MANAS Journal of Engineering
ISSN 1694-7398 | e-ISSN 1694-7398

Volume 12, Issue 2, (2024) Pages 198-207
https://doi.org/10.51354/mjen.1528980

Investigation of growth and survival of some pathogens in koumiss

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*This manuscript is derived from the PhD thesis of the first author.

ABSTRACT

The aim of this study was to investigate the growth and survival of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Staphylococcus aureus* in koumiss produced from raw mare's milk during the fermentation process. *E. coli* O157:H7 (Group 1), *L. monocytogenes* (Group 2) and *S. aureus* (Group 3) were added to the raw mare's milk that would used to producing koumiss and a combination of all three pathogens (Group 4) was inoculated. These microorganisms were introduced at 10^6 CFU/mL concentration into the raw mare's milk, which served as the base for koumiss production. During fermentation, microbiological and chemical analyses were carried out by taking one sample from each group at the 1st, 5th and 24th hours and at the 2nd, 3rd, 4th and 5th days. As a result of analyzes; pH, dry matter, and protein content declined, while titration acidity and alcohol content increased. A positive correlation was found between the bacterial count and the utilized pathogenic microorganisms. Conversely, a negative correlation was observed with the count of yeast molds. Remarkably, the counts of *E. coli* O157:H7, *L. monocytogenes*, and *S. aureus* reduced starting from the fifth hour of fermentation and diminished to undetectable levels by the second day. This decline in pathogenic microorganisms below detectable thresholds during the fermentation process was linked to the rise in titration acidity and alcohol content and the decrease in pH.

ARTICLE INFO

Research article

Received: 06.08.2024
Accepted: 08.12.2024

Keywords:
E. coli O157:H7, koumiss, mare's milk, *L. monocytogenes*, *S. aureus.*

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1. Introduction

Fermented foods play a crucial role as nutritional sources in societies residing in developing regions across the globe. Additionally, traditional fermented products serve as abundant reservoirs of diverse bioactive compounds [1]. Various fermented milk products are prevalent worldwide, including koumiss, kefir, shubat, yakult, acidophilus, bifidus, and others. Incorporating probiotic flora into fermented dairy items contributes to the desired texture and flavor as well as having positive physiological effects [2]. Lactic acid bacteria in these foods have a significantly impact on consumer health, inhibiting pathogenic bacteria and generating antibacterial effects [3].

Koumiss has been a popular beverage in Kyrgyzstan, Kazakhstan, Mongolia, and among the Turkic, Mongolian, and Caucasian peoples of Russia, including the Altay, Bashkortostan, Buryatia, Dagestan, Kabardino-Balkaria, Kalmykia, Karachaevo-Cherkessia, Tatarstan, Tyva, Chuvashia, and Saha (Yakutia) regions [4]. Koumiss, a fermented product, is an example of an alcoholic acid product traditionally derived from mare's milk fermentation [5-7]. The term "koumiss" is spelled similarly in multiple languages, such as kumiss, kuymiss, kymyz, qymyz, qimiź, and kumiz [8]. Notably, distinct terminology like chigo, chigee, airag, and kumiss is utilized in Mongolia [1,9]. Researchers have found that certain types of bacteria, including *Lactobacillus*, *Lactococcus*, *Acetobacter*, *Streptococcus*, *Serratia*, and *Leuconostoc*, as well as fungi, such as *Kazachstania*, *Kluyveromyces*, *Trichosporonaceae*, *Pichia*, and *Candida*, are most common in the traditional fermentation process [10].

Typically, koumiss contains approximately 0.7–1.8% lactic acid, 0.6–2.5% ethyl alcohol, and 0.5–0.9% carbon dioxide [11]. The concentrations of acid and alcohol in koumiss exhibit variations based on factors such as fermentation duration and incubation temperature during its production [6,12]. Throughout history to the present day, koumiss has been employed to address various health issues [13-16]. These include anorexia, pulmonary tuberculosis, gastritis, typhoid, paratyphoid, dysentery, constipation, diarrhea, anemia, indigestion, and fatigue [17]. Including mare's milk in the human diet has experienced rapid growth in numerous European countries such as Germany, France, Belgium, Austria, Croatia, and the Netherlands [18].

The nutrients in the milk blend create an optimal milieu for the growth and proliferation of microorganisms. Milk originating from a healthy animal's udder remains devoid of microorganisms. Nevertheless, milk is susceptible to contamination throughout the milking procedure, whether transportation or direct contact with the animal [19]. It has been demonstrated that the presence of lactic acid bacteria in fermented products, such as koumiss, not only serves to preserve these products but also enhances their probiotic potential, thereby conferring health benefits that include improved gut microbiota balance and immune system modulation [20].

The goal of this study was to find out how well pathogenic microorganisms like *Escherichia coli O157:H7*, *Listeria monocytogenes*, and *Staphylococcus aureus* could grow and stay alive during the fermentation process in koumiss, which is a traditional fermented product made from mare's milk.

2. Materials and Methods

The study sourced mare's milk from family farms in Kyrgyzstan, specifically in Bishkek, and utilized koumiss samples to obtain a starter culture. The production of koumiss was conducted using wooden barrels. As for the pathogens, *Escherichia coli O157:H7* (ATCC 43894), *Listeria monocytogenes* (ATCC 7644), and *Staphylococcus aureus* (NCTC 10654) were employed in the research.

Inoculum preparation and inoculation

In the nutrient broth medium, *L. monocytogenes* was subjected to a 24-h incubation at 30°C, while *S. aureus* and *E. coli O157:H7* were incubated at 35°C for the same duration. After growth, the bacterial strain underwent centrifugation at 3000 rpm for 5 min, facilitating the removal of the supernatant. The resultant pellet was rinsed using 9 ml of ¼ Ringer's solution and subjected to an additional centrifugation cycle. Following this centrifugation, the supernatant was once again collected, and the pellet was dispersed by adding 9 ml of ¼ Ringer's solution. This procedure yields the initial solution. To determine the count of pathogenic microorganisms, dilutions were conducted up to 10^{-6} dilutions, and the microbial count in the solution within the tube was quantified. After 24 h of storage at 4°C, the pellet underwent further dilution to 10^{-6} dilutions, and its count was determined. A direct correlation was established between the microbial counts derived from these measurements. Utilizing the acquired data, an inoculation strain averaging 10^6 CFU/mL was prepared for introduction into experimental milk [21]. This inoculation was done at 25°C, mirroring the fermentation conditions of the mare's milk [11].

Experimental production of koumiss

Following microbiological and chemical assessments koumiss derived from mare's milk was employed as a starter culture in the experimental production of koumiss [22]. Koumiss starter culture was introduced to the mare's milk in a proportion of 20%, the mixture homogenized using a blending apparatus, and subsequently incubated at 25°C for up to 5 days. The wooden barrel was intermittently stirred during this incubation period using an appropriate stirring device. For the research, all three pathogenic microorganisms were initially introduced separately and subsequently, in combination, into raw mare's milk. The initial concentration of pathogens inoculated into the experimental groups stood at 10^6 CFU/mL. The categorization was as follows: Group 1 represented *E. coli O157:H7*, Group 2 signified *L. monocytogenes*, Group 3 denoted *S. aureus*, and Group 4 comprised all three pathogens in simultaneous interaction. Microbiological and chemical analyses punctuated the fermentation course through sample collection at distinct intervals, including the 1st, 5th, and 24th hours, as well as the 2nd, 3rd, 4th, and 5th days.

Microbiological analyses

The mare's milk and koumiss, produced using traditional methods, were meticulously blended. Samples of 1 ml each were collected under aseptic conditions, and decimal dilutions (up to 10^{-7}) were meticulously prepared. These samples were subsequently incubated and subjected to a double serial plating method using petri dishes, with the pour plate technique employed.

For the enumeration of Total Mesophilic Aerobic Colonies (TMAB), Plate Count Agar (PCA) (Merck 1.05463) was utilized. The agar was inoculated and then incubated at 32°C for 48 to 72 h, after which the colonies were counted. In the case of total psychrophilic aerobic colonies (TPAB), PCA agar (Merck 1.05463) was employed. This medium was cultivated and incubated at 7°C for 7 days, and subsequent colony enumeration was performed [23]. For the enumeration of *Enterobacteriaceae* spp., Violet Red Bile Dextrose Agar (VRBD) agar (Merck 1.10275) was used. The agar was inoculated and incubated at 37°C for 24 h, followed by colony counting. Yeast and Mold enumeration involved using Yeast Extract Glucose Chloramphenicol Agar (YGC) agar (Merck 1.16000). This medium was cultivated and incubated at 22°C for 5 days, after which colony counting was carried out [23]. DeMan, Rogosa, and Sharpe (MRS) agar (Merck 1.10660) were used for enumeration of *Lactobacillus* spp. The agar was

plated and incubated at 30°C for 72 hours, followed by colony counting [24]. Regarding *E. coli O157:H7*, Sorbitol MacConkey Agar (SMAC) agar (Merck 1.09202) supplemented with Cefixime Tellurite Selective (Oxoid, SR 0172 E) was utilized. The agar was cultivated, and the colonies were counted after incubation at 35°C for 24 h [24]. Furthermore, samples were procured from the breeding colonies for additional testing. Indole, Methyl Red, Voges Proskauer, and Citrate (IMVIC) tests were administered [25].

For the enumeration of *L. monocytogenes*, Palcam Agar Selective Supplement (Merck 1.12122) was inoculated onto Palcam Agar (Merck 1.11755) medium. Following incubation at 35°C for 24 to 48 h, colonies displaying a distinctive brown-olive color with a black zone were enumerated [23]. Additional testing, including Gram staining, oxidase testing, catalase testing, and ß-hemolysis testing, was conducted on samples obtained from the growing colonies [26].

After inoculating *S. aureus* onto Baird-Parker agar (Merck 1.05406) and adding egg yolk tellurite emulsion (Merck 1.03785), we incubated the culture at 37°C for 48 hours. Distinctive bright black colonies measuring 1–3 mm in diameter were identified and quantified [24].

Samples were collected from actively growing colonies and subjected to the streak plate technique. These samples were then inoculated onto a pre-prepared blood agar medium. After inoculation, the plates were incubated at 37°C for 24 h. The appearance of clear zones surrounding the colonies on the blood agar was used to evaluate the occurrence of β hemolysis. After this, Gram staining, oxidase, and catalase testing were conducted on the colonies cultivated in the blood agar [26].

Chemical analysis

The pH analysis of the mare's milk and koumiss was executed using a pH meter (Thermo Scientific Orion 3-star benchtop, USA). The assessment of total acidity was conducted through the lactic acid percentage method. Nitrogen content (%) was gauged employing the micro-Kjeldahl method. Determination of dry matter content was executed gravimetrically. The determination of ash content involved quantifying the residual inorganic components in the samples after moisture evaporation and incineration of organic constituents [27].

Following the guidelines outlined by the Gosudarstvennyy Standard (GOST), the quantification of alcohol content in koumiss was conducted using the method specified in GOST 3629-47. This approach was also employed for determining the ethyl alcohol content in dairy products such as kefir and koumiss [28].

Statistical analysis

Bacterial counts were transformed into log10 CFU/mL using the SPSS software package. Subsequently, alterations in the population of pathogenic microorganisms were juxtaposed against the temporal progression of the experiments. Within this framework, the data underwent analysis of variance (ANOVA) testing based on the x-time model, with a thorough assessment of intervariable interactions. Duncan's multiple comparison test delineated variations between significant means. The statistical significance of the analysis results was evaluated at significance levels of $p<0.05$ and $p<0.01$.

3. Results

The physicochemical and microbiological characteristics of raw mare's milk, utilized in the production of koumiss, along with koumiss utilized as a starter culture and experimental koumiss samples, were subjected to investigation. Table 1 displays the physicochemical properties of the raw mare's milk and the koumiss employed in this study.

Table 1. Phytochemical characteristics of the raw mare's milk and the koumiss used in the study. (n=10)

	Mare's milk			Koumiss		
Parameters	**min**	**max**	**mean±SD**	**min**	**max**	**mean±SD**
ph	6.37	7.18	6.84±0.30	3.80	4.57	4.04±0.23
Titratable acidity (% LA)	0.07	0.09	0.08±0.01	0.93	1.08	0.99±0.06
Dry matter (%)	10.72	11.28	10.95±0.17	9.80	10.63	10.28±0.27
Protein (%)	2.14	2.52	2.38±0.13	2.09	2.18	2.12±0.03
Ash (%)	0.30	0.37	0.33±0.02	0.23	0.36	0.29±0.04
Alcohol (%)	-	-	-	0.60	1.20	0.80±0.17

Meanwhile, Table 2 presents the microbiological properties of the raw mare's milk and the koumiss utilized in the experimental procedures. Table 3 illustrates the microbiological transformations observed in the experimental koumiss samples throughout the incubation period, while Table 4 outlines the resultant physicochemical alterations.

Table 2. Microbiological properties of raw mare's milk and koumiss used in the experiments.(n=10)

Microorganism	Number of Microorganisms (log10 CFU/mL)	
	Mare's milk	**Koumiss**
TMAB	4.71±0.30	6.88±0.58
Enterobacteriaceae spp	1.07±1.54	ND
Yeast Mold	0.40±0.66	6.42±0.53
TPAB	4.08±0.30	5.71±0.45
Lactobacillus spp.	1.50±1.60	7.10±0.36

ND: Not Detected

Table 3. Microbiological changes during fermentation in experimentally produced koumiss samples (log10 CFU/mL).

Groups	Microorganism	Times						
		1. hour	**5. hour**	**24. hour**	**2.day**	**3.day**	**4.day**	**5.day**
1. group	***E. coli O157:H7***	6.60±0.30^{a}	5.46±0.40^{b}	2.00±2.23^{c}	<1.0	<1.0	<1.0	<1.0
	TMAB	6.94±0.36^{b}	7.08±0.34^{b}	7.74±0.53^{a}	4.29±0.50^{d}	6.70±0.72^{bc}	6.39±0.34^{c}	6.37±0.34^{c}
	TPAM	6.52±0.36^{b}	6.45±0.47^{b}	7.06±0.64^{a}	4.08±0.33^{d}	6.26±0.48^{b}	5.26±0.36^{c}	5.24±0.42^{c}
	Lactobacillus spp	5.90±0.47^{a}	7.00±0.44^{b}	7.40±0.35^{a}	5.62±0.38^{a}	6.76±0.34^{b}	5.51±0.44^{a}	5.62±0.39^{a}
	Yeast Mold	5.57±0.39^{c}	5.03±0.43^{d}	6.06±0.46^{b}	5.24±0.23^{cd}	6.72±0.47^{a}	6.32±0.32^{b}	6.39±0.34^{ab}
2. group	***L. monocytogenes***	6.44±0.34^{a}	5.22±0.52^{b}	3.19±2.23^{c}	<1.0	<1.0	<1.0	<1.0
	TMAB	6.86±0.36^{ab}	7.18±0.36^{a}	7.17±0.29^{a}	4.45±0.64^{d}	6.49±0.41^{bc}	6.19±0.56^{c}	6.39±0.39^{c}
	TPAM	6.42±0.36^{ab}	6.13±0.32^{b}	6.63±0.36^{a}	4.05±0.37^{d}	6.22±0.46^{b}	5.26±0.40^{c}	5.23±0.43^{c}
	Lactobacillus spp	6.29±0.37^{b}	6.65±0.36^{a}	6.66±0.25^{a}	5.53±0.41^{c}	6.72±0.29^{a}	5.46±0.47^{c}	5.48±0.47^{c}
	Yeast Mold	5.54±0.42^{c}	5.07±0.52^{d}	6.09±0.34^{b}	5.25±0.40^{c}	6.58±0.59^{a}	6.32±0.41^{ab}	6.36±0.42^{ab}
3.group	***S. aureus***	6.38±0.37^{a}	5.33±0.35^{b}	5.26±0.40^{b}	<1.0	<1.0	<1.0	<1.0
	TMAB	6.89±0.35^{ab}	7.21±0.43^{a}	7.24±0.26^{a}	5.80±0.37^{e}	6.72±0.76^{bc}	6.22±0.60^{de}	6.36±0.41^{cd}
	TAMP	6.51±0.36^{ab}	6.22±0.38^{b}	6.78±0.32^{a}	5.08±0.44^{c}	6.31±0.48^{b}	5.25±0.44^{c}	5.23±0.44^{c}
	Lactobacillus spp	6.30±0.30^{b}	6.78±0.35^{a}	7.03±0.31^{a}	5.61±0.38^{c}	6.79±0.51^{a}	5.42±0.34^{c}	5.53±0.36^{c}
	Yeast Mold	5.57±0.42^{b}	5.06±0.38^{c}	6.03±0.32^{a}	5.20±0.28^{bc}	6.46±0.83^{a}	6.32±0.36^{a}	6.36±0.45^{a}
4. group	***E. coli O157:H7***	6.48±0.37^{a}	5.72±0.36^{a}	1.48±2.03^{b}	<1.0	<1.0	<1.0	<1.0
	L. monocytogenes	6.38±0.34^{a}	5.17±0.53^{b}	3.05±1.71^{c}	<1.0	<1.0	<1.0	<1.0
	S. aureus	6.39±0.32^{a}	5.80±0.41^{b}	5.55±0.42^{b}	<1.0	<1.0	<1.0	<1.0
	TMAB	9.29±0.13^{a}	9.01±0.40^{a}	8.33±0.3^{b}	6.44±0.35^{c}	6.47±0.40^{c}	6.32±0.63^{c}	6.49±0.32^{c}
	TAMP	8.22±0.11^{a}	8.46±0.49^{a}	8.03±0.09^{a}	5.36±0.42^{c}	6.27±0.39^{b}	5.30±0.30^{c}	5.19±0.43^{c}
	Lactobacillus spp	5.98±0.13^{b}	6.81±0.42^{a}	7.05±0.14^{a}	5.67±0.29b^{c}	6.73±0.45^{a}	5.35±0.33^{c}	5.50±0.47^{c}
	Yeast Mold	4.75±0.49^{e}	4.67±0.38^{e}	5.77±0.38^{c}	5.27±0.29^{d}	6.53±0.31^{a}	6.01±0.26^{bc}	6.29±0.38^{ab}

a, b, c, d, e: Differences between means with different letters in the same row are statistically significant. ($p<0.05$)

Table 4. Physico-chemical changes during fermentation in experimentally prepared koumiss samples.

Groups	Parameters	Times						
		1.hour	**5. hour**	**24 hour**	**2. day**	**3. day**	**4. day**	**5. day**
1.group	**pH**	5.03±0.48^{a}	4.76±0.41^{b}	3.95±0.17^{c}	3.70±0.04^{d}	3.74±0.08^{cd}	3.69±0.05^{d}	3.68±0.06^{d}
	Titratable acidity (%LA)	0.38±0.01^{g}	0.67±0.05^{f}	1.54±0.03^{e}	1.66±0.18^{d}	2.05±0.03^{c}	2.44±0.03^{b}	2.57±0.03^{a}
	Dry matter (%)	10.90±0.18^{a}	10.91±0.25^{a}	10.51±0.26^{b}	10.43±0.29^{b}	10.43±0.31^{b}	10.42±0.26^{b}	10.41±0.27^{b}
	Protein (%)	2.39±0.14^{a}	2.38±0.14^{a}	2.12±0.02^{b}	2.12±0.05^{b}	2.12±0.02^{b}	2.12±0.03^{b}	2.08±0.05^{b}
	Ash (%)	0.30±0.05^{a}	0.30±0.05^{a}	0.30±0.05^{a}	0.29±0.03^{a}	0.29±0.03^{a}	0.30±0.04^{a}	0.30±0.02^{a}
	Alcohol (%)	0.30±0.06^{f}	0.51±0.10^{e}	1.77±0.21^{d}	1.82±0.15^{bc}	1.95±0.15^{c}	2.90±0.14^{b}	3.05±0.13^{a}
2.group	**pH**	5.05±0.56^{a}	4.83±0.35^{a}	3.90±0.09^{b}	3.71±0.04^{b}	3.73±0.05^{b}	3.69±0.02^{b}	3.69±0.03^{b}
	Titratable acidity (%LA)	0.38±0.01^{g}	0.67±0.03^{f}	1.52±0.03^{e}	1.69±0.04^{d}	2.05±0.04^{c}	2.45±0.03^{b}	2.56±0.02^{a}
	Dry matter (%)	10.71±0.29^{a}	10.85±0.24^{a}	10.41±0.27^{b}	10.46±0.27^{b}	10.45±0.28^{b}	10.42±0.19^{b}	10.41±0.23^{b}
	Protein (%)	2.36±0.13^{a}	2.41±0.13^{a}	2.12±0.02^{b}	2.09±0.03^{b}	2.12±0.03^{b}	2.11±0.01^{b}	2.11±0.02^{b}
	Alcohol (%)	0.30±0.04^{f}	0.54±0.05^{e}	1.77±0.12^{d}	1.83±0.12^{d}	1.96±0.11^{c}	2.93±0.09^{b}	3.08±0.11^{a}
	Ash (%)	0.31±0.04^{a}	0.31±0.04^{a}	0.30±0.04^{a}	0.30±0.02^{a}	0.30±0.03^{a}	0.29±0.03^{a}	0.30±0.03^{a}
3.group	**pH**	5.03±0.52^{a}	4.80±0.35^{b}	3.94±0.11^{c}	3.71±0.03^{cd}	3.73±0.06^{cd}	3.69±0.03^{d}	3.70±0.04^{d}
	Titratable acidity (%LA)	0.38±0.01^{g}	0.66±0.03^{f}	1.53±0.04^{e}	1.65±0.17^{d}	2.04±0.03^{c}	2.44±0.02^{b}	2.58±0.02^{a}
	Dry matter (%)	10.79±0.25^{a}	10.87±0.26^{a}	10.41±0.28^{b}	10.46±0.27^{b}	10.43±0.26^{b}	10.44±0.23^{b}	10.39±0.27^{b}
	Protein (%)	2.35±0.15^{b}	2.44±0.10^{a}	2.13±0.02^{c}	2.11±0.04^{c}	2.12±0.03^{c}	2.12±0.02^{c}	2.09±0.04^{c}
	Alcohol (%)	0.30±0.04^{f}	0.54±0.08^{e}	1.77±0.15^{d}	1.82±0.15^{d}	1.98±0.10^{c}	2.93±0.09^{b}	3.06±0.10^{a}
	Ash (%)	0.31±0.04^{a}	0.31±0.05^{a}	0.30±0.04^{a}	0.30±0.02^{a}	0.30±0.03^{a}	0.29±0.03^{a}	0.29±0.03^{a}
4.group	**pH**	5.07±0.28^{a}	4.79±0.14^{b}	3.90±0.12^{c}	3.71±0.04^{d}	3.70±0.02^{d}	3.69±0.04^{d}	3.69±0.03^{d}
	Titratable acidity (%LA)	0.38±0.01^{e}	0.65±0.03^{d}	1.51±0.03^{c}	1.59±0.24^{c}	2.04±0.02^{b}	2.45±0.01^{a}	2.57±0.02^{a}
	Dry matter (%)	10.84±0.21^{a}	10.90±0.19^{a}	10.46±0.17^{b}	10.28±0.26^{b}	10.44±0.10^{b}	10.41±0.22^{b}	10.22±0.25^{b}
	Protein (%)	2.40±0.11^{b}	2.48±0.03^{a}	2.11±0.02^{c}	2.10±0.03^{c}	2.12±0.03^{c}	2.12±0.02^{c}	2.09±0.02^{c}
	Alcohol (%)	0.31±0.05^{g}	0.59±0.04^{f}	1.86±0.14^{e}	1.73±0.13^{d}	2.02±0.08^{c}	2.92±0.08^{b}	3.09±0.10^{a}
	Ash (%)	0.31±0.02^{a}	0.31±0.01^{ab}	0.29±0.01^{b}	0.31±0.01^{ab}	0.30±0.02^{ab}	0.31±0.01^{ab}	0.30±0.01^{ab}

a, b, c, d: Differences between means with different letters in the same row are statistically significant. ($p<0.05$)

Table 5 illustrates the relationship between the microbiological and chemical attributes of koumiss within the experimental groups and the quantities of *E. coli O157:H7*. Among the koumiss samples, a positive correlation surfaced between the *E. coli O157:H7* count and the enumerations of *L. monocytogenes*, *S. aureus*, TMAB, TPAB, *Lactobacillus* spp., pH ratio, dry matter, and protein content. Conversely, a negative correlation was evident between the yeast mold count, alcohol content, and titratable acidity ($p < 0.01$).

Table 5. Correlation of *E. coli* O157:H7 numbers with microbiological and chemical properties of koumiss in experimental groups.

	r value	
	1. Group	**4. Group**
Escherichia coli O157:H7	1	1
Listeria monocytogenes	-	+0.936**
Staphylococcus aureus	-	+0.833**
TMAB	+0.389**	+0.865**
TPAB	+0.552**	+0.803**
Lactobacillus spp	+0.246*	+0.273
Yeast mold count	-0.510**	-0.778**
pH	+0.840**	+0.963**
Titratable acidity (%LA)	-0.891**	-0.887**
Dry matter (%)	+0.610**	+0.709**
Alcohol (%)	-0.846**	-0.861**
Protein (%)	+0.765**	+0.877**
Ash (%)	-0.009	+0.279

*p<0.05, **p<0.01 significant

Table 6 provides an overview of the relationship between koumiss microbiological and chemical attributes within the experimental groups and the quantities of *L. monocytogenes*. In the koumiss samples, a positive correlation emerged between the count of *L. monocytogenes* and the enumerations of *E. coli O157:H7*, *S. aureus*, TMAB, TPAB, *Lactobacillus* spp., pH ratio, dry matter, ash content, and protein content. Conversely, a negative correlation was evident between the yeast mold count, alcohol content, and titratable acidity ($p < 0.01$).

Table 6. Correlation of the number of *Listeria monocytogenes* to microbiological and chemical properties of koumiss in experimental groups.

	r value	
	2.Group	**4.Group**
Escherichia coli O157:H7	-	+0.936**
Listeria monocytogenes	1	1
Staphylococcus aureus	-	+0.913**
TMAB	+0.529**	+0.924**
TPAB	+0.546**	+0.868**
Lactobacillus spp	+0.435**	+0.376*
Yeast mold count	-0.451**	-0.721**
pH	+837**	+0.925**
Titratable acidity (%LA)	-891**	-0.893**
Dry matter (%)	+0.438**	+0.722**
Alcohol (%)	-0.853**	-0.856**
Protein (%)	+751**	+0.824**
Ash (%)	+0.180**	+0.145

*p<0.05, **p<0.01 significant

Table 7. Correlation of *Staphylococcus aureus* number to microbiological and chemical characteristics of koumiss in experimental groups.

	r value	
	3.Group	**4.Group**
Escherichia coli O157:H7	-	+0.833**
Listeria monocytogenes	-	+0.913**
Staphylococcus aureus	1	1
TMAB	+0.573**	+0.943**
TPAB	+0.632**	+0.930**
Lactobacillus spp.	+0.537**	+0.499**
Yeast mold	-0.418**	-0.675**
pH	+0.771**	+0.818**
Titratable acidity (%LA)	-0.884**	-0.872**
Dry matter (%)	+0.420**	+0.650**
Alcohol (%)	-0.832**	-0.818**
Protein (%)	+0.670**	+0.728**
Ash (%)	+0.099	+0.040

*p<0.05, **p<0.01 significant

The correlations between the microbiological and chemical attributes of koumiss within the experimental groups and the counts of *Staphylococcus aureus* are depicted in Table 7. Among the koumiss samples, a positive correlation surfaced between the quantity of *Staphylococcus aureus* and the quantities of *Escherichia coli O157:H7*, *Listeria monocytogenes*, TMAB, TPAB, *Lactobacillus* spp., pH ratio, dry matter, ash content, and protein content. Simultaneously, a negative correlation was apparent between the count of yeast molds, alcohol content, and titratable acidity ($p < 0.01$).

4. Discussion

Koumiss is a fermented product traditionally made from unpasteurized mare's milk. Research has investigated various physicochemical and microbiological properties of mare's milk and koumiss. It is believed that factors such as the season in which the milk is obtained, the animal breeds involved, the age of the mare, the lactation period, varied feed compositions, starter cultures, paddock, and milking conditions, as well as the incubation period and temperature, can all differ and influence the final product.

Several types of microorganisms were found in koumiss samples by Tegin [29]. These included TMAB counts ranging from 5.16±0.009 to 7.05±0.011 log10 CFU/mL, yeast mold counts ranging from 4.53±0.009 to 6.83±0.006 log10 CFU/mL, lactic acid bacteria count ranging from 5.13±0.026 to 7.10±0.004 log10 CFU/mL, and staphylococci-micrococci group microorganisms ranging from 0.77±0.249 to 4.17±0.044 log10 CFU/mL. The values noted by the researcher were consistent with those determined in this study for koumiss used as a starter culture. Out of the 25 samples used in Tegin's study, only one contained a coliform group microorganism detected at a level of 1.26±0.089 log10 CFU/mL. In contrast, this study did not detect coliform group microorganisms using koumiss as a starter culture.

Chaves-López et al. [30] reported that Colombian koumiss

produced from cow's milk contained lactic acid bacteria at levels of 7.05–9.53 log10 CFU/mL and yeast at levels of 6.26–8.65 log10 CFU/mL. This study observed that the koumiss used as a starter culture had counts close to those of lactic acid bacteria and yeast mold. Similarly, Mu et al. [31] found yeast counts in Chinese koumiss samples to be in the range of 5–7 log10 CFU/mL.

This study demonstrated a similar yeast mold count in koumiss used as a starter culture. During the experimental period, the pH of koumiss produced from mare's milk decreased steadily until the end of the 5th day of fermentation, when the analyses were conducted. This finding aligns with previous studies where a reduction in pH was a natural outcome of the fermentation process [22, 32-35]. It was observed that the growth of *Escherichia coli O157:H7*, *S. aureus*, and *L. monocytogenes* was significantly inhibited, particularly with a decrease in pH ($p < 0.01$).

In the koumiss samples, there was a clear positive correlation between pH and the tested pathogenic microorganisms. This correlation showed that as the pH value decreased, the number of pathogenic microorganisms also decreased in all four groups. *E. coli O157:H7* and *L. monocytogenes* also showed no growth in analyses performed on the second day. However, *S. aureus* showed no growth on the third day. The titratable acidity of the experimental koumiss samples increased at the end of the fermentation period. This finding was consistent with previous studies [22, 29, 32, 33, 36].

The increase in titratable acidity can be caused by Lacto-fermentation, which also showed a negative effect on *E. coli O157:H7*, *S. aureus* and *L. monocytogenes* numbers ($p < 0.01$). There was a negative correlation between the titratable acidity and the pathogenic microorganisms. An increase in the titratable acidity decreased the number of pathogenic microorganisms in all groups. Also, fermentation time showed a clear ($p < 0.05$) influence on protein content. This finding is consistent with the previous studies [22, 32, 33]. Proteolysis during fermentation may have caused the reduction in the protein content. Additionally, the study noted a decrease in the dry matter content after fermentation, which aligned with findings from various studies, including [22, 33, 36, 37]. The starter culture's fermentation caused this decrease in dry matter content.

The impact of fermentation time on ash content in experimentally produced koumiss samples from mare's milk was not deemed significant ($p > 0.05$). Likewise, the effect of ash content on the tested pathogens did not yield statistical significance. The ash content recorded in this study is consistent with values reported by various researchers [37, 38].

Table 4 demonstrates a consistent rise in alcohol content throughout the incubation period of koumiss ($p < 0.05$). This increase in alcohol content during koumiss fermentation is consistent with findings from multiple studies [12, 22, 34]. Notably, the reductionary impact of alcohol content on the tested pathogens was statistically significant across all four groups ($p<0.01$). The determined alcohol content in this study was 3.05±0.13 in the 1st group, 3.08±0.11 in the 2nd group, 3.06±0.10 in the 3rd group, and 3.09 in the 4th group. The variation in alcohol content outcomes among different studies may be predominantly attributed to varying incubation periods.

In the koumiss experimentally produced from mare's milk, the TMAB count closely aligned with the values reported by Tegin's study [29]. The impact of fermentation duration on the TMAB count within koumiss proved significant in this study ($p < 0.05$). The reduction in TMAB count observed by the end of the second day is attributed to the suppression of certain microorganisms' growth within the starter cultures. This phenomenon is linked to increased titratable acidity, elevated alcohol content, and diminished pH. In all three groups, the analyses on the fourth and fifth days indicated a partial stabilization in the increase TMAB levels, first observed at the end of the third day. This augmentation in TMAB count on the third day is attributed to the growth and flourishing of dominant species resilient to variations in the physicochemical properties of koumiss.

The quantity of *Lactobacillus* spp. detected in this study corresponded with previous research [22,29,34]. Table 4 illustrates the count of *Lactobacillus* spp. across all four groups. However, it decreased on the second day, followed by an increase on the third day, only to decline on the fourth and fifth days. The growth and proliferation of dominant species could be the cause of this elevation in the *Lactobacillus* spp. count. These hardy species are adaptable to fluctuations in koumiss's physicochemical properties, taking the place of suppressed strains. Notably, studies have reported that *L. plantarum* strains isolated from koumiss exhibit antibacterial effects against *E. coli* and *L. innocua* [35]. Similarly, Zhang et al. [39]. noted that *L. casei Zhang*, obtained from koumiss, demonstrated antibacterial properties against *E. coli*, *Escherichia coli* O157:H7, and K88. Another study showed that *L. plantarum* LB-B1 pediocin, which was taken from koumiss, could stop the growth of Listeria, Lactobacillus, Streptococcus, Enterococcus, Pediococcus, and Escherichia, even stopping *L. monocytogenes* [40].

Yinfeng et al. [41] reported that starter cultures present in koumiss had an inhibitory effect on *Listeria* spp., *S. aureus*, and *E. coli* growth. Ren et al. [42] documented that oral administration of *L. paracasei*, isolated from koumiss to *E. coli*-infected diarrheal mice treated and prevented *E. coli*-induced diarrhea, suggesting that koumiss might enhance immunity by improving gut microbiota. Similarly, Sanam et al. [43] reported that Lactobacilli isolated from mare's milk inhibited *S. aureus*, *E. coli*, and *Bacillus cereus*. These outcomes parallel the findings of the present study. Furthermore, Yinfeng et al. [41] mentioned that nine strains of isolated *Lactococcus* and 12 strains of *Lactobacillus* from koumiss had inhibitory effects on *Listeria* spp. but lacked a

preventive effect on *E. coli* and *S. aureus*. While their observations regarding *Listeria* are consistent with the findings of this study, their claims of effects on *E. coli* and *S. aureus* do not align. The observed yeast and mold count in the experiments had a significant impact on the number of pathogenic microorganisms ($p < 0.01$). A negative correlation was evident between the yeast mold count and the examined pathogenic microorganisms, suggesting that higher yeast mold counts correlated with decreased pathogenic microorganism counts across all four groups.

Chen et al. [44]. reported that *Saccharomyces cerevisiae* isolated from koumiss contained antibacterial compounds, which hindered *E. coli* O8 growth and replication by affecting its cell surface. Another study found that yeasts like *S. cerevisiae* and *Kluyveromyces marxianus* in koumiss starter cultures had antibacterial properties that kept mice from getting *E. coli* infections [45]. These findings resonate with the outcomes of the present study. Additionally, Yinfeng et al. [41] noted that four yeast strains isolated from koumiss had a preventive effect on *E. coli*, with two of these yeast strains also showing a preventive effect on *S. aureus*. However, they reported that the yeast strains had no inhibitory effect on Listeria.

5. Conclusion

The study involved inoculating raw mare's milk with *E. coli O157:H7* ATCC 43894, *L. monocytogenes* ATCC 7644, and *S. aureus* NCTC 10654 at a 10^6 CFU/mL concentration. Experimental groups were established using a 20% proportion of koumiss as a starter culture and were then incubated at 25°C. A decline in the count of pathogenic microorganisms was observed at the 5th and 24th hours within the experimental groups. Over the 2nd, 3rd, 4th, and 5th days, the tested pathogenic microorganisms' counts dropped to undetectable levels. The study unveiled a positive correlation between the rate of pH reduction and the initial concentration of inoculated pathogenic microorganisms at 10^6 CFU/mL.

In contrast, a negative correlation was identified between titratable acidity and alcohol content. Notably, the reduction in pathogenic microorganism counts in koumiss samples during experimentation, followed by their subsequent decline to undetectable levels, was attributed to pH reduction, increased titratable acidity, and heightened alcohol content. Consequently, the research suggests that koumiss produced from raw mare's milk and incubated at 25°C could potentially harbor pathogenic microorganisms up until the second day. The study also indicated that raw mare's milk possessed favorable microbiological quality. However, considering that raw mare's milk is consumed for therapeutic purposes, it is imperative to recognize that neglecting hygiene practices during milking and using milk from diseased mares could lead to serious health issues and negatively impact public health. Likewise, sensitizing farmers to use utilize high-quality milk for koumiss production and providing education on hygiene and sanitation practices would be prudent. It is understood that further comprehensive investigations are necessary to ensure standardized koumiss production and to determine its treatment and therapeutic attributes.

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