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Research Article

The Effect of Ultrasound-Treated Donkey Milk on some Serum Antioxidant and Cytokine Levels as well as ANAE-Positive Lymphocyte Ratios in Rat Trial Models

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Abstract

This study aimed to compare the effects of slow pasteurization and ultrasound treatment on the physicochemical and microbiological quality of donkey milk (DM) and to investigate how these processing methods influence specific antioxidant and immunological responses in rats. In the first stage, donkey milk samples were collected from twenty-two healthy donkeys and categorized into three groups: raw (Rm), pasteurized (Pst; 65 °C for 30 min), and ultrasound-treated (Ult; 100% amplitude for 6 min). Physicochemical parameters (e.g., pH, color, water activity, titratable acidity) and nutritional composition (dry matter, protein, fat, lactose) were determined. Microbial analyses included total aerobic mesophilic bacteria, coliforms, molds/yeasts, coagulase-positive *Staphylococcus*, and lactic acid bacteria counts. In the second stage, twenty-eight adult male Wistar albino rats were assigned to four groups (control, Rm, Pst, Ult) and each experimental group received 48 mL of the respective DM daily for four weeks. At the end of the feeding period, the rats were sacrificed, and blood samples were collected. Serum levels of malondialdehyde (MDA), glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), and various cytokines (IL-1β, TNF-α, IL-6, IL-10) were quantified. Differential leukocyte counts (lymphocytes, monocytes, neutrophils) and ANAE-positive lymphocyte percentages were also assessed. Although MDA and GSH levels were unaffected by DM type, SOD activities were significantly higher in all DM-fed groups relative to controls. Ultrasound-treated DM showed a more pronounced impact on pro-inflammatory cytokine levels (IL-1β, TNF-α, IL-6) than pasteurized milk, while IL-10 remained unchanged among the groups. Neither pasteurization nor ultrasound treatment affected the differential WBC or ANAE-positive lymphocyte percentages. Overall, ultrasound treatment offered a superior microbial load reduction without adversely impacting antioxidant or bioactive components. These findings suggest that ultrasound application may serve as a viable alternative to traditional pasteurization for preserving the functional properties of donkey milk.

Keywords: Antioxidants, Cytokines, Donkey milk, Rat, Ultrasound

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Introduction

Milk is a principal food source for many living beings (especially newborns) due to its well-balanced water, protein, carbohydrate, fat, vitamin, and mineral content (Li et al 2022). It is obtained from a wide variety of animals (cow, sheep, goat, camel, and donkey), and is intensively consumed by people in many parts of the world (Papademas et al 2022). One of them is donkey milk (DM) which is known as the milk closest to human milk (HM) in terms of total/whey protein and lactose ingredients. It was used as a health and beauty elixir in many civilizations from Ancient Greece to Egypt (Bertino et al 2022). Low fat and cholesterol levels, whereas high poly-unsaturated fatty acid (PUFA) and lysozyme contents are some of the most important features of DM compared to other mammalian milk types (Martini et al 2018). It has been revealed that the above-mentioned characteristics provide an advantage to DM because of the consumption in some special

conditions including children with cow's milk allergy, obesity, or diabetic patients (Trinchese et al 2018). Donkey milk can be a functional food in terms of these properties and nutritional content (Vincenzetti et al 2021).

On the other hand, raw milk can facilitate the growth or spread of a number of foodborne pathogens, including pathogenic viruses and coagulase-positive *Staphylococcus aureus*, *Escherichia coli* (STEC), *Listeria monocytogenes*, *Salmonella* spp., and *Brucella* spp. (Verraes et al 2015, Fusco et al 2020). International agencies have raised awareness of these foodborne infections over the past few decades, including the Food and Drug Administration (FDA), the European Food Safety Authority (EFSA), and the European Center for Disease Prevention and Control (ECDC) (EFSA 2018). Furthermore, the deterioration of raw milk and dairy products may also be caused by a number of spoilage microorganisms, such as several lactic acid bacteria species that are known to be employed in the fermentation of a variety of dairy products (Quigley et al 2013). According to the Food and Drug Administration (FDA) and the Pasteurized Milk Ordinance (PMO), heating is required to eliminate pathogenic or spoilage bacteria (Abdullah et al 2019). On the other hand, improper heat processing can lead to decreased nutritional content, poor quality, and the production of allergenic proteic fragments (Roth-Walter et al 2008, Lima et al 2018, Choi and Oh 2020). Specifically, donkey milk contains immune-related bioactive peptides that are more heat-sensitive than those reported in milk from other mammals (Miao et al 2020). The loss of energy required to make dairy products healthier has recently become a global problem. The FAO report from 2015 states that the annual global production of milk is 703,996,079 tons. Each ton of milk requires an estimated 600 MJ of heat energy and 200 MJ of electrical energy to pasteurize. Researchers looked into various alternative techniques, including microfiltration, X-rays, ultraviolet light, oscillating magnetic fields, ohmic heating, and ultrasound application, in response to growing environmental consciousness, increased energy consumption, and increased costs brought on by the poor performance of the heat exchangers used in plate pasteurizers (Bansal and Chen 2006). One of the fastest developing technologies is ultrasound technology, which uses sound waves at frequencies higher than the human hearing threshold (>20 kHz) (Soria and Villamiel 2010). The technique is designed to reduce physical and chemical alterations, improve food quality, and preserve food safety (Mohammadi et al 2017). According to Salleh-Mack and Roberts (2007), there is evidence that the ultrasonic method may effectively eliminate bacteria and meet FDA regulations, which call for a 5-log reduction in the microbial population.

The study was planned in two stages. In the first stage, the physicochemical and microbiological parameters of slowly pasteurized and ultrasound-treated donkey milk samples were compared. Then, raw, pasteurized, and ultrasound-treated donkey milk was given orally to Wistar rats at a dose of 48 mL/day for 4 weeks. In order to evaluate the effects of the administrations on rats, serum antioxidant levels [malondialdehyde (MDA), glutathione (GSH), catalase (CAT), superoxide dismutase (SOD)], various cytokines [interleukin-1-beta (IL-1β), tumor necrosis factor-alpha (TNF-α), IL-6, IL-10)], differential leukocyte counts (lymphocytes, monocytes, neutrophils), and ANAE-positive lymphocyte percentages were evaluated. For this purpose, we aimed to compare the effects of slow pasteurization and ultrasound treatment on the physicochemical and microbiological quality of DM and to investigate how these processing methods influence specific antioxidant and immunological responses in rats.

Material and Methods

Milk Sample Preparation

Twenty-two healthy donkeys bred at Marmara Donkey Farm in the Edremit region of Balıkesir, Turkiye province provided DM samples for collection. Milking from donkeys was performed 7 times in total at 4-day periodic intervals. A total of 5 liters of milk was put into sterile containers. Then, they were transmitted (+ 4°C) to the laboratory as quickly as possible for analysis. Then, DM samples were divided into three groups as raw (Rm), pasteurized (Pst), and ultrasound-applicated (Ult). During the storage period, DM samples were stored at +4°C in a refrigerator. Slow pasteurization was applied to 100 mL (30 jars) of DM at 65 °C for 30 minutes. Also, Bandelin Hd 2200.2 was applied to 100 mL (30 jars) of DM with a probe TT 13 (AMPLICHRON® Germany) device at 100% amplitude-pulsation for 6 minutes (Tavsanli et al 2022). Temperature changes (°C) of DM samples were determined with a stud thermometer (Knmaster thermometer PS-300, Türkiye during the pasteurization and ultrasound applications (Figure 1).

Physico-chemical Analysis

Color measurement of DM samples was performed by using Lovibond® SV 100 colorimeter (Amesbury, United Kingdom) according to Commission Internationale d'Eclairage (CIE, 1978; L value black/whiteness; a, red/green color feature; b, yellow/blue color feature). The results were calculated by taking the average of

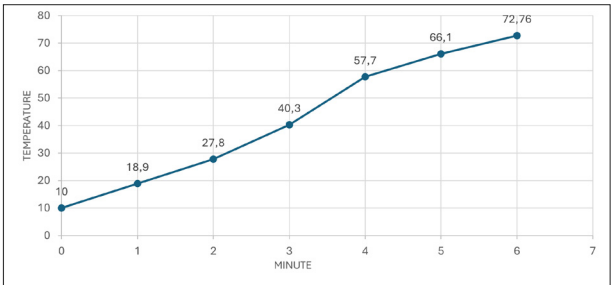

Figure 1. Temperature (°C) changes of raw donkey milk (DM) samples during the ultrasound application

5 measurements in 3 repetitions with an interval of 5 minutes. Besides, the pH levels of different DM samples were defined with a digital pH meter (Hanna HI 2211, Germany). In addition, the water activity (aw) value was defined by the aw meter device (Novasina LabTouch-aw, Switzerland). Furthermore, titration acidity (SH) was also detected according to TS 1330/2006 standard method. The milk ingredients such as dry matter, protein, fat, and lactose analysis of different DM samples were done by using an automatic milk analyzer (Bentley Combi FTS 600, USA) that had been calibrated with raw DM.

Microbial Analysis

Ninety mL of sterile Buffered Peptone Water (BPW) was added to 10 ml of DM sample and homogenized in the stomacher (IUL 400) for 2 minutes. Then, 1 ml of the homogenate of DM samples diluted 1:10 was transferred to tubes containing 9 ml of sterile BPW, and serial decimal dilutions were prepared. Then, serial dilutions were inoculated onto appropriate media.

For total aerobic mesophilic bacteria (TAMB) count, Plate Count Agar (1.05463, Merck, Darmstadt, Germany), TS ISO 4833-1:2013 method; Violet Red Bile Agar for coliform counting with TS ISO 4832 method; to Dichloran Rose Bengal Chloramphenicol Agar for mold and/or yeast counting by TS ISO 6611 method; For coagulase-positive *Staphylococcus* count / *Staphylococcus-micrococcus* count, they were inoculated on Baird-Parker Agar with TS ISO 6888 method. For lactic acid bacteria enumeration, they were cultivated on MRS agar (Merck 1.10660) for lactobacilli and M17 agar (Merck 1.15108) for lactococci (Mathara et al 2004).

Animals and Administration of Different DM Samples

Balıkesir University Animal Experiments Local Ethics Committee's decision dated 25.03.2021, with the ethics committee approval number 2021/3-6, was obtained for the execution of this study. In the present study, twenty-eight adult male Wistar Albino rats were obtained from Balıkesir University Experimental Medicine Research and Application Center (BAUEMRAC). They were healthy and approximately two months old (~2). Their respective body weights (350 ± 5.0 g) were quite similar. Four groups were developed for the experiment: pasteurization (Past; n = 7), ultrasonography (Ult; n = 7), raw milk (Rm; n = 7), and control (C; n = 7). Although the animals were divided into different groups, they were each kept in unique cages. During the experiment, animals were housed in standard plastic rat cages. The room temperature was approximately 23 ± 2 ºC, and it had 55 ± 10% relative humidity. Twent-hours day/night light period was also performed in a special unit of BAUEMRAC. The technique used by Trinchese et al (2015) was implemented to feed the animals with various DM samples. Every administration was carried out in a way that ensured end-of-day control. The C group animals received standardized rat pellets and fresh drinking water on an ad libitum basis for the duration of the trial (four weeks) following the two-week adaption phase. For four weeks, each animal in the Rm group received 48 milliliters of milk each day. Besides, pasteurized milk samples (at the rate of 48 mL/day) were given to each of the rats that were reared in the Pst group for 4 weeks. In addition, ultrasound-applied milk samples (at the rate of 48 mL/day) were also applied to each of the rats in the Ult group in the same period. After the controlled -milk applications, fresh water, and standard rat food were accessed to all rats.

After the experiments, the life of the rats was terminated using the cervical dislocation procedure by sedation with ketamine and xylazine (0.1 mL/100 mg/b.w). After that, blood samples were drawn into tubes with and without anticoagulant from the heart using the cardiac puncture technique. Following the centrifugation of the acquired blood samples (3000 rpm, 25 min, Hermle Z380, Rösler, Germany), plasma and serum samples were obtained. Up to the time of the analysis, they were kept in a freezer at -80 ºC.

Detection of MDA Concentrations, GSH Values, and Some Antioxidant Enzyme Activities

Standard commercial kits (BT- LAB; Shanghai, China) were used to measure the concentration of MDA (Code:SH0020), GSH (Code: EA0142Hu) values, CAT (Code: E0869Ra), and SOD (Code: E1444Ra) enzyme activity in serum samples using an ELISA instrument (Biotek® ELX800, USA) (Bulut et al 2023, Durmus et al 2024).

Detection of Some Serum Cytokine Levels

Using commercial kits (BT-LAB®, Shanghai, China) and following the user manuals, the levels of cytokines IL-1β (Code: E0119Ra), TNF-α (Code: E0764Ra), IL-6 (Code: E0135Ra), and IL-10 (Code: E0108Ra) were determined from the serum samples mentioned above. Rat blood samples were used for this test, which is based on a

Table 1. Colour analysis of the different preservation methods applied donkey milk samples

Experimental Groups	L	a	b
Rm	75.5a	-0.35	-1.25
Pst	76.25a	-0.56	-1.85
Ult	79.6b	-0.45	-2.55

[a,b]$p < 0.05$, The means with different letters in the same column are significant with each other. C: Control; Pst: Pasteurized-Milk; Rm: Raw-Milk; Ult: Ultrasound-Applicated Milk. L value, black/whiteness; a, red / green color feature; b, yellow / blue color feature.

double-antibody sandwich ELISA assay to find the levels of IL-1β, TNF-α, IL-6, and IL-10. To summarize, the kit's standards and serum samples were extracted onto an extraction plate, derivatized using an equalizing reagent, and then ELISA was performed on pre-coated microtiter strips containing IL-1β, TNF-α, IL-6, and IL-10. In fifteen minutes, the absorbance of the fluid in the wells was measured using a microplate reader (Biotek® ELX800, USA) at 450 nm. Using a standard curve, the optical density was utilized to determine the cytokine levels that were previously described (Hatipoglu and Keskin 2022).

Admi Detection of Differential Leucocyte (WBC) and ANAE-Positive Lymphocyte Percentages

ANAE demonstration was shown by according to Donmez and Sur (2008). In this process, air-dried blood smears were fixed in a glutaraldehyde acetone solution at –10 °C for 3 min (pH 4.8). For the preparation of the incubation solution, 20 mg of substrate, α naphthyl-acetate (N-8505, Sigma, Steinheim, Germany) dissolved in 0.8 mL of acetone (Merck, Darmstadt, Germany), 4.8 mL of hexazotized pararosaniline (hexazotization was carried out by mixing equal volumes (2.4 mL each) of 4% sodium nitrite (Merck) and 2% pararosaniline (Merck), and 80 mL of PBS (pH 5). The incubation solution was filtered after its final pH was brought to 5.8 using 1 N NaOH. Following a 4-hour incubation period at 37°C, the smears underwent three rounds of distilled water rinsing, and the nuclei were stained for 20 minutes using 1% methyl green made in acetate buffer (pH 4.2). To create control specimens, the smears were incubated in an incubation solution devoid of α-naphthyl acetate. ANAE-positive lymphocytes were investigated under the Leica DM 2500 (Wetzlar, Germany) by counting 200 lymphocytes. Also, the prepared smears that were stained with the May Grünwald Giemsa, were used to define the ratios of lymphocyte (LYM), monocyte (Mon), and neutrophile (Neu) (Figure 2).

Statistical Analysis

The data were statistically evaluated using Duncan's test and analysis of variance (ANOVA) using the SPSS 25.0 program (SPSS, Inc., Chicago, IL). Data were considered significant at $p < 0.05$.

Results

Properties of Different Preservation Methods Applied DM

Physico-chemical and microbial analysis results of DM samples that were applied to rats were shown in Tables 1, 2, and 3.

Serum MDA Concentrations, GSH Values, and Some Antioxidant Enzyme Activities

In this study, MDA concentrations and GSH values of the rats were not affected by the different preservation methods applied to DM samples, statistically ($p > 0.05$). Although serum CAT enzyme activities were also not

Table 2. The average physico-chemical properties of the different donkey milk samples

Experimental Groups	pH	SH	Aw	Dry matter	Fat	Lactose	Protein
Rm	7.02	0.64	0.992	8.87	0.7	6.5	1.5
Pst	6.98	0.67	0.993	8.88	0.7	6.5	1.5
Ult	6.99	0.68	0.994	8.86	0.7	6.5	1.5

C: Control; Pst: Pasteurized-Milk; Rm: Raw-Milk; Ult: Ultrasound-Applicated Milk. SH: titration acidity; Aw: water activity.

Table 3. The average microbial characteristics of the different donkey milk samples (log 10 cfu /mL, Mean ± SEM)

Experimental Groups	TAMB	Lactobacillus	Lactococcus	Yeast/mold	Staphylococcus spp	Coliform
Rm	5.6 ± 0.06[a]	5.34 ± 0.06[a]	4.30 ± 0.04[a]	4.37 ± 0.01[a]	3.16 ± 0.31[a]	2.34 ± 0.13[a]
Pst	3.12 ± 0.75[b]	1.55 ± 0.35[b]	1.35 ± 0.25[b]	1 ± 0.00[b]	1 ± 0.00[b]	1 ± 0.00[b]
Ult	1 ± 0.00[c]	1 ± 0.00[c]	1 ± 0.00[c]	1 ± 0.00[b]	1 ± 0.00[b]	1 ± 0.00[b]

[a,b,c]p < 0.05, The means with different letters in the same column are significant with each other. C: Control; Pst: Pasteurized Milk; Rm: Raw-Milk; Ult: Ultrasound-Applicated Milk. TAMB: total aerobic mesophilic bacteria. SEM: standard error of mean.

affected by the different DM administrations, only serum SOD activities significantly increased in Rm, Pst, and Ult groups compared to C ($p < 0.05$), shown in Table 4.

Some Serum Cytokine Levels

In the present study, serum IL-6 levels were defined as the highest in the Rm and Ult groups compared to Pst ($p < 0.05$). Besides, serum TNF-α cytokine levels were higher in the Ult group than in the Pst group ($p < 0.05$). In addition, serum IL-1ß levels were detected as the highest in the Pst and Ult groups when compared to the Rm group, statistically ($p < 0.05$). However, based on IL-10 levels in the present investigation, no significant difference was determined between the experimental groups ($p > 0.05$), as indicated in Table 5.

Differential WBC and ANAE Positive Lymphocyte Percentages

As can be shown in Table 6, we were unable to find any statistically significant differences between the experimental groups in terms of differential WBC and ANAE positive lymphocyte percentages ($p > 0.05$).

Discussion

Many foodborne pathogens, including coagulase-positive *S. aureus*, *E. coli* (STEC), *L. monocytogenes*, *Salmonella* spp., and *Brucella* spp. can grow and spread in raw milk (EFSA 2018, Fusco et al 2020). However, the deterioration of raw milk and other dairy products may also be caused by a wide variety of spoilage microorganisms, including some lactic acid bacteria that are known to be employed in the fermentation of different dairy products (Quigley et al 2013). According to the FDA and the PMO, heating is needed to get rid of harmful or spoilage bacteria (Abdullah et al 2019). Nevertheless, improper heat treatment can result in a reduction in nutritional value, a deterioration in quality, and the emergence of allergic proteic moieties (Roth-Walter et al 2008, Lima et al 2018, Choi and Oh 2020). Previous researches have shown that the pasteurization process causes significant losses in functional foods. Additionally, it has been discovered that all of the milk's biological components including T cells, B cells, macrophages, and neutrophils are totally rendered inactive during the pasteurization process. In addition, it has been reported that it significantly reduces many other immunoreactive components (IGA, IGE, lactoferrin, lysozyme, erythropoietin, Insulin-like growth factor-1, and insulin-like growth factor-2) (Ewaschuk et al 2011). In this case, the effects of ultrasound applications, which are shown as an alternative to thermal pasteurization, on bioactive components need to be investigated. There are many studies on the application

Table 4. Serum MDA concentrations, GSH values, SOD and CAT enzyme activities of the experimental groups (Mean ± SEM)

Groups	n	MDA(nmol/mL)	SOD (ng/mL)	GSH (mmol/L)	CAT (CU/L)
C	7	1,86 ± 0,36	2,64 ± 0,42[b]	0,27 ± 0,01	232,47 ± 37,02
Rm	7	2,47 ± 0,32	4,66 ± 0,57[a]	0,25 ± 0,01	321,83 ± 50,25
Pst	7	2,37 ± 0,32	4,68 ± 0,68[a]	0,26 ± 00,1	298,37 ± 69,72
Ult	7	2,88 ± 0,30	4,66 ± 0,62[a]	0,25 ± 0,01	330,40 ± 55,92

[a,b]p < 0.05, The means with different letters in the same column are significant with each other. C: Control; Pst: Pasteurized-Milk; Rm: Raw-Milk; Ult: Ultrasound-Applicated Milk. MDA: malondialdehyde; SOD: superoxide dismutase; GSH: glutathione; CAT: catalase; SEM: standard error of mean

Table 5. Some serum cytokine levels (IL-6, IL-10, TNF-α, and IL-1-ß) of the experimental groups (Mean ± SEM)

Groups	n	IL-6 (ng/L)	IL-10 (pg/mL)	TNF-α (ng/L)	IL-1-ß (pg/mL)
C	7	14,56 ± 1,35^{ab}	235,88 ± 27,92	257,60 ± 67,34^{bc}	2693,21 ± 222,74^{ab}
Rm	7	18,11 ± 0,82^{a}	207,73 ± 24,31	419,26 ± 83,28^{ab}	2265,98 ± 343,84^{b}
Pst	7	13,25 ± 1,51^{b}	270,67 ± 30,21	177,56 ± 43,56^{c}	3047,07 ± 129,49^{a}
Ult	7	17,80 ± 1,33^{a}	301,21 ± 42,61	555,07 ± 66,61^{a}	3041,81 ± 94,23^{a}

$^{a,b,c}p < 0.05$, The means with different letters in the same column are significant with each other. C: Control; Pst: Pasteurized-Milk; Rm: Raw-Milk; Ult: Ultrasound-Applicated Milk. TNF-α: Tumor necrosis factor- alpha; IL-6: Interleukin-6; IL-10: Interleukin-10; IL-1ß: Interleukin 1-beta. SEM: standard error of mean.

of ultrasound for different purposes in food production including homogenization, emulsification, extraction, degassing, crystallisation, cutting, and microbial inactivation (Scudino et al 2020, Manyatsi et al 2024). In our study, the purpose of ultrasound application was to provide microbial inactivation equivalent to or even superior to pasteurization. However, in addition to providing microbial inactivation, ultrasound can also cause modifications in foods that result in tissue and cell degradation, fiber breakage, isomerization, and micellization. This increases the extractability of bioactive compounds in food ingredients and/or causes their degradation. Moreover, the resulting new matrix interacts differently with the human body, affecting the accessibility or digestibility of the compound. This situation may cause different reactions in living beings (Rojas et al 2021). Color values in milk are shaped as a result of its physicochemical structure (Chudy et al 2020). In the present study, the measured 'L' value showed that there was a difference between Ult treated with raw and pasteurized donkey milk. The L value in the Ult group was closer to the 100 whiteness value compared to others. The lack of difference in the basic chemical composition (dry matter, protein, fat, lactose) of the donkey milk used in the study can be explained by the release of micro-level nutritional contents and bioactive compounds under the effect of cavitation occurring in ultrasound (Perera and Alzahrani 2021). In this study, a much better microbial inactivation of the microbial flora of donkey milk was determined than pasteurization. The results of the present study were similar to previous studies (Ma et al 2019, Alcantara-Zavala et al 2021, Tavsanli et al 2022). The interest in the consumption of DM is increasing day by day due to its well-known anti-oxidant, anti-inflammatory, anti-aging, anti-cancer, and anti-microbial properties (Aspri et al 2017).

Therefore, it is important to determine "how the different preservation methods affect anti-oxidant and anti-inflammatory properties of DM and, also health status of the DM-consumed rats". In our study, the administration of different preservation methods applied to DM samples (at the rate of 48 mL/day for 4 weeks, p.o) did not affect the serum MDA concentrations in Wistar rats. Similarly, Tastekin et al (2018) reported that administration of DM (25 mg/kg/day for 10 days, p.o) in addition to standard rat food did not alter tissue (gaster) MDA (0.14 ± 0.03 µmol/mL) concentrations in rats. On the other hand, Trinchese

Table 6. Differential WBC and ANAE positive lymphocyte percentages of the groups (%)

Groups	n	NEU	MON	LYM	ANAE positive LYM
C	7	27,98 ± 1,20	3,61 ± 1,72	68,40 ± 1,63	49,14 ± 0,34
Rm	7	32,21 ± 3,24	3,03 ± 1,57	64,67 ± 4,30	49,00 ± 0,30
Pst	7	25,48 ± 1,12	3,08 ± 1,37	71,32 ± 1,26	48,85 ± 0,34
Ult	7	30,77 ± 2,59	4,12 ± 1,62	65,08 ± 2,91	48,42 ± 0,48

C: Control; Pst: Pasteurized-Milk; Rm: Raw-Milk; Ult: Ultrasound-Applicated Milk. Neu: Neutrophil; Mon: Monocytes; Lym: Lymphocyte; ANAE: Alpha-naphthyl acetate esterase.

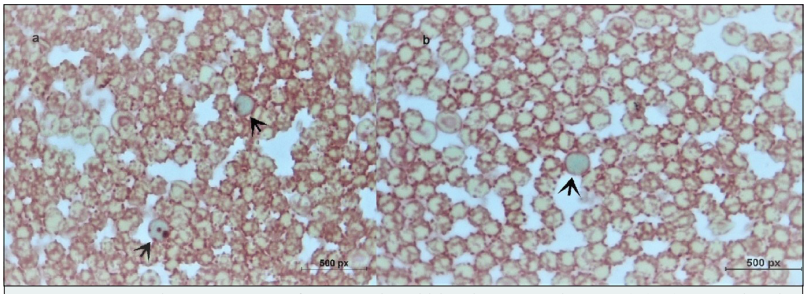

Figure 2. ANAE demonstration of rat peripheral blood in control group (C); a) Arrowheads: ANAE positive lymphocytes; b) Arrowhead: ANAE negative lymphocyte.

et al (2021) suggested that administration of DM (48 mL/day for 4 weeks, p.o) and/or HM (22 mL/day for 4 weeks, p.o) decreased the tissue (heart) MDA concentrations, within the non-pathological measurement range, in rats. Different results can be occurred due to using of different kits and organs, or administrated-milk content. In the present study, we could not observe any alterations in serum GSH values of all experimental groups which were normal ranges for rats. Li et al (2020) informed that Dezhou donkey's DM (3 g/kg /b.w/day for 28 days) administration did not cause significant changes in plasma GSH values in rats. Trinchese et al (2015) also stated that GSH values of liver tissue significantly increased due to DM (48 mL/day for 4 weeks, p.o) or HM (22 mL/day for 4 weeks, p.o) administrations when compared to cow milk (CM) (21 mL/day for 4 weeks, p.o)-treated or C groups. Similarly, GSH values and GSH/GSSG ratio of the rat skeletal muscle increased due to DM administration in rats (Trinchese et al 2018). It may be observed due to using of different dosage regimens, milk content, and texture. In our study, only serum SOD enzyme activities considerably increased in Ult, Pst, and Rm groups compared to the C group. Li et al (2020) reported that Dezhou donkey's DM (3 g/kg/b.w/day for 28 days) administration significantly increased the plasma SOD enzyme activity in diabetic rats which was consistent with our results. Trinchese et al (2018) also informed that SOD enzyme activity in the rat skeletal muscle was significantly higher in both DM- and HM-treated rats compared to C group. Conversely, Trinchese et al (2015) found a significantly lower SOD enzyme activity in the heart tissue of DM- and HM-treated groups compared to the C, interestingly. When the SOD enzyme activities are considered, the obtained results are of a quality proving the antioxidant effect of DM. In the present study, it was not detected any substantial alterations among the groups (C, Pst, Rm, and Ult) according to serum CAT enzyme activities. Trinchese et al (2018) revealed that DM and HM administration decreased mitochondrial hydrogen peroxide (H_2O_2) release and increased CAT enzyme activity in the cardiac tissue of rats. Although there are many studies about the effects of different domestic animal's (cow, camel, and goat) milk on CAT enzyme activity, we could not to reach any information about the effects of different preservation methods applied to DM samples on serum CAT enzyme activity in rats in the literature. It might be due to different milk content, kits, or tissues (Aspri et al 2017, Martini et al 2018, Trinchese et al 2018).

Cytokines are small, non-storage proteins that are secreted in a very short time from stimulated cells that mediate immunity, inflammation, and hematopoiesis. Its main purposes are to guarantee the lymphoid system's and some other cells' growth and differentiation, as well as to activate and draw inflammatory cells to the response region. Moreover, by directly preventing the proliferation of cancer cells, cytokines can result in the regression of tumors. Additionally, cytokines contribute to the body's increased anticancer actions (Akdoğan and Yöntem 2018). Generally, they are examined as pro-inflammatory cytokines (IL6, TNF-α, and IL-1β) and anti-inflammatory cytokines (IL-10). Cytokines are primarily involved in host defense against infections and diseases. However, their excessive production causes clinical symptoms such as shock, tissue damage, and weight loss in the host. The obtained cytokine levels were within normal reference for Wistar rats in the present study. In this study, IL-6 levels were detected as the highest (between normal ranges for rats) in Ult and Rm, however, lowest in the Pst group. When our study is evaluated in terms of IL 6, Ult-treated and raw donkey milk have a similar effect on rats. As reported by (Malissiova et al 2016), the fact that consumers traditionally prefer raw donkey milk, maybe a reflection of the knowledge that humanity has acquired from the ancient world history of its effects on the immune system (Mao et al 2009, Akca et al 2019). Taghiloo et al (2021) suggested that DM significantly increased IL-6 levels in PBMCs (in-vitro/human cell line). Also, Amati et al (2010) informed that serum IL-6 levels increased (23.0 pg/mL) following DM administration in humans. Besides, Mao et al (2009) also reported that active fractions of DM could stimulate cytokine production of IL-6 in A549 human lung cancer cells.

In our investigation, TNF-α levels were shown to be

greater in the Ult group when compared to other trial groups. Besides, TNF-α levels were found the lowest in the Pst group that was similar to IL-6 values in the present study. Tafaro et al (2007) showed that DM has an ability to release of TNF-α from PBMCs at different intervals from lactation. On the other hand, Trinchese et al (2015, 2018) exhibited that TNF-α levels decreased due to DM administration (48 mL/day) in the cardiac and skeletal muscle tissues. The rapid intestinal transit of micromolecules formed by ultrasound application could lead to these changes. In the present study, IL-10, an anti-inflammatory cytokine, levels of the rats were not affected by different types of DM administrations. It was reported that DM administration significantly increased IL-10 levels in PBMCs of humans in two previous studies (Tafaro et al 2007, Taghiloo et al 2021). In addition, Albenzio et al (2018) suggested that the cytokine IL-10 (PBMCs) did not differ among milking species in children with generalized epilepsy that corresponding with our results. These results can be obtained due to different study techniques (*in-vivo* or *in-vitro*). IL-1β, plays a main role in stimulation of the innate immune system, inflammation, and various chronic inflammatory diseases. Serum IL-1β levels (within normal ranges for rats) were significantly higher in the Pst and Ult groups than the Rm and C groups in the present study. Amati et al (2010) reported that DM administration (200 mL/day for one month) slightly increased serum IL-1ß levels, ranging within normal ranges, in healthy aged people. Tafaro et al (2007) also informed that DM administration induced the release of IL-1β from human PBMCs. Similarly, Mao et al (2009) suggested that DM could induce a marked increase in cytokine production of IL-1β from lymphocytes and macrophages of humans in vitro. The alterations that detected in this study can be interpreted as powered-immuno-modulatory activity of DM by using ultrasound or pasteurization techniques.

In this study, different types of DM administrations did not cause any alterations in differential WBC and ANAE-positive lymphocyte percentages of the experimental groups. According to Mao et al (2009), the active ingredients in DM have the potential to kill tumors indirectly via activating lymphocytes in addition to directly suppressing tumor proliferation in vitro. Also, it was reported that splenic lymphocyte proliferation and natural killer cell (NK) activity were significantly increased by DM (5 g/kg) in conjunction with *L. rhamnosus* ZDY114 (5×10^7 cfu mL^{-1}) as opposed to DM or *L. rhamnosus* ZDY114 alone (Peng et al 2014). Furthermore, Kisadere et al (2022) reported that DM (pasteurized or ultrasound-applied) administration increased the lymphocyte counts in rats. This might be related to the administration dose or ingredients of DM.

The literature does not provide any noteworthy information regarding the effects of various preservation techniques used on donkey milk samples on variations in the percentages of WBC and ANAE-positive lymphocytes.

Conclusion

It was determined that the composition of donkey milk (at the dose of 48 mL/day) stimulated the antioxidant system in rats. Also, antioxidative components of the donkey milk were not affected by heat. It was also found that donkey milk increased the production of cytokines that are effective in humoral immunity in rats. However, no immune modular effect was detected in rats fed pasteurized donkey milk, as the heat treatment applied to donkey milk during pasteurization denatures the bioactive components that have an immune modulator effect. As a result, ultrasound application provided a more effective antimicrobial effect on donkey milk than pasteurization. In addition, no negative effects on antioxidative and especially bioactive components were detected. Besides, ultrasound-treated DM showed a more pronounced impact on pro-inflammatory cytokine levels (IL-1β, TNF-α, IL-6) than pasteurized milk. Especially in functional foods, ultrasound can be used instead of pasteurization.

Declarations

Competing Interests

Authors declares that there are no conflicts of interest related to the publication of this article.

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Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author.

Ethical Statement

Balıkesir University Animal Experiments Local Ethics Committee's decision dated 25.03.2021, with the ethics committee approval number 2021/3-6, was obtained for the execution of this study.

Author Contributions

Motivation / Concept: IK, MFA, TEG, BA; Design: IK, MFA, TEG, BA; Control/Supervision: IK, MFA, TEG, BA; Data Collection and Processing: IK, MFA, TEG, BA; Analysis and Interpretation: IK, MFA, TEG,BA; Literature Review: IK, MFA, TEG, BA; Writing the Article: IK; Critical Review: IK, MFA, TEG, BA.

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