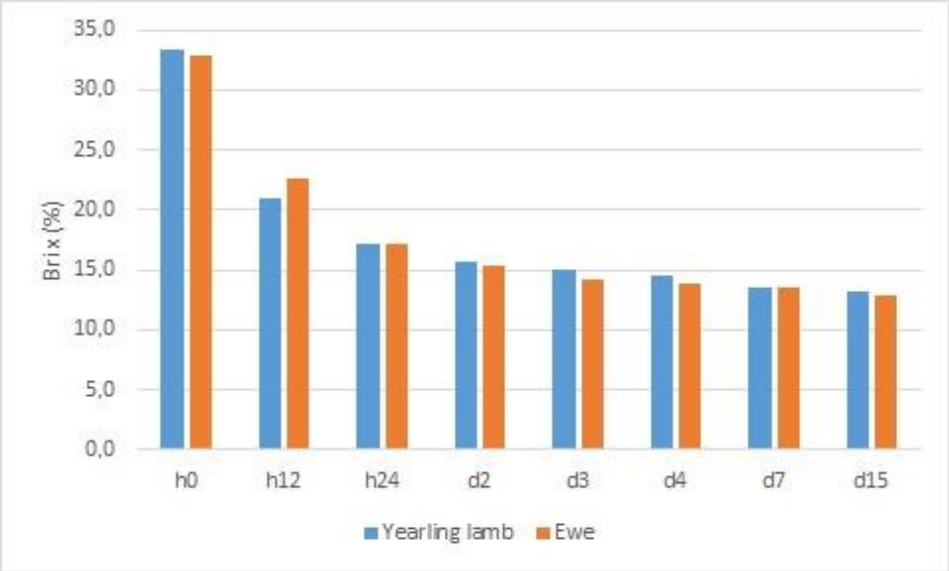
RESULTS

The changes in the colostrum and milk Brix percentages over time are presented in Table. The colostrum Brix percentages of the ewes immediately after birth, and at 12 and 24 hours and 2 and 3 days after birth were determined as 32.8±1.7%, 22.6±1.2%, 17.2±1.0%, 15.4±0.8%, and 14.2±0.3%, respectively. The colostrum Brix percentages of the yearling lambs immediately after birth, and at 12 and 24 hours, and 2 and 3 days after birth were determined as 33.3±1.8%, 21.0±1.9%, 17.2±1.4%, 15.6±0.6%, and 15.0±0.4%, respectively. There was no difference between the ewes and yearling lambs for the quality of colostrum determined with the Brix refractometer. The Brix percentages of the colostrum samples collected immediately after parturition ranged from 24.7% to 41.2%. The colostrum and milk Brix percentages of the Karya ewes and yearling lambs throughout the 15 days after lambing are presented in Figure. There was no difference between the Brix percentages of the ewes' milk on days 4, 7 and 15 (13.9 ± 0.3%, 13.5 ± 0.3%, 12.9 ± 0.3%, respectively) and the yearling lambs' milk (140.6 ± 0.4%, 13.6 ± 0.2%, 13.3 ± 0.1%, respectively). The colostrum Brix percentages decreased significantly until the 24th hour after birth. However, there was no difference between the Brix percentages of the colostrum and milk samples after 24 hours.

Table. Colostrum and milk Brix percentages of Karya ewe and yearling lambs throughout the 15 days after lambing

Group (G)	Time (T)									p		
	Birth	Hour 12	Day 1	Day 2	Day 3	Day 4	Day 7	Day 15	LS Mean (Group)	Group	Time	G*T
Yearling lambs	33,29 ± 1,77	21,01 ± 1,88	17,18 ± 1,38	15,64 ± 0,64	15,03 ± 0,38	14,59 ± 0,38	13,60 ± 0,16	13,27 ± 0,10	17,95 ± 0,57	0,865	<0,001	0,781
Ewes	32,81 ± 1,68	22,58 ± 1,22	17,23 ± 0,98	15,41 ± 0,77	14,21 ± 0,30	13,87 ± 0,26	13,47 ± 0,30	12,91 ± 0,29	17,81 ± 0,59			
LS Mean (Time)	33,05 ± 1,22[a]	21,79 ± 1,12 [b]	17,21 ± 0,85 [c]	15,53 ± 0,50 [c]	14,62 ± 0,24 [c]	14,23 ± 0,23 [cd]	13,53 ± 0,17 [de]	13,09 ± 0,16 [e]				

Figure. Colostrum and milk Brix percentages of Karya ewes and yearling lambs throughout the 15 days after lambing. h, hour; d, day.



DISCUSSION

Higher colostrum IgG concentrations are expected in older animals due to both their increased lifetime exposure to pathogens (Devery-Pocius and Larson, 1983), and the age-related increase observed in the secretory capacity of ovine udder epithelial cells (Campion et al., 2019). However, it was determined similar colostrum quality by means of digital Brix refractometry in ewes and yearling lambs. The current study results agree with studies reporting the age or parity of ewes not being associated with colostral IgG concentrations (Campion et al., 2019; Sarıca and Aydoğdu, 2024) and colostrum IgG concentrations of primiparous and multiparous ewes being similar (Alves et al., 2015). In contrast, there are also study results reporting higher (Gilbert et al., 1988; Torres-Rovira et al., 2017) and lower (Chniter et al. 2016) colostral IgG concentrations in primiparous ewes, compared to multiparous ewes. The reason for the difference between the results of these studies could be differences in the analytical methods used, the breed of the study animals or the system of feeding management.

In agreement with a previous study by Eğdir and Öcal (2023), reporting high colostral IgG concentrations in Karya ewes, the present study demonstrated high Brix percentages for colostrum samples collected immediately after parturition. The mean postpartum colostrum Brix percentage

(33.1%) determined in the present study is higher than the colostrum Brix percentages reported to have been measured at the first hour after parturition in a study evaluating the colostrum quality of different breeds of ewes using the Brix refractometer (Uysal et al., 2024). Similarly, the mean Brix percentage determined in the present study is higher than the mean Brix percentages reported in several other previous studies (Santiago et al., 2020; Kessler et al., 2021; Hamer et al., 2024; Sarıca and Aydoğdu, 2024). A high Brix value of colostrum or milk is closely related to the concentration of total protein (especially IgG) contained in colostrum/milk (Santiago et al., 2020; Todaro et al., 2024). It has been reported that colostral IgG concentrations vary among different breeds of sheep (Campion et al., 2019; Uysal et al., 2024) and also with the increased or decreased milk production capacity of ovine udder epithelial cells (Boutinaud et al., 2004). Karya sheep used in the present study are prolific and have high milk yields (Karaca et al., 2018). It is considered that Karya sheep may have genetically more functional mammary tissue epithelial cells, and therefore, may produce colostrum with higher Brix percentages.

Consistent with previous studies, colostrum Brix percentages decreased from hour 0 to hour 24 postpartum in the present study. Santiago et al. (2020) reported that colostrum Brix percentages gradually decreased until 48 h post-lambing. Decreasing Brix percentages in relation to the time elapsed after lambing were also reported by Uysal et al. (2024). The decrease in the amount of colostrum in the udder due to suckling by lambs and, concurrently the continued secretion of transition milk in the epithelial cells are thought to be effective in the decrease of colostrum Brix percentages. In the present study, the Brix percentages of milk on days 4, 7 and 15 after lambing (14.2, 13.5, and 13.0, respectively) were consistent with the Brix value (13.9%) reported by Todaro et al. (2024).

CONCLUSION

This study showed that while there was no difference between the Karya ewes and yearling lambs for the colostrum qualities determined with the Brix refractometer, the colostrum quality decreased significantly from the time of birth to 24 hours after lambing. The colostrum/milk Brix percentages decreased steadily from 24 hours to 15 days after birth.

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Ethics Approval

Milking is not subject to local ethics committee approval in accordance with the provisions of "Regulation on Working Procedures and Principles of Ethics Committee of Experiments on Animal (Official Gazette No: 28914, 15 February 2014)"

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Conflict of Interest

The author declares that there is no conflict of interest.

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2 Zero Hunger
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Effect of Centrifugation Speed and Blood Tubes with Different Contents on Certain Serum/Plasma Biochemical Parameters in Cocks

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ABSTRACT

This study was carried out to investigate the effect of blood tubes with different anticoagulants and different spinning speed applications in the blood centrifuge on biochemical analyses. The study conducted with a total of 16 Gerze cocks, a native breed of Türkiye. In the first part of the study, 8 roosters were slaughtered and the blood taken was transferred to lithium heparinized tubes only. Then, the blood was centrifuged at 3000, 4000 and 5000 *rpm* for 10 minutes. Blood samples were taken into plain-tubes, K_3EDTA and lithium heparin tubes, and centrifuged at 3000 *rpm* for 10 minutes. The study results indicated that, centrifugation speed had no statistically significant effect on the levels of plasma glucose (p = 0,691), triglyceride (p = 0,451), total cholesterol (p = 0,993), high-density lipoprotein [(HDL-cholesterol) (p = 0,917)], low-density lipoprotein [(LDL-cholesterol) (p = 0,990)], total protein (p = 0,860), inorganic phosphorus (p = 0,885), calcium (p = 0,980), amount of plasma (p = 0,903) and, aspartate aminotransferase activities [(AST) (p = 0,692)]. In the second part of the study 8 different cocks apart from first part of the study, were slaughtered and the blood taken into tubes with different contents as plain tubes (serum), lithium heparinized tubes (plasma) and K_3EDTA tubes (plasma). Same parameters with first part of the study were investigated. The results showed that no statistical difference found between serum or plasma obtained from blood tubes with different contents (p>0.05). Additionally, the calcium levels could not be analyzed with K_3EDTA plasma. Consequently, we observed that centrifugation speeds up to 5000 *rpm* did not affect the parameters frequently investigated in scientific research and routine analysis in poultry Similarly, in the tube content experiment, no statistical difference was found in other parameters.

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INTRODUCTION

Blood plasma tests are widely used in the laboratory to diagnose of various diseases, and monitor treatments. Plasma is isolated from whole blood by using centrifugation (Anderson & Anderson, 2003). The purpose of centrifugation is to remove blood cells without changing the plasma composition. Cadamuro et al., (2018) reported that centrifugation speed, centrifugation time, and temperature were found to affect sample quality. Prolonged centrifugation at high speed can lead to hemolysis or structural damage to the measured sample, while short centrifugations at low speed can lead to insufficient separation of plasma or serum from cellular blood components (Lippi et al., 2008).

Anticoagulants are additives that prevent blood or plasma from clotting and it has been reported that this substances don't cause a significant difference in analytical process (Guder, 2010). Anticoagulation is achieved by binding calcium ions (ethylenediaminetetraacetic acid [EDTA]) or inhibiting thrombin (heparin). It has been known that the most preferred anticoagulant in chemical analyzes is heparin. Conversely, EDTA is especially useful for hematology. Laboratory tests may also require biochemical analyses other than hemograms. Therefore, analysis should be performed with anticoagulants for plasma parameters (Mohri et al., 2007). As anticoagulants, except for EDTA, heparins (low molecular weight and standard), warfarin, and recently developed oral anticoagulants are also used (Işık & Kozak, 2024).

Heparin is a substance that prevents the conversion of fibrinogen to fibrin (Baien et al., 2018), and used to exeminate presence of electrolyte, gas, and, alcohol in blood. However, due to acidic structure of heparin, it is not recommended for use in morphologic analyzes such as white cell and platelet counts, and polymerase chain reaction (PCR) diagnosis. EDTA is recommended as an anticoagulant in hematologic tests because it best preserves cellular components and blood cell morphology (Vertiprakhov et al., 2021).

It has been reported that blood samples for coagulation studies in birds should be collected in plastic or silicone tubes containing 3.8% sodium citrate. The principle is based on inhibiting clotting in the blood sample by binding calcium with sodium citrate. (Kaneko et al., 2008).

There is sufficient information in the literature about the effect of different types of anticoagulants on blood parameters in humans (Li et al., 2013) or animals (Rezaei et al.,2022), but there is limited information about the use of tubes for analyzing bird blood samples (Guzman et al., 2008). Keskin (2020) reported that he separated the serum by applying the centrifugation speed at 3.000 *rpm* for 5 minutes in his study in rats. Kara et al., (2024) used K_2EDTA as a coagulant in their study with Kangal Dogs for hematological analyses. It is known that, unlike mammals, mature erythrocytes in birds have nuclei and are larger than mammalian erythrocytes (Jones, 2015). In this context, in order to obtain accurate analysis results, it is essential to select the right tubes during blood sample collection. Therefore, the present study was aimed to compare the effect of different blood tubes and increasing centrifugate speeds on blood parameters in poultry.

MATERIAL and METHOD

Animals and Keeping

In the present study, 16 Gerze breed cocks at the age of 35 weeks were bred at Bahri Dağdaş International Agricultural Research Institute were used.

The decision of the ethics committee of the study was obtained from the Bahri Dağdaş

International Agricultural Research Institute Animal Experiments Local Ethics Committee, with the decision dated 31.03.2023 and numbered 155. The animals were kept in a cage system measuring in 4 m width × 3 m length × 1.5 m height, and 80 cm from the ground for 6 weeks. The cocks were fed *ad libitum* with the ration given in Table 1 and had uninterrupted access to water with a nipple system.

Table 1. Ration content given to cocks

Raw materials	%	Nutrients	Diet
Wheat	27.5	DM, %	90
Corn	32.5	CP,%	22
Sunflower oil	1.2	ME,kcal/kg	2800
Soybean meal, %48	30	Ca,%	1
Marble powder	1.35	Ash %	0.43
Dicalcium phosphate	1.6	Na,%	0.2
Salt	0.35	Cl	0.26
Vitamin-mineral mixture	0.25	Met+Sis,%	0.75
		Lysine,%	1.12
		Threonine,%	0.81
		Tryptophan,%	0.3

Tube Content

In this part of the study, it was investigated the effects of blood tubes with different contents on biochemical parameters. Fort his, 8 cocks were slaughtered. Only the left *vena jugularis* was cut with a sterile lancet to avoid mixing with arterial blood. In this study, 6 ml capacity tubes with plain-tubes, 6 ml capacity Lithium Heparin tubes, and 6 ml capacity K_3EDTA tubes were used. Approximately 3 ml of blood was taken into each tube. The obtained blood was rapidly centrifuged at 3000 *rpm* for 10 minutes, and, the obtained serums and plasmas were sent for biochemical analysis rapidly.

Centrifugation Speed

In this part of the study, 8 cocks, which were different from the first part, were slaughtered. Only the left *vena jugularis* was cut with a sterile lancet to avoid mixing arterial blood, and blood was taken from each animal into 6 ml lithium-heparinized tubes only, to not exceed 3 ml. The excess blood in the tubes was then removed with an Ependorf pipette. Thus, a net of 3 ml of blood was obtained in each tube. To investigate the effect of centrifugation speeds on the samples obtained, plasmas were obtained by centrifugation at 3000 *rpm*, 4000 *rpm*, and 5000 *rpm* for 10 minutes. Then the plasmas were collected with an insulin syringe, and the amount of plasma was determined and sent for biochemical analysis rapidly.

Blood Analysis

Glucose, triglyceride, total cholesterol, HDL- cholesterol, LDL- cholesterol, total protein, inorganic phosphorus, calcium levels and, ALT (alanine aminotransferase), AST (aspartate aminotransferase) activities were analyzed in the serum and plasma samples. In the study, attention was paid to the presence of carbonhydrate, lipid, protein, lipoprotein, enzymes, and mineral substances in the parameter set, which is commonly investigated in poultry studies. Analyses were performed with an auto analyzer. (Abbott-Architect C8000- USA).

Statistical Analysis

The SPSS 25.0 package program was used for statistical analysis. The distribution of the data set was analyzed by Shapiro-Wilk and it was determined that the data set was not normally distributed. For this reason, a nonparametric Kruskall-Wallis test was applied to determine the differences. The pairwise comparisons of groups were made using Mann-Whitney U test with Bonferonni correction.

RESULTS

In the effects of tube contents on biochemical parameters, no statistical difference was observed in the parameters value ($p>0.05$). Although there was no statistically significant difference in other parameters; the total protein value was found to be lowest in the plain-tube; glucose, HDL-cholesterol, inorganic phosphorus levels, and AST activities were found to be lowest in the K_3EDTA tube; triglyceride, total cholesterol, and LDL- cholesterol levels were found to be lowest in the lithium heparin tube (Table 2).

Table 2. Effect of tube content on biochemical parameters (n=8)

	Plain Tube (Serum)	K_3 EDTA (Plasma)	Lithium Heparin (Plasma)	p value
Total Protein (mg/dl)	44.47 ± 1.28	47.08 ± 1.42	47.32 ± 1.58	0,267
Glucose (mg/dl)	275.25 ± 10.19	273.50 ± 10.74	280.75 ± 10.66	0,696
Triglyceride (mg/dl)	48.62 ± 8.53	57.37 ± 8.58	40.75 ± 5.40	0,177
Total Cholesterol (mg/dl)	99.37 ± 4.19	96.12 ± 4.20	94.25 ± 4.60	0,357
LDL-cholesterol (mg/dl)	203.12 ± 31.08	177.12 ± 29.94	174.01 ± 26.24	0,727
HDL-cholesterol (mg/dl)	69.33 ± 2.80	66.93 ± 2.65	68.70 ± 2.83	0,605
Inorganic phosphorus (mg/dl)	3.85 ± 0.24	3.75 ± 0.23	3.78 ± 0.26	0,75
Calcium (mg/dl)	10.63 ± 0.14	< 2	11.07 ± 0.19	0,792
AST (U/L)	240.50 ± 8.66	219.12 ± 7.61	235.75 ± 11	0,226
ALT(U/L)	< 6	< 6	< 6	-

Although there was no statistically significant result on the effect of centrifugation speed on biochemical parameters; the total protein value was found to be the lowest at 4.000 *rpm*; with glucose, total cholesterol, LDL-cholesterol, inorganic phosphorus, and calcium values at 3.000 *rpm*, and triglyceride, HDL-cholesterol, and AST activites at 5.000 *rpm* (Table 3).

Table 3. Effect of centrifugation speed on biochemical parameters (n=8)

	3000 *rpm*	4000 *rpm*	5000 *rpm*	p value
Total Protein (mg/dl)	47.0 ± 1.52	46.22 ± 1.38	46.52 ± 1.29	0,86
Glucose (mg/dl)	246.5 ± 6.18	250.62 ± 5.51	252.0 ± 7.36	0,691
Trigliserid (mg/dl)	34.0 ± 4.15	32.12 ± 4.66	31.25 ± 4.81	0,451
Total cholesterol (mg/dl)	93.50 ± 3.79	93.87 ± 3.80	94.12 ± 3.03	0,993
LDL-cholesterol (mg/dl)	197.0 ± 27.08	205.37 ± 27.10	214.25 ± 30.88	0,99
HDL-cholesterol (mg/dl)	67.0 ± 3.41	66.91 ± 3.49	66.45 ± 3.34	0,917
Inorganic phosphorus (mg/dl)	3.32 ± 0.2	3.41 ± 0.19	3.41 ± 0.2	0,885
Calcium (mg/dl)	10.36 ± 0.13	10.65 ± 0.06	10.75 ± 0.12	0,98
AST (U/L)	245.0 ± 14.12	237.12 ± 12.77	229.37 ± 10.93	0,692
ALT (U/L)	< 6	< 6	< 6	-
Amount of Plasma (ml^{-2})	126.62 ± 5.73	128.75 ± 6.55	129.50 ± 7.10	0,903
Visible Hemolysis	-	-	-	

DISCUSSION

Ethylenediaminetetraacetic acid (EDTA) is an organic chemical compound used in biochemical analysis, especially in whole blood counts, which prevents clotting by binding calcium ions in plasma. EDTA also has the function of chelating metallic ions. EDTA is the preferred anticoagulant because it does not change erythrocyte morphology, so ideal for use in hematology (Riba et al., 2020). Calcium and phosphorus levels in poultry are very important parameters in fertility, egg production, and shell quality. In the present study, centrifugation speed did not affect calcium, and phosphorus levels. However, it was observed that K_3EDTA binds calcium, and the calcium analysis could not be performed (Table 2). Therefore, tubes that contain EDTA should not be preferred for the measurement of cations such as calcium, zinc, iron, and copper which have +2 valence in reactions. In the current study, ALT activites could not be measured by the biochemistry autoanalyzer, which is used in routine analysis, in different centrifugation speed trials, both in tubes with different contents. For this reason, ELISA or RIA methods which, are more sensitive, can be used for ALT analyzes. Additionally, ALT analysis can also be performed by spectrophotometric method which is more economic.

For glucose, total protein, total cholesterol, HDL-cholesterol, inorganic phosphorus levels, and ALT activites were analyzed in different tube contents (Table 2), it was observed that the levels were very close to each other, and it can be said that these measurements were not affected by the tube content. However, when triglyceride and LDL-cholesterol levels were examined, some numerical differences were observed, although there was no statistical difference. It is thought that the numerical difference between EDTA-tube and lithium heparinized-tube measurements in triglyceride levels may be important for scientific research. Also, in LDL-cholesterol levels, although there was no statistical difference between plain-tube and Lithium-Heparin tube measurements, it was thought that the numerical difference may affect scientific research.

In the present study, it was investigated whether blood tubes and centrifugation speed have an effect on routine analyses commonly used in scientific studies in poultry. It is known that mature erythrocytes in birds, unlike mammals, have nuclei and are larger than mammalian erythrocytes (Jones, 2015). The disintegration of erythrocytes and the spread of hemoglobin content outside the cell is called hemolysis (Türkmen et al., 2007). In the present study, it was thought that hemolysis might increase with increasing centrifugation speed, but according to the results, visible hemolysis was not observed at increasing centrifugation speeds ($p>0.05$). Except for the biochemical parameters, the increase in centrifugation speed did not affect the amount of plasma.

CONCLUSION

As a result, it was observed that the parameters frequently investigated in scientific research and routine analysis in poultry were not affected by centrifugation speeds up to 5000 *rpm* (Table 3). Similarly, except for calcium, no effect of tube content on other parameters was observed. The study's findings suggest that centrifugation speeds more than 3000 *rpm* are unnecessary. Blood tubes containing lithium heparin are approximately three times more expensive than plain-tubes. The using of plain-tubes in routine biochemical assays would be more cost-effective.

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Ethical Statement

Since retrospective data were used in this study, there was no need to obtain ethical permission.

Author Contributions

Research Design: Halil HARMAN (%40) Abdullah BİLİR (%30), Halil YAVUZ (%30)
Data Collection: Halil HARMAN (%100)
Research - Data analysis – Validation: Halil HARMAN (%40) Abdullah BİLİR (%30), Halil YAVUZ (%30)
Writing the Article Halil HARMAN (%40) Abdullah BİLİR (%30), Halil YAVUZ (%30)
Revision and Improvement of the Text Halil HARMAN (%40) Abdullah BİLİR (%30), Halil YAVUZ (%30)

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Conflict of Interest

There is no conflict of interest between the authors.

Sustainable Development Goals (SDG)
9 Industry, Innovation and Infrastructure

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Determination of the Prevalance of Toxoplasmosis in Cats with Immunochoromatographic Rapid Tests Kits in Kırıkkale University Veterinary Faculty Animal Hospital

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ABSTRACT

Toxoplasmosis is a zoonotic disease caused by *Toxoplasma gondii* that can cause disease in all warm-blooded animals. In the transmission cycle of the disease, cats serve as the primary/definitive hosts, and transmission occurs through direct and indirect oral ingestion of oocysts spread by the definitive hosts. To diagnose the disease, a variety of methods are employed, including fluorescent diagnostic techniques, indirect hemagglutination tests, modified agglutination tests, ELISA, polymerase chain reaction (PCR), Sabin-Feldman dye tests, and immunochromatographic rapid diagnostic test kits. In clinical settings, rapid diagnostic test kits are the preferred option due to their ease of access, cost-effectiveness, and rapid results. The objective of this study is to ascertain the prevalence of toxoplasmosis in cats in the Kirikkale region and to highlight the efficacy of rapid diagnostic kits in this regard. The study material consisted of 50 cats brought to the Kırıkkale University Veterinary Faculty Animal Hospital for diagnosis and treatment of various disease presentations. Toxoplasma was detected using rapid diagnostic kits. The diagnostic tests performed on the blood samples taken from the 50 cats for the purposes of diagnosis and treatment revealed that three of them were positive. The screening revealed a prevalence of toxoplasmosis in the sample population of 6%. It has been determined that cats can harbor this disease despite exhibiting symptoms compatible with toxoplasma. The use of rapid diagnostic kits for screening cats is a viable and practical solution. The study objective was to contribute to the development of control policies for cats in the context of public health and disease control policies. The results of this study will serve as a source of information for future studies.

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INTRODUCTION

Toxoplasmosis is a disease caused by the *T. gondii* and has a global distribution. It has a zoonotic potential and has been observed in all haematophagous animals, including birds and humans (Dubey 2010). This infection represents a significant public health concern due to its epidemiology and pathogenesis. It is commonly reported in humans and animals due to its easily transmissible nature (Dumanlı & Aktaş, 2010; Marquert et al., 2000). Despite the prevalence of the disease, the number of clinically diagnosed cases remains low (Dumanlı et al., 2013). Of particular concern is the congenital transmission potential of *T. gondii*, particularly in the asymptomatic form. This underscores the importance of addressing the disease for the benefit of future generations (Jones et al., 2003).

Toxoplasma has a complex life cycle that includes 3 stages as trophozoits, bradyzoits and sporozoits (Mevelec et al, 2020). Trophozoites are responsible for acute infections in tissues and can spread to almost all organs quickly. Most of the pathologies are composed by trophozoites (Bernal and Gennari, 2019). Bradyzoites are found in cysts formed by trophozites. They cause a life-long chronic inflamation and procure the hosts immunity stability (Di Cristina et al, 2008). Sporozoits include sporants and oocycts in the intestinum, and has the ability to actively infect (Dubey and Frenkel, 1972; Tenter et al, 2000; Dumanlı et al, 2013).

Toxoplasmosis has two hosts in its transmission cycle. Felidae serve as the definitive hosts, while other warm-blooded animals act as intermediate hosts. Additionally, toxoplasmosis can result in asexual proliferation in felidae, with this family serving as an intermediate host (Bernal and Gennari, 2019). The disease is transmitted via direct oral ingestion of oocysts, which are spread in definitive hosts by water or food contaminated with oocysts. In addition, the disease can be transmitted by ingestion of tissues containing trophozoites or bradyzoites (Karakavuk et al., 2021). Following ingestion of oocysts and cysts by intermediate hosts, sporozoites and trophozoites are released into the intestinal lumen. Once the oocysts have passed the intestinal epithelial barrier, they undergo endodyogeny in the parasitophorous vacuoles of various cells. Consequently, the trophozoites develop and spread throughout the organism. It is also possible for trophozoites to infect the fetus, which can result in disruption of the placental barrier. (Courret et al., 2006; Persson et al., 2009). The acute clinical picture is caused by trophozoites, while the latent, chronic, lifelong presentation is caused by encysted bradyzoites. (Chen et al., 2022).

Toxoplasmosis can be identified by detecting *T. gondii* antibodies, which has remained a relevant diagnostic method over the past decade. Numerous factors that influence the prevalence of the disease, with a particularly high prevalence observed in feral cats (Dubey et al., 2020). It should be noted that the prevalence of the agent varies from region to region within the same country. In studies employing indirect and immunofluorescent antibody tests, the antibody seroprevalence of the agent has been reported to range from 15% to 82% in Brazil (Munhoz et al., 2017; Neto et al., 2018; Cardia et al., 2013). In China, the rate was found to be between 11% and 63% using indirect agglutination methods (Qiu et al., 2020; Hou et al., 2018; Cong et al., 2018). In Egypt, the rate is approximately 95% using a modified agglutination test (Al-Kappany et al., 2011). A study conducted with 1,490 animals in Thailand revealed a prevalence rate of 4.8% (Jittapalapong et al., 2010). In Turkey, the prevalence rate has been reported to range from 34% to 66% using the Sabine Feldman Dye method (Yücesan et al., 2019; Ercan & Kırmızıgül, 2019).

There is still no clarity regarding the diagnosis of toxoplasmosis (Dubey, 1995). Toxoplasmosis can be identified through several tests, including flourescent tests, indirect hemagglutination tests, modified agglutination tests, ELISA, PCR, Sabin-Feldman dye, and immunochromatographic rapid tests

(Lappin et al. 1989; Liesenfeld et al. 1996). The materials used in rapid tests for detecting *T. gondii* antigens or antibodies vary, but the underlying working principle is consistent across all tests. The objective of this study is to identify the prevalence of *T. gondii* with lateral flow immunochromatographic rapid tests (RIDXTM Toxoplasma Ab Test, Korea), which include surface antigen (SAG 1: p.30) + dense granule protein (GRA 1: p.24). These rapid tests are designed to detect antibodies to *T. gondii* in blood samples. The objective was to evaluate the prevalence of toxoplasmosis in Kırıkkale and assess the threat to human and animal health.

MATERIAL and METHOD

This study was approved by the Kırıkkale University Animal Experiments Local Ethics Committee (Approval no: 22.07.2024-E.265544).

Study Material

The animal material of the study was comprised of 50 cats from Kırıkkale University Veterinary Faculty Animal Hospital. The study population was consisted of domestic and shelter cats. All cats' ages are >1 year and the presence of any clinical symptoms compatible with toxoplasmosis was not sought for incorporation in the animals to be included in the study

Sample Collection and Implement Rapid Tests

Blood samples were collected from animals' vena cephalica antebrachiums to collecting tubes as 2 milliliters. These samples were centrifugated at 3000 rpm for 10 minutes to obtain serum. Rapid diagnostic tests were performed in the same day for detecting toxoplasmosis antibodies. 10 microliters of the obtained serum samples were added to the sample well on the rapid diagnostic test kits (Asan Easy Test® Seoul, Korea) and then 15 microliters of toxoplasma reagent was applied to the same well. 10 minutes later results were recorded. Seeing the control line on the result area was searched all rapid test kits for determine the activation of test kits. If the test section line was seen, the result was evaluated as positive.

Statistical Analyses

In this study the prevalance of toxoplasmosis was calculated with descriptive statistical methods. Positive results were calculated as a percantage

RESULTS

The efficacy of immunochromatographic rapid tests was evaluated in a cohort of 50 cats. The test results are presented in Table. Three cats had positive results with toxoplasmosis (%6) and they were older than 3 years old and youger than 4 years old. In the study, nine cats' blood samples were collected for the purpose of detecting whole blood results prior to the administration of routine vaccinations. No positive results for toxoplasmosis were identified in these cats. The positive cats had different clinical symptoms consistent with various diseases. Stranguria was seen on the first positive cat. The second cat showed diarrhea, and the third cat had ocular lesions.

Table. The ratio and numbers of antibody positive cats

	Animals	**Rapid Test Result Positive**	**Rapid Test Result Negative**
n	50	3	47
% (ratio)	100	6	94

DISCUSSION

Cats are considered the definitive host for toxoplasmosis. This disease is the most common protozoal pathogen in humans and is found worldwide in both humans and warm-blooded animals. (Jones et al 2018). There are many studies on the seroprevalence of toxoplasmosis. According to these studies, *T. gondii* antibody ratio was %62.3 in Albania with IFAT (Silaghi et al 2014). In Algeria seroprevalence of toxoplasmosis was found to be %50 (Yekkour et al 2017). In Iran, using different serologic diagnostic methods, the seroprevalence was between %2.7 and %82.2 (Derakhshan ve Mousevi 2014, Hamidinejat et al 2011, Asgari et al 2018). It was reported that the percentage of toxoplasmosis antibodies in Iraq was between %30.4 and %45.5 (Al-Rahmani et al 2010, Switzer et al 2013). In Türkiye, the presence of antibodies ranging from %34.2 to %66.6 was detected in studies conducted using IFAT, ELISA and dye test methods (Yücesan et al 2019, Can et al 2014, Ercan and Kırmızıgül 2019, Erkılıç et al 2016). It is reported that this situation reaches up to %81 in Europe (Dubey et al 2020). In another study, the seropositivity of toxoplasmosis was found to be 48% using the Sabin Feldman test in previous years in Türkiye (Yasa Duru et al, 2017). In this study, the ratio of toxoplasma positive animals was found to be %6. We thought that this proportional difference between the two studies was related to the diagnostic method. In a recent study conducted with a rapid diagnostic kit in another region of our country, 5.5% of cats were found seropositive (Aktemur, 2021). It seems that the results of this study are compatible with studies performed with the same method. Studies show that toxoplasmosis is still active in our region. It is reported to be found at very high rates in neighboring regions, as well as in humans and animals in European countries with similar climates (Dubey et al. 2020, Molan et al. 2019).

It was reported that toxoplasmosis was seen in cats living in urban areas more than those living alone (Abbas et al 2021). The agent continues to exist in central areas in cities over cats living in periurban regions. According to studies, it has been seen that cats can carry the agent even if they live alone at home (Sroka et al 2018). For cats that are definite hosts, regardless of what was the enviromental living conditions, the factor can persist to continue living. Enviromental conditions of Türkiye are suitable for the toxoplasmosis life cycle. The stray cat's population is not known exactly except that domestic cats number is detected as more and less because there is no system to find real population of domestic cats. As a definitive host for toxoplasmosis, Türkiye’s cat's living standards allow the disease to become a public health problem.

Toxoplasmosis occurs in cats of all ages, regardless of gender and breed (Dubey 2020). Pneumonia is the most common clinical symptom (Dubey 2010). Icterus, anorexia, vomiting, paresis and dermatitis take place in toxoplasmosis clinical table (Dubey 2020). Besides that oculer lesions are identified in infected cats. It was reported that retinochoroiditis, chorioretinitis, optic neuritis and anterior uveitis were detected (Ali et al, 2021). In the diagnosis of the disease, the presence of Ig G and Ig M is usually releaved serologically (Remington et al. 1995). Disease pathogenesis blocks the identification of agents. Antibodies can be found animals that haven’t any clinical symptoms (Dubey et al 2020, Ali et al 2021). In line with the aforementioned information, in this study, the presence of

antibodies to the agent was investigated regardless of clinical complaints. In the findings obtained, clinical complaints were detected in animals with positive disease, while antibodies could not be detected in healthy animals.

The diagnosis includes the serological presence of IgG and IgM antibodies in toxoplasmosis. There are several ways to detect antibodies serologically, including MAT, ELISA, PCR, IMX, Sabin-Feldman, and dye tests. It's also included in immunochromatographic-based diagnostic test kits (Luo et al., 2018). Onosakponome et al. (2020) found that the rapid tests had a specificity of 46.7% and a sensitivity of 81.7%. Hassaneina and Shehata (2018) also found that the rapid tests had a specificity of 54.4% and a sensitivity of 100% in humans. A study on using immunochromatographic rapid diagnostic test kits to diagnose toxoplasmosis in cats found that they had a specificity and sensitivity of 98.63% and 100%, respectively (Villanueva-Saz et al., 2023). Both human and veterinary studies show that rapid tests are useful diagnostic tools when laboratory techniques aren't available. In clinical settings, rapid tests can be a great way to diagnose toxoplasmosis.

CONCLUSION

In conclusion, we determined the prevalence of toxoplasmosis in cats in Kırıkkale. Determining the prevalence of toxoplasmosis, which has zoonotic potential for the region, is important for the existence of the disease, as assumes a role for cats in human cases as a source, and for establishing the control methods of toxoplasmosis. We recommended that larger studies be conducted on this subject using advanced molecular and serological diagnostic methods.

Ethical Statement

This research article has not been published anywhere before.

Ethics Committee Approval

This study was approved by the Kırıkkale University Animal Experiments Local Ethics Comuttee (Approval no: 22.07.2024-E.265544)

Author Contributions

Research Design (CRediT 1) Author 1 (%40) – Author 2 (%30) – Author 3 (%30)
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Determination of Some Technological Characteristics of *Streptococcus thermophilus* and *Streptococcus macedonicus* Isolated from Classical White Cheese Production Stages

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ABSTRACT

Lactic acid bacteria (LAB) are classified within the taxonomic family Lactobacillaceae family and are renowned for their pivotal roles in food safety and public health. Additionally, they contribute to the formation of aroma, flavour and texture in fermented foods. Among LAB, *Streptococcus* spp. is considered at the generally recognized as safe (GRAS) level, particularly for use as starter cultures in the dairy industry. The aim of this study was to ascertain the technological properties of *S. thermophilus* BST2001-BST2007 (7) and *S. macedonicus* BSGM2471-BSGM2471 (2). These were isolated from samples obtained at various stages of classical white cheese production, including acid forming capacity, proteolytic activity, viability at different pH, temperature and salt concentrations, and diacetyl production. The findings of the study demonstrated that the acid forming capacities of all isolates were similar during the initial 6th hour. However, at the 12th hour, the *S. thermophilus* BST2006 and BST2007 isolates decreased the pH of the medium to 4.45 and 4.46, respectively, resulting in the formation of a clot. At the end of the 24-hour period, it was observed that all *S. thermophilus* isolates had formed clots, whereas the *S. macedonicus* isolates had demonstrated a markedly reduced capacity for acid production and did not form any clots. In contrast, both species showed high proteolytic activity (zone diameter >10 mm) and were able to growth within a temperature range of 15-45°C. Isolates of *S. macedonicus* were observed to be unable to grow at a salt concentration of 7%. Additionally, diacetyl production was observed in four *S. thermophilus* isolates and one *S. macedonicus* isolate. These results indicate that there may be significant differences in technological properties even among the same LAB species. This highlights the importance of researching and introducing new LAB species to the sector to develop products with specific, targeted properties in the fermented food industry, particularly in cheese production.

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INTRODUCTION

LAB found in fermented foods are an important member of the Lactobacillaceae family. LAB play a significant role in the formation of aroma, flavor, and texture in the production of fermented foods, as well as exerting beneficial effects on food safety and public health (Widyastuti et al., 2014).

Although some species of *Streptococcus* spp. in the LAB group have been demonstrated to exhibit pathogenicity in humans and animals, others have been GRAS for human consumption. The most important species in this genus is *Streptococcus thermophilus*, which is widely used as a starter culture in production of various dairy products (Blaiotta et al., 2011). *S. thermophilus* is a homofermentative lactic acid bacterium that is employed as a starter in the manufacture of fermented milk products, including yoghurt and ripened cheese (Cui et al., 2016).

Another species, *Streptococcus macedonicus*, was first isolated from Greek Kasseri cheese, a naturally fermented product (Tsakalidou et al., 1998). *S. macedonicus*, a member of Streptococcus spp., is a Gram-positive and catalase-negative bacterium. It was isolated during the identification of lactic acid bacteria from different stages of classical cheese production without the addition of starter culture. It has been reported that the optimum temperature for the growth and bacteriocin production of this bacterium is 42.3°C and 20-25°C and the optimum pH is 6.4 and 6.0, respectively (Van den Berghe et al., 2006).

The current study isolated *Streptococccus* spp. from classical cheese production stages and characterized them genotypically using MALDI-TOF. A total of nine isolates belonging to two species were identified, along with some technological characteristics.

MATERIAL and METHOD

The aim of this study was to ascertain the technological properties of two species of *Streptococcus*, namely *S. thermophilus* (7) and *S. macedonicus* (2), isolated from the classical white cheese production steps. These species were evaluated for their acid formation, proteolytic activity, ability to survive at different pH levels, temperatures, and salt concentrations, among other characteristics.

Acid-Forming Capacity

To determine the acidification capacity of LAB strains, the protocol proposed by Terzić-Vidojević et al. (2015) was modified. In brief, the acidification capacity was determined by calculating the percentage of acidity in lactic acid through pH measurement and titration. For this purpose, skimmed UHT milk was incubated at 30°C for 15 days and 55°C for 7 days, and no leakage or bombing of the packaging was observed after incubation. To 10 mL of UHT skimmed milk added into sterile test tubes, 0.1 mL (v/v) of 18-hour-old fresh cultures (0.5 McFarland) were added and incubated at 30°C for 3, 6, 12 and 24 hours. At the end of each incubation period, including the initial 0-hour period, 2 mL samples were aseptically taken from the tubes, and pH was measured with a pH meter (HANNA HI2211). Subsequently, titration was performed.

Determination of Proteolytic Activity

The proteolytic activity was evaluated in accordance with the protocol proposed by Raveschot et al. (2020). In brief, LAB were cultured in M17 broth (Biolife, LB.BL.4017202,) and the final optical density at 600 nm (OD600) were adjusted for each fresh culture. From each prepared cell suspension,

20 µl was added to wells (well diameter 4 mm) in Skim Milk Agar (10%) and incubated at 37°C for 72 h. At the end of the incubation period, the proteolytic activity was evaluated based on the zone diameters formed around the wells: A zone larger than 10 mm was considered to indicate very high activity, a zone between 3 and 10 mm indicated high activity, and a zone smaller than 3 mm indicated low activity (Alapont et al., 2015).

The Ability to Grow at Different Temperatures

To assess the capacity to exhibit growth at varying temperatures, LAB isolates were inoculated into 10 mL M17 broth media at a rate of 1% and incubated at 10˚C, 15˚C and 45˚C for a duration of 48 h. At end of this period, the isolates that showed growth by forming turbidity in the medium were designated as positive, and those that did not form turbidity were designated as negative.

The Ability to Grow at Different NaCl Concentrations

To determine the growth ability of lactic acid bacteria at different NaCl concentrations, 1% of fresh bacterial cultures were inoculated into M17 broth media containing 2% and 6.5% NaCl, respectively, and incubated at 37°C for 24 h. After the incubation period, turbidity formation in the media was considered positive and those without turbidity formation were considered negative.

The Ability to Grow at Different pH

In this study evaluated the ability LAB cultures to grow at two different pH values (3.9 and 9.6). To this end, 10 ml of M17 broth with pH values of 3.9 and 9.6 were inoculated with 1% of active cultures and then to incubation at 30°C for 48 h. The results were considered positive in the tubes with turbidity and negative in the tubes without turbidity.

Diacetyl Production

Diacetyl production was evaluated in accordance with the protocol proposed by Franciosi et al (2009). Briefly, 0.1 mL of fresh cultures of LAB were transferred to 10 mL of sterile skimmed milk (10% w/v) and then incubated at 30°C for 24 h. Following incubation, 1 mL of each bacterial culture was transferred to sterile tubes, 0.5 mL of α-naphthol (1% w/v) and KOH (16% w/v) were added and incubated again at 30°C for 10 minutes. Observation of a red ring at the top of the tubes was considered positive for diacetyl production.

RESULTS

The technological properties of *S. thermophilus* and *S. macedonicus* isolates obtained from different stages of classical white cheese production were determined for their use as natural (autochthonous) starter cultures in some fermented milk products, such as yoghurt and cheese. According to the results of the acid-forming ability test, the pH of the medium changed between 5.82-6.23 at the 6th hour of incubation. Meanwhile, *S. thermophilus* BST2006 and BST2007 isolates decreased the pH of the medium to 4.45-4.46 and formed clots at the 12th hour of incubation, respectively. At the end of the 24th hour of incubation, it was observed that *S. thermophilus* isolates decreased the pH of the medium to 4.31-4.99 and complete coagulation occurred in the medium. On the other hand, the titration acid ratios of the medium in terms of lactic acid were found to vary between 0.18-0.27 at the 3rd and 6th hours of incubation and between 0.25-0.40 at the end of the 12th h. (Table 1).

It was determined that all strains had high proteolytic activity (zone diameter >10 mm) and could grow in the temperature range of 15-45°C. It was observed that isolates of *S. macedonicus* BSGM2471-

BSGM2471 were unable to grow at a salt concentration of 7%. Diacetyl formation was found to be dependent on the strain, rather than the species. A discrepancy in diacetyl production was observed between *S. thermophilus* and *S. macedonicus* strains belonging to the same species (Table 2).

DISCUSSION

LAB are widely used in cheese and yoghurt production due to their contribution to the flavour, texture and nutritional value of fermented products (Mokoena et al., 2021). LAB are well adapted to environmental conditions such as low pH, high NaCl, anaerobiosis and the presence of fermentable carbohydrates. Therefore, significant differences in their technological characteristics may occur between members of the same species. It is of paramount importance to investigate novel species with novel and advantageous characteristics and to disseminate them throughout the industry (Montel et al., 2014; Cui et al., 2016).

The acid-forming capacity of *S. thermophilus* is a determining factor in the duration and quality of dairy production, while its proteolytic activity affects the formation of flavouring substances (Cui et al., 2016). The results of this study indicated that *S. thermophilus* BST2006 and *S. thermophilus* BST2007 isolates reduced the pH to 4.45 and 4.46 by the end of the 12th h, respectively, and coagulation was observed in the medium. This suggests that these two isolates were more effective at acidifying the medium. Additionally, it was observed that these two isolates had high proteolytic activity (Table 1; Table 2). Galia et al. (2019) reported a close correlation between proteolytic activity and acidifying capacity of *S. thermophilus* strains. Some *S. thermophilus* strains cannot lower the pH below 5.2 and are therefore often used in combination with other starter bacteria. *S. thermophilus* proteolytic activity plays a role in releasing peptide sequences from caseins and whey proteins during lactic fermentation (Rodríguez-Serrano et al., 2018). Gaglio et al. (2014) reported that the pH values and diacetyl formation of the *S. thermophilus* strains identified in the Vastedda cheese, a raw sheep's milk product produced without the addition of a starter culture and protected by the European Union in Italy, exhibited strain-specific variations. These findings are like those of the current study.

In the present study, two isolates of *S. macedonicus* were identified from samples obtained at different stages of the production of a classical white cheese. It was determined that both *S. macedonicus* BSGM2470 and *S. macedonicus* BSGM2471 isolates decreased the pH of the medium to 5.06 and 4.69, respectively, by the end of the 24th h. Furthermore, an observation was made that the technological properties of both isolates were like each other (Tables 1; Table 2). Among the *S. macedonicus* (L36-L37) isolates that were isolated from white cheese samples, the L36 isolate was found to have high acid-forming capacity (Meral Aktaş and Erdoğan, 2022). It has been reported that *S. macedonicus* species has weak acidification, low proteolytic and citrate catabolizing activity in milk. However, it was found to have moderate lipolytic activity (Georgalaki et al., 2000). In contrast to the findings of Georgalaki et al. (2000), the *S. macedonicus* BSGM2470 and BSGM2471 strain identified in this study was found to have high both acidification and proteolytic activity. De Vuyst and Tsakalidou (2008) reported that *S. macedonicus* has lipolytic and proteolytic activity, and some strains of *S. macedonicus* were also characterised by exopolysaccharide and bacteriocin production. Gaglio et al. (2014) reported that the pH values of *S. gallolyticus* subsp. *macedonicus* strains identified from Vastedda cheese exhibited variation depending on the strain the end of 8th and 24th h. The findings of this study are like to the findings of our study. The same researchers also found that diacetyl production varied depending on the strain. De Leonardis et al. (2013) stated that diacetyl production may vary depending on the milk structure and the strain of lactic acid bacteria. In their study, they reported that *L. paracasei* and *L. rhamnosus* produced diacetyl better than *S. thermophilus*. Pacini et al. (2006) detected *S. macedonicus* in traditional Italian cheese samples, while Renye et al. (2011) detected the same species in traditional

Mexican cheese samples produced from both raw milk and pasteurized milk. Demir and Kaptan (2025) isolated *S. macedonicus* from Edirne white cheese and reported that further research on the technological properties of this bacterium is needed. In this study, some technological properties of *S. macedonicus* (e.g. acid-forming capacity, proteolytic activity) were revealed. The findings of both studies are like the findings of our study. On the other hand, according to the literature review, it was observed that there are limited number of studies on the technological properties of both bacteria.

CONCLUSION

In the last decades, there has been an increasing focus on scientific research investigating the identification and technological characteristics of indigenous starter culture bacteria in the production of fermented dairy products, especially in Europe. This study showed that the technological properties of *S. thermophilus* and *S. macedonicus* strains isolated during the white cheese production process exhibited species-dependent variations. The findings of this study suggest that the technological properties of these bacteria can be used as autochthonous starter cultures in the production of fermented milk products such as yoghurt and cheese.

Ethical Statement

According to Article 8(k) of the Regulation on the Working Procedures and Principles of the Ethics Committees on Animal Experiments, Ethics Committee Approval is not required.

Author Contributions

Research Design (CRediT 1) Author 1 (%100)
Data Collection (CRediT 2) Author 1 (%100)
Research - Data analysis - Validation (CRediT 3-4-6-11) Author 1 (%100)
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Conflict of Interest

Author declares that there is no conflict of interest.

Sustainable Development Goals (SDG):

3 Good Health and Well-Being
12 Sustainable consumption and production

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Green Synthesis of Silver Nanoparticles (PA-AgNPs), Characterisation, Antibacterial and Proliferative Effects

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ABSTRACT

Currently, the creation of several novel materials has accelerated the fields of nanoscience and nanotechnology. The production of nanoparticles is being developed using a variety of physical, chemical and even biological processes. The biological approach is becoming increasingly popular among these methods because it is easy to use, requires modest operating conditions, and produces waste and products in a more environmentally friendly manner. Green nanoparticles can be synthesised using a variety of components and biochemicals found in plants, which can act as stabilising and reducing agents. There is great potential for AgNPs, particularly in the medical applications or biomedical field. The most common application of nanoparticles has been as antibacterial agents. In this study, AgNPs were synthesised using the green synthesis method. The antibacterial and proliferative effects of these synthesised nanoparticles were investigated. In this direction, yellow cherry (*Prunus avium* L.) belonging to Konya/Ereğli region was harvested at appropriate time and conditions. The extract of the collected yellow cherry was used as a reducing agent to obtain Ag nanoparticles. The extract obtained from the collected yellow cherry, rich in polyphenols and flavonoids, served as a reducing agent for AgNP synthesis. The Prunus avium-AgNPs (PA-AgNPs) were found to be spherical with an average size of 40.98 ± 12.45 nm based on SEM analysis. The synthesised Ag-NPs were shown to be effective against S. aureus and E. faecalis bacteria based on their antibacterial activity, which was assessed using the disc diffusion technique. At the same time, a healthy cell line (L929) was used to assess the proliferative effect using the MTT technique. The results showed that the IC50 (concentration that inhibits 50%) of the cells was 46 µg mL-1 at 24 hours, 92.5 µg mL-1 at 48 hours and 105 µg mL-1 at 72 hours. In this study, silver nanoparticles were synthesized with an environmentally friendly approach using the extract obtained from the fruits of Prunus avium, which is rich in bioactive content. Compared to AgNPs synthesized by chemical methods, particles synthesized by biosynthesis (green synthesis) method showed much lower cytotoxic effects.

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INTRODUCTION

Nanotechnology is one of the emerging branches of science with a variety of applications in the biomedical field (Mustapha et al., 2022). The aspects that green nanotechnology, a subset of nanotechnology, has recently revealed at the molecular level have attracted much attention (Uyaner et al., 2024). These products continue to be a developing field of study with their use in the treatment of various diseases in many fields, especially in medicine (Ramazanli and Ahmadov 2022). A new class of useful materials are nanoparticles. Among the useful superior properties that NPs offer during synthesis is their resistance to high temperature fluctuations (Syafiuddin et al., 2017). The field of biomedical applications is very interested in metallic NPs. Metallic nanoparticles can be created using a variety of chemical, physical and biological methods (Keskin et al., 2023). Physical and chemical methods are time and energy consuming, expensive and not environmentally friendly. Various enzymes, algae, microbes and plants are used for the biological synthesis approach. Green synthesis of NPs using plant extracts has become a promising technique for the production of metallic NPs, especially in recent years. It offers significant advantages, including the ability to synthesise NPs in a straightforward, rapid, inexpensive and environmentally friendly manner, as well as ease of scaling, a less biohazardous structure and protection of cell lines (Jabir et al., 2021). Nanometals, particularly gold (Au), zinc (Zn), silver (Ag), palladium (Pd) and titanium (Ti), are often preferred in areas such as drug delivery systems, biological markers and the manufacture of optical devices. Furthermore, these metals are not only used in the synthesis of NPs, but also in some processes such as increasing their stability (Emanuel et al., 2015). In addition, NPs are useful substances with applications in food and cosmetics, medical applications, bioremediation research, etc. (Khalilov, 2023). Silver NPs are being studied by scientists in many different fields due to their unusual physicochemical structures among metal nanoparticles (Khan et al., 2023). In a variety of scientific fields, including biomedical research, AgNPs are extensively used in antibacterial, antiviral, anti-inflammatory and anti-cancer treatments. Several biomedical articles that inhibit infection are made with AgNPs (Jumah et al., 2020). Silver was used to heal infected wounds before antibiotics were used in modern medicine, but its use has declined due to its high toxicity and the accessibility of antibiotics (Singh et al., 2018). Antibiotics have become the most preferred group of drugs for the treatment of infections in human and animal health, and even in aquaculture production. However, excessive use of this group of drugs causes antibiotic resistance in organisms and their presence in large quantities in wastewater has been analysed (Aydın et al., 2024). Scientists have recently become interested in AgNPs, partly because of their potential use as antibacterial agents (Gunashova, 2022). Much research has used biological methods and plant sources (leaf, flower, root, fruit or whole plant) to produce AgNPs (Namburi et al., 2021). In particular, the fact that AgNPs produced in synthesis studies using plant sources are environmentally friendly and do not require special conditions, together with the fact that the synthesis process is simple, inexpensive and produces a larger quantity of product, are some of the factors that are driving interest in biological approaches (Rather et al., 2022). Due to its non-toxicity, environmental friendliness, affordability, ease of scalability, and greater yields than chemically synthesized AgNPs, green synthesis AgNPs have gained more attention from researchers in the literatüre (Asif et al., 2022). Plants, especially their fruit parts, are rich sources of potentially bioactive compounds such as phenolic acids, flavonoids, coumarins, and various organic acids. In extracts derived from plant sources, phenolic compounds, alcohols, flavonoids, and phytochemicals with carboxyl groups are substances that decrease the positively valued silver in the aqueous structure, create silver nanoparticles, and also have an impact on stability (Keskinkaya et al., 2023; Srikar et al., 2016). The use of NPs to be obtained using the green synthesis method as reducing agents, such as minimizing carbon footprint, sustainability, and reducing product toxicity, supports environmental protection efforts compared to chemical reducing agents (Kazak, 2024).

In addition to providing nutrients, fruits and vegetables also naturally contain antioxidants through secondary metabolites. Due to their positive impact on human health, antioxidants are gaining attention. They help prevent the damaging free radicals that cause cancer, heart disease, and many other disorders (Shui et al., 2006). Both naturally occurring oxidation and reduction processes in living things and dangerous sources including radiation, viruses, air pollution, and toxic byproducts of cell metabolism can produce free radicals. Lipids, proteins, nucleic acids, and other pathogenic processes are all oxidatively damaged by these reactive species. The relevant reactive species are known to contribute to aging, cell damage, and tissue damage by causing molecular alterations and gene mutations in cells (Ugur et al., 2023). The human body benefits from antioxidants because they neutralize free radicals and stop them from forming by giving up their electrons. They delay or stop the oxidation of the substrate that causes oxidative damage and are present in foods and the body in lower quantities than oxidizable substrates (Buyuktuncel et al., 2013). The fruit of the genus Prunus, which belongs to the Rosaceae family, is *Prunus avium* (Usenik et al., 2008). *P. avium* is one of the most popular temperate climate fruits. According to reports, one of the natural antioxidants is the white cherry, Prunus avium (Pszczola, 2001 It is a fruit with high consumption due to its sweetness, rind color, and sugar content. It has been reported that sweet cherries contain various phenolics, anthocyanins, flavonols and flavan-3-ols, and hydroxycinnamate (Gonçalves et al., 2004; Usenik et al., 2008). Based on this, we hypothesized that *P. avium* fruits containing flavonoids, ellagitannins, and anthocyanins could be applied in the green synthesis of AgNPs.

In this study, non-toxic, environmentally friendly, cost-effective, easily scalable PA-AgNPs were collected during the harvest of Yellow Cherry (Prunus avium L.), extracted and produced by green synthesis method. The functional groups of the synthesized nanoparticles were evaluated by FTIR measurements and their morphological properties were evaluated using SEM. Using this cherry from Ereğli district of Konya province, high bioactive content and efficient PA-AgNPs were obtained. The antibacterial activity of these green nanoparticles (PA-AgNP) was evaluated using disc diffusion method (*E. coli, S. epidermidis, S. aureus and B. Subtilis*). MTT method was applied to evaluate the proliferative effect on the cell line L929 (Mouse Fibroblastic Cell Line) in accordance with ISO standards. In this study, AgNP, which is synthesized for the first time by green synthesis method of Prunus avium extracts, one of the natural foods with high bioactive content, was synthesized and its antibacterial and proliferative effect potential was successfully evaluated. Moreover, this study is an innovative approach in obtaining many products with high bioapplicability in the environmentally friendly medical field thanks to green nanotechnology by using plants with various biocomponents.

MATERIAL and METHOD

Preparation of Prunus avium Extract and Synthesis of PA-AgNP

Prunus avium (yellow cherry) species was obtained from Konya/Ereğli public markets and after the samples were sorted and cleaned, they were stored at -20^{o}C. The fruits were brought to room temperature and squeezed with a press to extract the juice for use in the green synthesis study. The prepared extract was filtered using filter paper. About three hours were spent stirring a 100 mL volume solution with 0.1 M concentration AgNO3 salts (metal nitrate) and 20 mL of cherry extract at 60^{o}C using a magnetic stirrer (Atacan et al., 2023). Then, this precipitate nanoparticle was pyrified and dried at 60 ^{o}C.

Characterization Analysis of PA-AgNPs

The biomolecules' functional groups were identified using FTIR measurements in order to assess how well the produced Ag NPs were stabilized. SEM, or scanning electron microscopy, was used to examine the morphological characteristics of the produced nanoparticles.

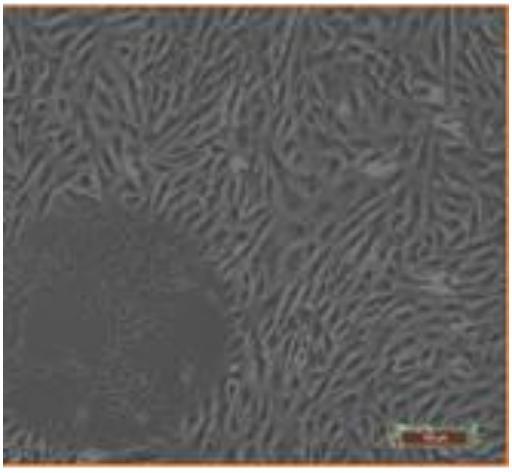
Assessment of PA-AgNPs' Antibacterial Activity

In our study, the disk diffusion method, which is one of the most preferred methods to evaluate the antibacterial activity of the PA-AgNPs, was used. In the study, standard strains of Escherichia coli, Bacillus subtilis, Staphylococcus epidermidis and Staphylococcus aureus, which are commonly found on surfaces and cause hospital infections, were obtained from Sakarya University Microbiology Laboratory. Results were interpreted according to the National Committee for Clinical Laboratory Standards.After dissolving the prepared PA-AgNPs in 1% DMSO, the concentration was adjusted to 1 mg mL-1. Then, as a control group, 30 µL of 1% DMSO, PA-AgNP and yellow cherry extract were impregnated onto sterile discs with a diameter of 6 mm. Using a densitometer, suspensions with a density of 0.5 Mcfarland (108) were prepared from fresh cultures (24 h old). Using a swab, samples from this solution were added to Mueller Hinton agar medium. Pre-prepared sample-impregnated discs were placed in Petri dishes into which bacteria were inoculated. After incubation (24 hours at 37 °C), the inhibition zones formed in the Petri dish were measured using a digital caliper and the results analysed (Semerci et al., 2020). Gentamicin-loaded discs were used as positive controls. All studies were performed in 3 replicates under aseptic conditions and the results were given as mean.

Cell Line and Cell Culture Medium

The cell line L929 (Mouse fibroblastic cell line), which complies with the UNI EN ISO 10993/2009 standard, was used to investigate the proliferative effect of green synthesis PA-AgNPs (Figure 1) (Torabi et al., 2023; Cannella et al., 2019). DMEM High Glucose (2 mM L-glutamine) was used as the culture medium. To avoid contaminating the culture media, Penicillin/Streptomycin was added at a dosage of 1 mg mL-1 in addition to supplementing the culture medium with fetal calf serum (10% (v/v)) (fetal bovine serum, FBS). The mouse fibroblastic cell line was multiplied by incubating in an incubator (at 37°C, 5% CO2). When the monolayer (adherent) cells filled 85% of the medium in which they were grown, they were enzymatically separated from the surface of the culture vessel and passaged. Thus, the storage and continuity of the cell line was ensured.

Figure 1. Inverted microscope image of L929 in (Mouse Fibroblastic Cell Line) that after 24 hours of seeded and after confuluent the flask at 10x.



Investigation of Cytotoxic Effect of PA-AgNPs

MTT is a yellow, water-soluble tetrazolium dye (Cat. No. 32030, Serva, TURKIYE). This dye is reduced to formazan crystals by dehydrogenase enzyme groups active in the mitochondria of living cells and turns purple. The purple colour can be obtained by dissolving the formazan crystals formed with DMSO (dimethylsulfoxide). The purple colour formed by the dissolved formazan crystals is evaluated spectrophotometrically (Korzeniewski and Callewaert, 1983). The MTT method is a colorimetric assay to evaluate metabolically active cells by reducing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt to insoluble formazan crystals by dehydrogenase, which can work with mitochondrial activity (Boyraz et al., 2021). In the study, L929 cells (2x105 in 1 ml) were seeded onto the 96-well cell culture dish for analysis and then incubated for 24 hours at 37°C in an incubator with 5% CO2. At the end of the incubation period, PA-AgNPs were prepared at a concentration of 10 mg mL-1 using DMSO at a ratio of 1/1000. Concentrations between 250 µg mL-1 -4 µg mL-1 were applied using the serial dilution method (Concentrations determined by serial dilution method: 4-8-16-32-64-125-250 ug mL-1). To evaluate the proliferative effect, MTT was applied after incubation at these concentrations for 24-48-72 time periods. The proliferative effect of each determined concentration was determined by the MTT method after 24-48-72 hours. Cell proliferation was analysed at 570 nm in a microplate reader (Thermo Scientific, USA) (Kars et al., 2006).

Statistical Analysis

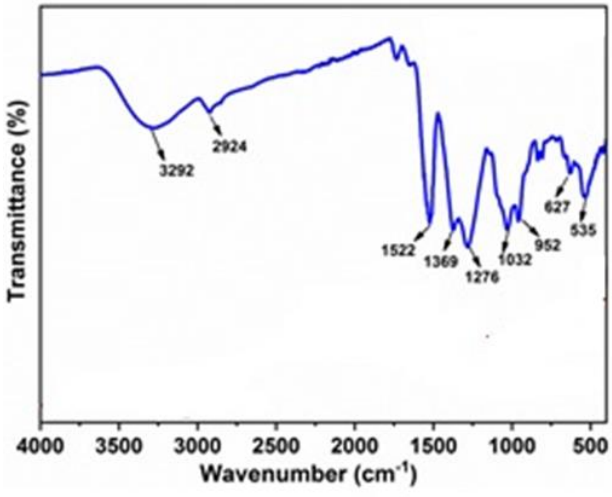
Analyses regarding cell viability were repeated at least three times. Analyses were performed as a result of the proliferation values determined for each concentration using the formula %cell Proliferation=(control/treatment)*100. Significant differences between the concentrations determined at 24, 48 and 72 hours were evaluated as a group for different time periods. Significant differences between proliferative effects at different concentrations were examined using Student's t-test ($p<0.05$) (Selvi, 2024). GraphPad Prism was used to statistically analyze the data (GraphPad Prism version 8.0.2, USA).

RESULTS

Synthesis and Characterization Analyses of PA-AgNPs

The FTIR analysis results of the Ag NPs synthesized by the green synthesis method using yellow cherry fruit extract are shown in Figure 2.

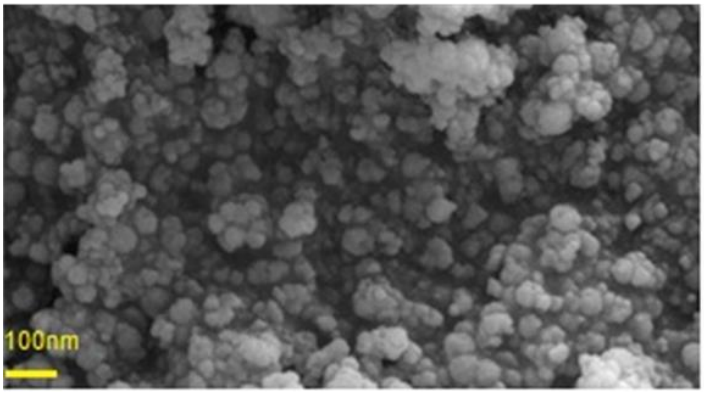
Figure 2. FTIR Spectra of PA-AgNP



The stretching indicated by the peak at 2924 cm-1 is the -CH stretching vibration of alkanes (Saiful et al., 2019). The C=O bond at 1717 cm-1 can be considered as its origin. The CO vibration is responsible for the peak at 1032 cm-1 corresponding to the 952-1276 cm-1 region. The results of the FTIR analysis of this study are in agreement with those of other studies (Albeladi et al., 2020; Bagyalakshmi and Haritha, 2017).

In order to evaluate the surface properties and quantify the size of PA-AgNPs generated at the ideal concentration ratio of white cherry extract and AgNO3 combination, an SEM picture was taken. Figure 3 shows the resulting image and the synthesis of PA-AgNPs was indicated by the white dots present in the image. The average size of the spherical particles was 40.98 ± 12.45 nm**.**

Figure 3. SEM image of PA-AgNP



Evaluation of Antibacterial Activity of PA-AgNPs

The results of the antibacterial activity of AgNPs prepared from yellow cherry extract against Escherichia coli, Bacillus subtilis, Staphylococcus aureus and Staphylococcus epidermidis bacteria are shown in Table 1. In order to determine whether the yellow cherry extract has an effect on the antibacterial activity of AgNPs, their antibacterial activity was also evaluated. It was found that the synthesised AgNPs produced an inhibition zone diameter of 13 mm on S. epidermidis and S. aureus bacteria. It was observed that there was no antibacterial activity in the discs impregnated with 1% DMSO and yellow cherry extract. When PA-Ag NP was compared with the antibiotic gentamicin, it was determined that PA-Ag NPs showed moderate antibacterial properties on E. coli, S. epidermidis and S. aureus.

Table 1. Antibacterial activity of synthesized Ag NPs and yellow cherry extract on test bacteria

	Test bacteria [(Inhibition zone diameter: IZD (mm)] (±SD) (n=3)			
	E. coli	***S. epidermidis***	***S. aureus***	***B. subtilis***
Ag NP	**12±0.1**	**13±0.3**	**13±0.1**	**11±0.1**
Yellow cherry extract	**0**	**0**	**0**	**0**
%1 DMSO	**0**	**0**	**0**	**0**
Gentamicin	**17.5±0.4**	**21±0.1**	**20±0.1**	**20±0.1**

Investigation of Cytotoxic Effect of PA-AgNPs

Using Prunus avium, green synthesised silver nanoparticles were added to L929 cell lines at a dose of 10 mg mL-1 for 24-48-72 hours. Viability analysis (MTT), determined by dehydrogenase enzyme activity dependent on mitochondrial activity, was used for each determined time period. Percentage cell proliferation graphs were plotted according to the results obtained. The graphs showed a decreasing trend between 62.5 µg mL-1 -250 µg mL-1 concentration at 24-48-72 hour periods (Figure 4abc). However, it was observed that the cells showed a proliferative effect between the concentrations of 8 µg mL-1-62.5 µg mL-1. As a result of the proliferation graph prepared, the concentration that inhibited 50% of the cells, IC50, was determined to be 46 µg mL-1 in 24 hours, 92.5 µg mL-1 in 48 hours and 105 µg mL-1 in 72 hours on average (Figure 4d). In addition, the experimental groups whose incubation periods ended as a result of PA-AgNP application were photographed under an inverted microscope as a function of dose and compared with the MTT test results. The % cell viability graph and the inverted microscope images produced as a result of the MTT method used in our research confirm and support each other (Figure 5).

Figure 4. Proliferative effect of green synthesis PA-AgNP. **a)** 24 hour % cell viability analysis, **(b)** 48 hour % cell viability analysis results, **c)** 72 hour cell viability analysis and **d)** concentration that inhibits fifty percent of the cells (24-48-72). The p values were respectively found to be 0.001/0.001/0.001 at 24/48/72 hours GraphPad Prism version 8.0.2 was used to statistically analyze the data. The threshold for statistical significance was set at $p<0.05$.

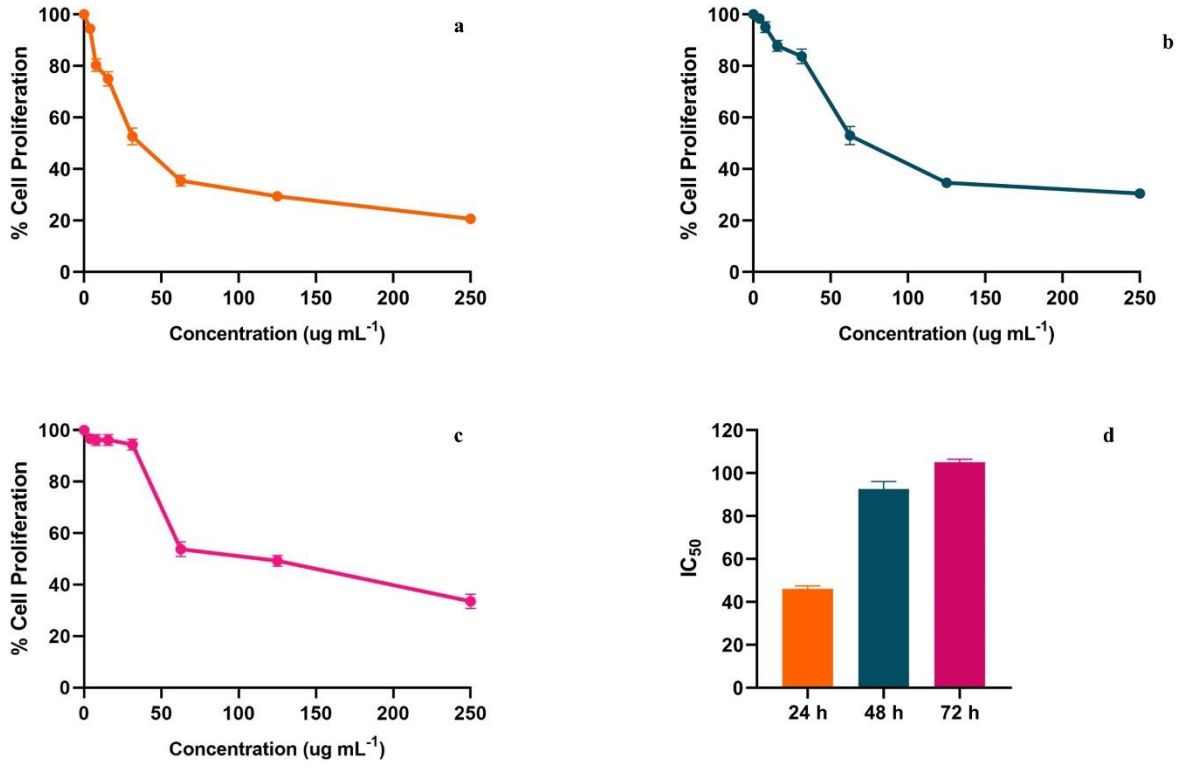
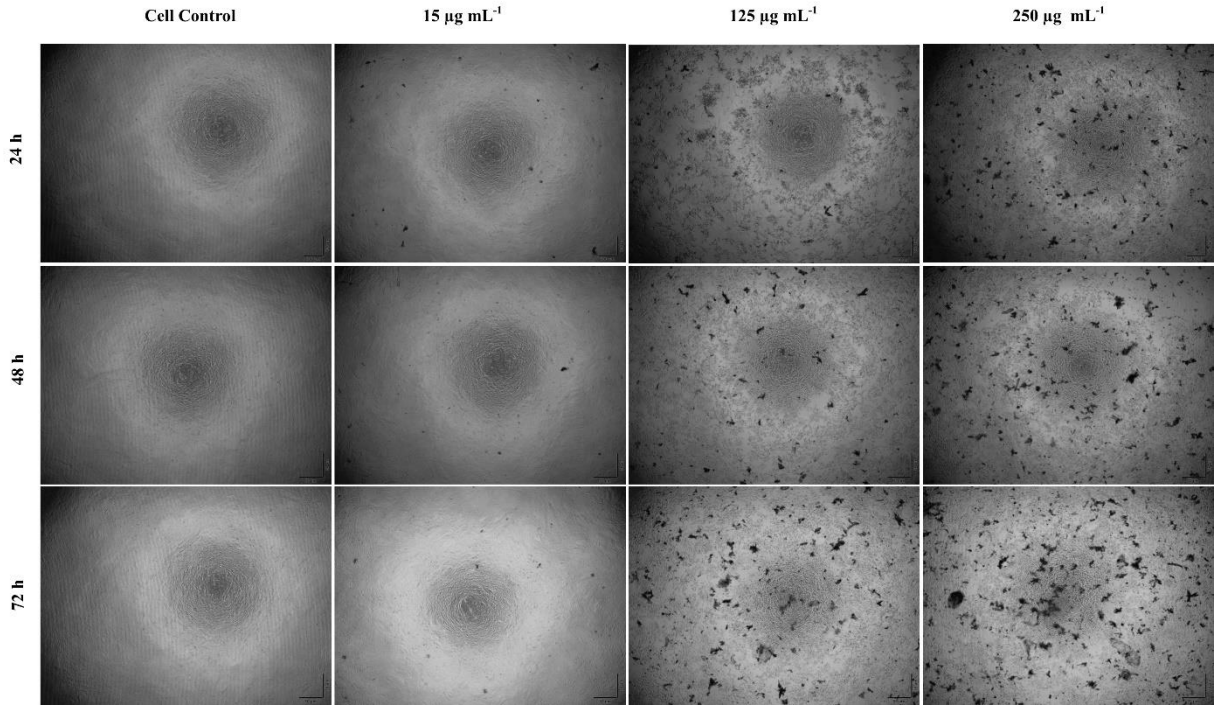


Figure 5. Inverted microscope (10X) images of green synthesis PA-AgNP.



As a result of the statistical analysis, p values were respectively found to be 0.001/0.001/0.001 at 24/48/72 hours (Table 2). The P value less than 0.05 indicates that the analysis results are significant ($p<0.05$).

Table 2. Student's t-test analysis ($p<0,05$)

	t value	**p value (p<0.05)**
24 h	5,635	0,001
48 h	6,115	0,001
72 h	7,078	0,001

DISCUSSION

Silver nanomaterials have great potential to be used directly or indirectly in medical applications (Uygur et al., 2009). However, the research conducted has led to the search for new synthesis methods due to the production of AgNPs by the known classical chemical/physical synthesis method and its toxic effects on living cells. In the age of ecologically friendly development, green synthesis methods for producing metal nanoparticles from plant extracts have drawn attention because of their affordability, ease of use in comparison to chemical and physical methods, and environmental sustainability (Alshameri and Owais, 2022). The reduction of metal ions to zero-valent metals and the stability of metal nanoparticles are caused by terpenoids, flavonoids or alkaloids, polyphenols, phenolic acids, and other secondary metabolites that are present in different plant sections. Furthermore, fatty acids, carbohydrates, or amino acids can be used as reducing agents in the creation of metal oxide nanoparticles (Behravan et al., 2019).

AgNPs have been reported in the literature to exhibit cytotoxicity, which is mostly concentration dependent (Akter et al., 2018). In the study conducted by Park et al. (2010), it was reported that 0.2 µg

mL-1 (0.2 ppm) AgNP concentration decreased cell viability by 20% and 1.6 µg mL-1 (1.6 ppm) AgNP concentration decreased cell viability by 40% when AgNPs were applied to the RAW 264.7 cell line (Park et al., 2010). Similarly, 25 µg mL-1 (25 ppm) was reported to be the most toxic concentration of AgNPs applied to the rat liver cell line BRL 3A, and toxicity was observed at concentrations ranging from 1 to 25 µg mL-1 (1-25 ppm) (Akter et al., 2018).

Nowadays, the development of green nanotechnology reduces the toxic effects of highly toxic metals such as Ag, especially by using green synthesis method with plant extracts. Veeraraghavan et al. (2021) obtained green synthesis SB-AgNPs using Scutellaria barbata extract and applied green synthesis AgNPs at concentrations of 2.5, 5, 7.5, 10, 15 µg mL-1 for 24 hours using L929 cell line. In this period, the viability rates of AgNPs at the concentrations used are over 50% compared to the chemical and physical method (Veeraraghavan et al., 2021). A similar study was carried out by Maghimaa and Alharbi. CL-AgNPs obtained from Curcuma longa L. extracts were applied to the L929 cell line for 24 h and it was reported that they showed no toxic effects at concentrations between 5-35 µg mL-1 (Maghima and Alharbi, 2020).

In the study by Ghasemi et al. (2024), RD-AgNP synthesis was carried out using an extract from the leaves of the Rubus discolor plant. It was found that it showed anticancer activity at concentrations between 11.2 µg mL-1 and 49.1 µg mL-1 in different types of cancer cells (HepG2-MCF7-A432), while it showed cytotoxic effect at a concentration of 158 µg mL-1 in the healthy cell line HU02. In comparison, PA-AgNPs were reported to have much lower cytotoxic activity in the healthy cell line (Ghasemi et al., 2024). In another study, DK-AgNPs were obtained by Keskin et al. (2023) by green synthesis method using Diospyros kaki L. The cytotoxic effects of DK-AgNPs were investigated on glioblastoma (U118), human ovarian sarcoma (Skov-3), human colorectal adenocarcinoma (Caco-2) cancer cell lines and healthy human dermal fibroblast (HDF) cell line by MTT technique. Experimental results showed that after 48 hours of incubation with DK-AgNPs for cancer cell lines, it was found to be highly cytotoxic (25-50 µg mL-1), while it was reported that the cytotoxic effect was less in the healthy cell line (25-200 µg mL-1) (Keskin et al., 2023).

In this study, PA-AgNPs obtained from Prunus avium extracts by green synthesis method were applied to the healthy mouse fibroblastic cell line L929 for 24-48-72 hours and 50% cell viability was determined as 24h-46 µg mL-1, 48h-92.5 µg mL-1 and 72h-105 µg mL-1 by MTT method. In the study, concentrations between 250 µg mL-1 -4 µg mL-1 (Concentrations determined by serial dilution method: 4-8-16-32-64-125-250 ug mL-1) were used, which are much higher values compared to the literature. When the obtained IC50 values were compared to the chemical method in the literature, no cytotoxic effect was observed at high concentrations. In the study to evaluate the proliferative effect, PA-AgNPs were evaluated on L929 separately at three different incubation times. IC50 values at different times were also compared within themselves.

Studies in the literature show that AgNPs are mostly spherical and oval in shape and their average diameters are up to 10-50 nm (Rafique et al. 2017). In our study, AgNPs synthesized using yellow cherry fruit extract were found to be in this range. Similarly, AgNPs synthesized using fruit extracts including Carissa macrocarpa and Momordica charantia fruit extracts were determined to be spherical in shape and to have antimicrobial activity (Soman and Ray, 2016; Rashid et al., 2017). Roy et al., (2014) reported that Ag-NPs synthesized by green synthesis method using Malus domestica extract were spherical in appearance and the average diameter of the spheres was 20 nm.

Numerous studies have demonstrated the antibacterial properties of Ag NPs prepared using plant products and the green synthesis approach. Reportedly, Ag NPs made with Abutilon indicum extract

exhibited strong antibacterial activity against bacteria such as S. typhi, E. coli, S. aureus and B. subtilis (Ashokkumar et al., 2015). Ag NPs prepared from Aloe vera extract were found to be effective against P. aeroginosa, S. aureus and E. coli bacteria in a related investigation. In our study, Ag NPs synthesised using yellow cherry extract were found to have antibacterial activity against the test bacteria used, similar to studies in the literature.

It is thought that PA-AgNPs exhibit strong antibacterial activity depending on the nanoparticle size (average 41 nm) prepared in our study. The actual mechanisms of the antibacterial and antifungal activities of AgNPs are among the issues that are attempted to be elucidated in detail. The antimicrobial activity of Ag + ions contained in the nanoparticle structure is explained by many mechanisms. The silver ions in the structure have the ability to bind preferentially to the phosphate group of the nucleic acid, rather than to its nucleosides, and to form various complexes with nucleic acids, including DNA and RNA. Some research suggests that positively charged nanoparticles and negatively charged bacterial cells are electrostatically attracted to each other, making them the best proposed bactericidal agent. In addition, they have been shown to not only diffuse within the cell, but also accumulate in the membrane and intercellular space, causing denaturation of the membranes or bacterial wall (Biswas et al., 2019; Abada et al., 2024).

CONCLUSION

In recent years, it has been observed that the bioactivity of metal nanoparticles has been increased by obtaining them by green synthesis method using biological materials. In our study, PA-AgNPs were found to have IC50 values of 46 µg mL^{-1} at 24 h, 92.5 µg mL^{-1} at 48 h and 105 µg mL^{-1} at 72 h in L929 cells, which indicates that the biological (green synthesis) synthesis method has low cytotoxicity when compared to chemical and biological synthesis methods. Thus, in our study, they were found to be much less cytotoxic, even at an average concentration 10 times higher, especially in the healthy cell line L929. In addition, this study suggests that it is a wound healing agent thanks to its bioactive content, especially with metals with high antibacterial properties such as AgNP. Long-term toxicity assessments, anticancer activity, and in vitro and in vivo wound healing models are anticipated in future research to confirm the possible medicinal uses of PA-AgNPs.

Ethical Statement

Local Ethics Committee Approval was not obtained because experimental animals were not used in this study.

Author Contributions

Research Design (CRediT 1) Author 1 (%50) – Author 2 (%40)- Author 2 (%10)
Data Collection (CRediT 2) Author 1 (%50) – Author 2 (%40)- Author 2 (%10)
Research - Data analysis - Validation (CRediT 3-4-6-11) Author 1 (%60) – Author 2 (%40)
Writing the Article (CRediT 12-13) Author 1 (%60) – Author 2 (%40)
Revision and Improvement of the Text (CRediT 14) Author 1 (%60) – Author 2 (%40).

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Conflict of Interest

Authors declare that there is no conflict of interest.

Sustainable Development Goals (SDG)

13 Climate Action

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Effect of Boron Mineral on Health

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ABSTRACT

Boron is a mineral that plays important roles in humans, animals and plants. However, the exact mechanism by which this mineral performs its functions in humans and animals is not fully understood. As a result, it is not classified as an essential element for humans and animals. People and animals live in the areas of Eskişehir, Kütahya, Bursa and Balıkesir provinces that have been boron resources for thousands of years. The total reserves in Türkiye have been estimated at 3.3 million tonnes. The water that comes from these provinces is consumed by the plants, vegetables, fruits and animals that feed on the plants grown here and by humans who consume them as food. It is an important mineral that has been consumed continuously with plants, animal products and water since the existence of our country. It is found in very high concentrations in precious foods such as dates, plums, grapes, almonds, hazelnuts, peanuts and honey. Many studies carried out in countries without boron resources and using high doses of boron have led to it being included in the group of toxic minerals. The mineral boron, which is important in terms of dosage and duration of use, and which we believe is present in physiological foods and which we are trying to clarify with our studies, is one of the essential trace elements. The benefits of boron mineral to living organisms have been demonstrated in many studies to date. However, it is a promising mineral that needs to be supported by further biological studies.

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INTRODUCTION

Boron is a naturally occurring element that exists as borates in oceans, coal, shales, sedimentary rocks, and various soils. It is widely found in nature, with an average concentration of approximately 10 mg/kg in the Earth's crust. (WHO, IPCS, 1998). Boron is a ubiquitous element present in rocks, soil, and water. While most soils globally contain less than 10 ppm of boron, elevated concentrations are observed in certain areas, particularly in parts of the western United States and regions stretching from the Mediterranean to Kazakhstan (Shorrocks, 1997; DPT, 2001).

The average concentration of boron in soil is typically in the range of 10 to 20 ppm and boron deficiency exists in large areas of the world. Economically significant boron mineral deposits, which always occur as oxygen-bound boron compounds, are rare and are generally found in arid regions with a history of volcanic or hydrothermal activity. These deposits are actively mined in Türkiye, the United States and various other countries (Matterson, 1980). In Türkiye, which has 73% of the world's boron reserves (Table 1), major deposits are found at Bursa-Kestelek, Eskişehir-Kırka, Kütahya-Emet and Balıkesir-Bigadiç (Eti Maden, 2024).

Table 1. Distribution of world boron reserves **(**Tenmak, 2017**)**

Country	**Total Reserve (Thousand tons B_2O_3)**	**Total Reserve (% B_2O_3)**
Türkiye	948712	73.4
Russia	100000	7.7
U.S.A	80000	6.2
Chile	41000	3.2
China	36000	2.8
Peru	22000	1.7
Serbia	21000	1.6
Bolivia	19000	1.5
Kazakhstan	15000	1.2
Argentina	9000	0.7
Total	1310300	100

Studies on boron in both animals and humans have shown that approximately 90% of orally ingested boron is absorbed (Trumbo et al., 2001). Boron is mainly excreted in the urine, with a smaller proportion excreted in the faeces (2%) and minimal amounts excreted in the breath, sweat and bile. Studies in animals show that boron does not accumulate significantly in soft tissues; rather, it concentrates much more in bone than in blood or other soft tissues. (Moseman, 1994).

In a 2009 screening of boron in the Polatlı region, it was found that boron was found in chamomile at 8.41 ppb; in water at 1.94; 2.02; 2.26; 2.33; 2.42; and in milk at different dairy farms at 0.74; 4.13; 4.97 ppb (ug/L).

Effects of Boron Mineral on Health

Many studies have shown the benefits of boron mineral in living organisms in bone development, brain function, macro mineral metabolism, reproduction, energy substrate utilisation, immune function, cardiovascular health and insulin secretion, and even anti-aging effects.

A deficiency in boron has been linked to weakened immune function and a higher prevalence of osteoporosis, which can elevate the risk of mortality. Conversely, excessive boron intake may lead to cellular damage and toxicity in both humans and various animal species. Recent studies have explored the diverse effects of boron, including its role in immune system activation, support for antioxidant detoxification, influence on bone metabolism, enhancement of animal performance, and regulation of multiple physiological systems. Additionally, boron has been recognized as an agent that mitigates heat stress in plants, with similar benefits proposed for animals. In addition, boron supplementation in dairy cows has been found to induce significant changes in liver metabolism (Abdelnour et al., 2018).

A review of studies in both animals and humans suggests that boron may play a vital role in the hydroxylation of steroid rings. This is based on the hypothesis that boron affects the metabolism of various nutrients and steroid hormones, including $1,25(OH)_2$ vitamin D, 17-β-estradiol, and testosterone. This function becomes especially significant when dietary intake of vitamin D, magnesium or both is insufficien (Rondanelli et al., 2020).

Boron affects the activity of at least 26 different enzymes, many of which are essential for the metabolism of energy substrates (Hunt, 1998). Boron's unique chemical properties allow it to interact with various metabolites and enzymes, which can affect mineral and energy metabolism in both humans and animals (Deviran and Volpe, 2003). In addition, boron plays a regulatory role in the metabolism of several essential minerals, including phosphorus, magnesium and calcium (Wilson and Ruszler, 1996).

The most studied effect of boron on health is its effect on bone development. Boron is essential for osteogenesis, and its deficiency has been shown to negatively affect bone growth and regeneration (Demirer et al., 2012). In addition, boron improves the utilisation of vitamin D. Research has shown that boron supplementation can promote bone growth in animals suffering from vitamin D deficiency and help alleviate the mineral metabolism disorders associated with this deficiency (Hunt, 1994).

The effects of boron on reproduction are the most debated areas of toxicity. Boron was once classified in the EU as a reproductive toxic mineral in labelling, packaging and classification. Claims that boron is reproductive toxic have been supported by animal toxicity studies.

Although epidemiological studies conducted in later years in communities in countries with high boron exposure, such as our country and China (Duydu and Üstündağ, 1994; Scialli et al., 2010; Yalçin et al., 2019), have shown that boron does not harm reproduction, this perception still persists and other benefits of boron in living organisms are ignored. In addition, unlike the animal toxicology studies conducted in the past with limited resources, there are studies in animals that have used boron and examined its effects on reproductive function. These studies have found that boron improves semen quality and enhances reproduction (Armstrong et al., 2002; Elkomy et al., 2015; El-Sadany, 2017; Ibrahim et al., 2019; Krishnan et al., 2019).

It has been reported in studies that boron causes changes in immune response, including inflammatory processes. In this context, a study examining the effects of boron on immune function found that boron acts as a regulator of immune and inflammatory responses, as well as macrophage polarization. This strengthens the role of boron in enhancing both innate and adaptive immunity, with potential implications for cancer and other diseases (Routray and Ali, 2016). Additionally, another study indicated that food-appropriate boron supplementation can serve as an immune regulator for both humans and animals (Jin et al., 2017).

A study investigating the relationship between insulin metabolism and boron indicated that exposure to borax reduces blood lipid levels (Basoglu et al., 2000). Moreover, research has shown that

a lack of boron raises the insulin requirements necessary to sustain plasma glucose levels when vitamin D and magnesium nutrition is inadequate in chicks and rats (Bakken and Hunt, 2003). Furthermore, dietary boron supplementation has been shown to decrease body weight, leptin, and insulin levels while increasing plasma T3 levels, thereby enhancing the metabolic activity of rats (Kucukkurt et al., 2015).

Research also suggests that boron may influence cardiovascular health. One study highlights the potential role of boron-containing compounds in regulating signalling pathways associated with inflammation, oxidative stress and lipid metabolism. The potential cardioprotective effects of these compounds provide new and exciting opportunities for their use in dietary supplements and possibly pharmaceuticals (Donoiu et al, 2018).

To further explore boron's functional role, researchers conducted additional studies on brain electrophysiology and cognitive performance in response to dietary boron manipulation among healthy older men and women. The results indicated that low dietary boron intake significantly impaired performance in a variety of cognitive and psychomotor tasks, particularly those emphasizing manual dexterity, compared to high boron intake. Overall, the findings from these studies indicate that boron might be crucial for human brain function and cognitive performance, offering further proof of its importance as an essential nutrient for humans (Penland, 1994).

Five studies examining electrical brain activity in both animals and humans have found that boron deficiency leads to reduced electrical brain activity, similar to the effects of general malnutrition. Furthermore, evaluations of cognitive and psychomotor function in humans indicate that boron deficiency is associated with reduced performance in tasks involving motor speed, attention, dexterity, and short-term memory. These findings emphasize the significance of boron supplementation in supporting brain function and mental health (Penland, 1998).

In appropriate nutritional doses, boron supports bone strength and cognitive function, regulates immune and inflammatory responses, and impacts the body's reaction to oxidative stress. Its diverse effects appear to stem from its influence on cellular signaling pathways or its role in the formation and function of key biochemical components. Research from both human and animal studies suggests that a daily boron intake of around 1.0 mg could be sufficient to ensure its beneficial effects (Nielsen, 2017). Insufficient boron levels have been associated with weakened immune function, increased mortality risk, osteoporosis, and cognitive impairment. On the other hand, excessive boron intake has been linked to cellular damage and toxicity in both humans and various animal species (Haliq et al., 2018).

According to a 2023 study, boron-containing compounds have shown effects on neurons, with new boron-containing compounds being synthesised and their effects on neuronal activity reported. These compounds exert their effects by modulating inflammation and oxidative processes. The study highlights the intention to investigate the use of boron-containing compounds as targeted drugs for the treatment of neurodegenerative diseases in future research (Barrón-González et al., 2023).

Research into the role of boron in supporting healthy living and longevity is limited but promising. Boron has bioactive properties that influence the formation and activity of NAD^+ and S-adenosylmethionine (SAM), both of which are associated with ageing and longevity. Evidence suggests that boron has beneficial effects on oxidative stress, inflammatory responses, DNA damage detection and repair, and the role of SAM in methylation and regulation of homocysteine levels. These effects suggest that adequate boron intake may alleviate several age-related pathological conditions, cognitive decline, including cancer, sarcopenia and bone health. Therefore, a boron-rich diet could support healthy ageing and longevity (Nielsen, 2018).

In addition, boron compounds and complexes other than boric acid and its salts are used for skin rejuvenation. These compounds have demonstrated benefits in reducing wrinkles, increasing skin thickness, improving hydration, softness and elasticity, improving skin tone and minimising the number and size of age spots. These compounds are often formulated in appropriate solvent systems, such as microemulsions or macroemulsions, and can be applied in various forms, including creams, bath salts, cosmetics and shampoos (Miljkovic and Pietrzkowski, 2000). Moreover, the findings indicate that boron supplementation enhances bone strength and improves the microstructure of both cortical and trabecular bone in diabetic animals as well as in supplemented control groups (Dessorti et al., 2017).

CONCLUSION

Boron is a mineral that has long been used in agriculture as a fertiliser and pesticide, and is also used in insulation and building materials, glass, cement, metal and defence industries. Humans and animals regularly receive boron through their daily diet. However, information on the biological effects of this element is still limited.

Despite the numerous studies conducted in recent years on the effects of boron and its mechanisms of action, further research is required to fully elucidate its effects and the underlying mechanisms by which it acts in living organisms. It is known that this mineral acts on many enzymes and enzyme systems, affecting fat and lipid metabolism, mineral metabolism and vitamin D, as well as having benefits for bone development, the immune system, cardiovascular health and even healthy ageing and longevity. Although much of the current research and information on boron is useful, much more is needed.

Although the benefits of boron are known and supported by studies, the toxicity studies conducted with very high doses of boron in the past are still valid today, and the claims that boron is toxic and the perception that is being created should be removed with the studies that will be conducted.

In Türkiye, which has 73% of the world's boron reserves, work in this area is unfortunately below what it should be. In America, which has about 7% of the world's reserves, the boron market is much more developed and its use in the health sector is much higher. In addition, the production of high value-added boron products should be increased and their use promoted.

In our country, which is the world's repository of this valuable mineral, whose use should be much greater in the future, further research and support of the use of boron and its benefits to living organisms with studies will be beneficial to our country and world in terms of both health and economy.

Ethical Statement

This is a review article and does not necessarily need ethics approval.

Author Contributions

Data Collection (CRediT 2) Gültekin YILDIZ (%100)
Research - Data analysis - Validation (CRediT 3-4-6-11) Gültekin YILDIZ (%100)
Writing the Article (CRediT 12-13) Gültekin YILDIZ 1 (%100)
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3 Good Health and Well-Being

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