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ORIGINAL PAPER

An in-vitro assessment of antifungal and antibacterial activity of cow, camel, ewe, and goat milk kefir and probiotic yogurt

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Abstract

In the present study, the antimicrobial activity of kefir and probiotic yogurt produced from cow, camel, ewe, and goat milk on pathogenic bacteria (*Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella enterica*) and fungi (*Aspergillus niger*, *Penicillium* sp., and *Fusarium* sp.) during 20-day storage period at 4 ºC was investigated. The carbohydrate content and pH of milk samples decreased during the fermentation process to produce probiotic yogurt and kefir, also the acidity increased significantly. The results revealed that kefir samples had stronger antifungal and antibacterial effect than probiotic yogurt samples. Among kefir samples, the ewe and cow milk kefir expressed the highest and the lowest antimicrobial activity, respectively. *A. niger*, *S. aureus*, and *L. monocytogenes* were the most sensitive and *Penicillium* sp. and *E. coli* were the most resistant microorganisms against treatment by kefir and probiotic yogurt.

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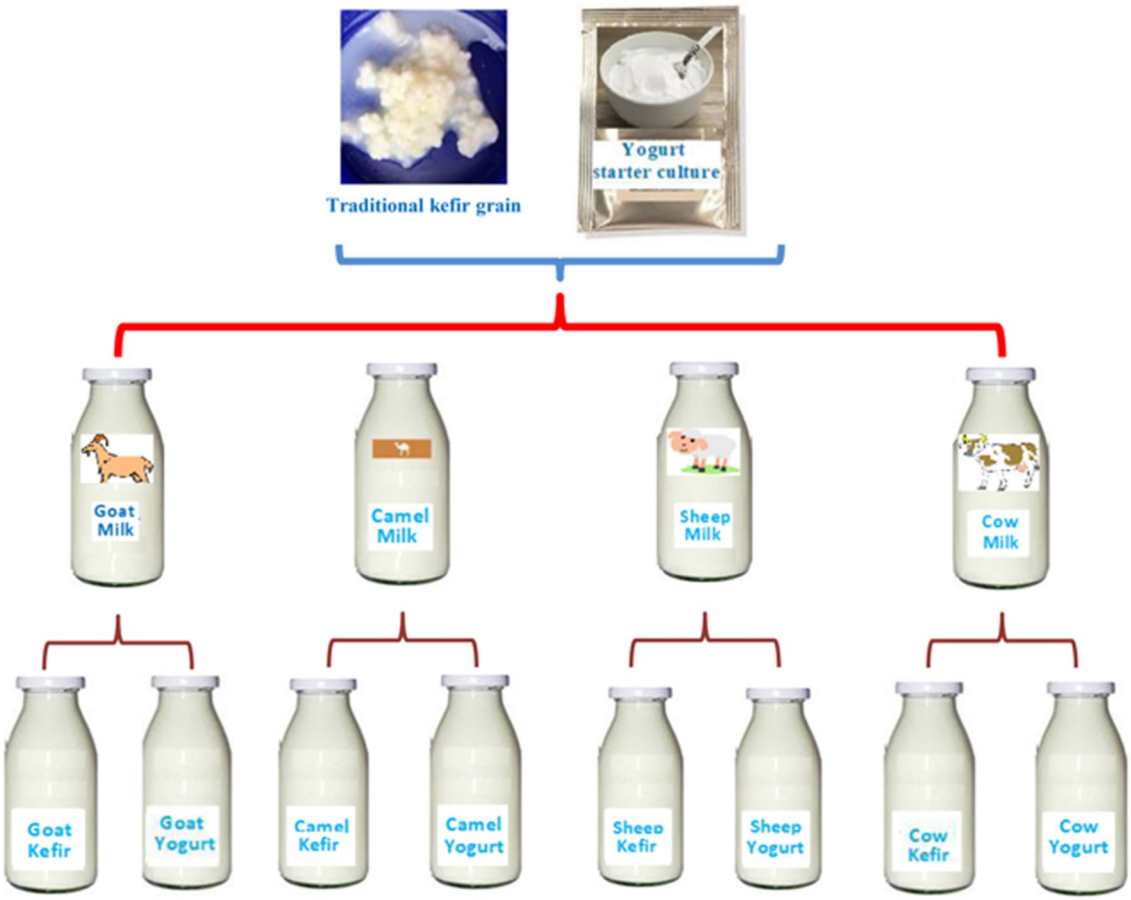
Graphic abstract



Keywords Antibacterial · Antifungal · Kefir · Probiotic yogurt

Introduction

Lactic acid bacteria (LAB) are considered as one of the most important groups of probiotic microorganisms, have a significant effect on the technological process and quality of the fermented dairy products. LAB originated from different types of animal or plant foods and are known as natural food preservatives. This great preservative potential is attributed to the production of several types of inhibitory metabolites such as organic acids, bacteriocins, hydroxylated fatty acids, diacetyl, hydrogen peroxide, and reuterin [1, 2]. In the last decade, several studies have been conducted to find and use LAB strains as bio-preservatives in the food and beverage industries.

Due to the presence of high potential natural inhibitory mechanisms such as lactoferrins, immunoglobulins, lysozyme, and the lactoperoxidase/thiocyanate/hydrogen peroxide system, variable degrees of protection against pathogen and spoiling microorganisms has been found in the milk of different mammals [3]. It is reported that camel milk possesses antimicrobial and bactericidal properties [4] and has higher inhibitory activity compared to cow's milk as the concentrations of lactoferrins and lysozyme are three and two times higher than those of cow's milk, respectively [5]. Camel's milk protein includes a heterogeneous complex of α-lactalbumin, lactophorin, serum albumin, immunoglobulins, and peptidoglycans. It is worthy to note that camel's milk has a longer shelf-life at room temperature than other mammals' milk [6]. Some recent studies investigated the antimicrobial potential of proteins of ewe's milk and cheese [7, 8]. It has been reported that four bioactive peptides with antifungal and antibacterial activity were produced through hydrolysis of αs_2 ewe milk casein and pepsin [7, 9] but little information is found on antimicrobial activity of fermented ewe milk as yogurt or kefir. The fatty acids profile of goat milk shows medium and short-chain fatty acids, like caproic, caprylic and capric acid that have biofunctional and beneficial effects on human health, such as reducing the level of

cholesterol. Also, it is reported that αS_1-casein in goat milk shows lower allergenic potential than cow milk [10, 11].

Kefir and yogurt are among the most popular fermented dairy products in the world. Yogurt is produced using a bacterial starter culture to ferment the milk. Kefir drink originated from the Caucasus, Eastern Europe, and Russia is fermented milk produced by kefir grains that are consisted of a symbiotic mixture of lactic and acetic acid bacteria, several genera of yeasts, and mycelial fungi aggregated in a polysaccharide matrix named kefiran [12]. Yogurt has been introduced in the Iranian diet since centuries ago and is well accepted as a rich source of calcium. It includes both types of food microbial cultures, starters and probiotic microorganisms [10]. The microorganisms of the yogurt starter culture and kefir grains produce lactic acid and natural bioactive compounds, bacteriocins, and antibiotics during fermentation. Kefir and yogurt have been reported to be beneficial to human nutrition and health, such as improving the function of the immune system and digestive organs, helping the treatment of blood hypertension, allergies, metabolic defects, and heart diseases [13]. Namaei et al. reported the antibacterial activity of non-industrial yogurt against *Salmonella* and *Shigella* [14]. Also, de Lima et al. showed that Brazilian kefir produced from fermented sheep's milk was a rich source of antimicrobial and antioxidant metabolites [7]. In a study by Said et al., the antimicrobial activity of goat milk kefir on *Escherichia coli* and *Salmonella enteric* subsp. *enterica serovar typhimurium* was demonstrated [15].

The publication is scarce on antibacterial and antifungal activity of kefir and probiotic yogurt produced from camel, ewe, and goat's milk, probably because of the restricted and low availability of these species' milk on the market. It is assumed that fermented dairy products possess different antimicrobial activity based on their milk source, starter culture, and shelf-life; therefore, the aim of the present work was to determine and compare the pH, acidity, antibacterial, and antifungal activity of the probiotic yogurts and kefirs produced from cow, camel, ewe, and goat milk at different stages of the storage at 4 ºC.

Materials and methods

Chemicals and culture media

All the chemicals, reagents, consumables (such as filter papers), and culture media used in this work were obtained from Merck (Darmstadt, Germany).

Preparing inoculums of kefir and probiotic yogurt

Probiotic yogurt samples were prepared using commercial starter culture (containing *Lactobacillus delbrueckii* subsp. *bulgaricus, Streptococcus thermophiles*, and *bifidobacteria*) as direct vat set culture purchased from Danisco (Denmark). To produce kefir, traditional kefir grains were obtained from the rural producers in Semnan countryside (Semnan, Iran). Active kefir grains were recovered by transferring the grains into the pasteurized low-fat cow milk (approximately 0.45% fat content) and incubated at 25 ± 1 °C for 24 h and it was repeated for 7 consecutive days [16]. Then, the kefir grains were filtered to separate the milk curd and washed with sterile distilled water 3 times. The grains were inoculated into pasteurized cow milk and stored at 25 ± 1 °C until used.

Producing kefir and probiotic yogurt samples

Raw cow, ewe, and goat milk were obtained from the dairy farm of Bandpei (Mazandaran, Iran) and camel milk was purchased from a camel farm in Kalaleh (Golestan, Iran). All milk samples were heated to 85 ± 1 °C for 10 min in the water bath and cooled to the temperature appropriate for inoculation (43 °C for probiotic yogurt and 25 °C for kefir). Kefir samples were prepared by inoculating kefir grains (5% v/v) to each individual milk and incubating (Memmert Incubator 400, Switzerland) at 25 °C for 20 h. The probiotic yogurt samples were produced by mixing milk samples and starter culture (2% v/v) followed by incubation at 43–45 °C until reaching a pH 4.6 ± 0.1. At the end of the fermentation period, the kefir samples were filtered through a sterile metal sieve (1.5 mm pore size) in order to separate the kefir grains and then filled into 250 mL bottles. All samples were kept at 4 ± 1 °C until analysis. The samples were analyzed on the 1st, 5th, 10th, 15th, and 20th days of the storage period [17].

Chemical compositions

In order to evaluate the effect of the fermentation on the chemical composition of milk, the moisture, ash, fat, protein, and total carbohydrates contents of pasteurized milk, probiotic yogurt, and kefir samples were determined after 24 h fermentation according to AOAC procedures [18].

Preparing the kefir and probiotic yogurt samples for the assays

For the chemical assays, 2 g of each sample was added to 20 mL of extracting solvent (methanol/water, 70:30 v/v) and mixed thoroughly using a magnetic stirrer (model RSM-03-10K, Phoenix, Germany) for 4 h at 20 ± 1 °C in a dark place. Then the mixture was centrifuged (model Z206A, Hermle, Germany) at 3000 rpm for 12 min and filtered using a Whatman™ Grade 2 cellulose qualitative filter paper (Diameter: 12.5 cm, Pore Size: 8 µm). The supernatants were used to determine pH and acidity [17].

In order to prepare samples for antimicrobial experiments, the fermented products were filtered through a metal sieve of 1.2-mm^2 mesh size and the filtrate was centrifuged at 13,500 × *g* for 15 min to precipitate the microorganisms. The obtained kefir/yogurt cell-free supernatants were sterilized using a nitrocellulose filter (0.22-µm pore size) and kept at − 20 °C until used for antimicrobial assays [19].

pH measurement

The pH of the kefir and probiotic yogurt extracts was measured using a pH-meter model 913 (Metrohm, Switzerland). The pH-meter was calibrated before use, by pH 4.00 and 7.00 standard buffer solutions.

Measuring total acidity

The acidity of the samples was determined according to AOAC procedure and reported as grams of lactic acid per liter of the product [18].

Antifungal activity

Antifungal activity assay was performed according to the method described by Eddine et al. and Gamba et al. [21, 22] with some modifications. *Aspergillus niger* (ATCC 9142), *Penicillium* sp., and *Fusarium* sp., as the most common fungi causing contamination and spoilage in food and feed, were obtained from the Organization of Scientific and Industrial Research (Tehran, Iran). The fungi were cultured on the slant medium of potato dextrose agar (PDA) and incubated at 30 ± 1 °C for 7 days. Then 10 ml of sterile sodium lauryl sulfate (0.01% w/v in NaCl 1%) was added to the slant PDA to obtain monospore suspension. The suspensions were filtered through Whatman paper (of 180 µm pore size). The conidia were counted using a Neubauer chamber and the fungal population was adjusted to 5×10^5 conidia/mL. The antifungal experiment was performed on Petri dishes and the basal medium consisted of malt extract (1% w/v), yeast extract (2% w/v), and agar (2% w/v). The culture media were sterilized in an autoclave at 121 °C for 15 min and after cooling to 45 °C the agar medium was mixed with the kefir or probiotic yogurt extract (10% v/v) [20]. Then, 20 mL of the supplemented medium was transferred into the Petri dishes [90 mm diameter).

The plates were inoculated by micro-pipetting of 10 µL of the conidia suspensions at 5×10^5 conidia/mL, in the center of the solidified culture medium. Positive control plates were agar media without any kefir or probiotic yogurt extract, inoculated with the same volume of fungal suspension. Negative control plates contained agar media supplemented with the same amount of kefir or probiotic yogurt extract and no fungal inoculation. The diameter of the inoculums was measured and considered as the fungal colony's initial diameter. Inoculated Petri dishes were put in the plastic boxes containing bottles of water to prevent dehydration and incubated at 25 ± 1 °C for 7 days. As filamentous fungi that grow on solid culture media extend a circular formed colony around the initial inoculation area, the diameter of colonies was measured. The mean diameter of each colony was calculated from four diameter measures taken from the center of each individual colony [21, 22]. The diameter of growth inhibition zone was calculated as:

$$\text{Growth inhibition zone} = D_{\text{control}} - D_{\text{sample}}$$

where D_{control} was the mean diameter (mm) of the fungal colony in control and D_{sample} was the mean diameter (mm) of the treated samples. The antifungal activity was evaluated on days 1, 5, 10, 15, and 20 of the storage period of the samples.

Antibacterial activity

The most important foodborne bacterial pathogens, *Staphylococcus aureus* (ATCC 29213), *Listeria monocytogenes* (PTCC 1298), *Escherichia coli* (ATCC 25922), and *Salmonella enterica* (ATCC 14028) were kindly provided by the Department of Microbiology (Faculty of Veterinary Medicine, University of Tehran).

The initial cultures of bacteria were transferred into brain heart infusion broth and incubated at 37 ± 0.5 ºC for 18–24 h. The bacterial suspension was centrifuged at 12,000 × *g* for 15 min, the obtained pellet was washed 2 times with sterile phosphate buffer saline (PBS) and the supernatant was discarded. The pellets were re-suspended in PBS and the bacterial count was subsequently adjusted to 5×10^{10} cfu/mL using McFarland turbidity standard solutions [4]. The standard well agar diffusion technique described by Cheesbrough [23] was performed to determine the antibacterial activity of probiotic yogurt and kefir samples against pathogenic bacteria (*S. aureus, L. monocytogenes, E. coli*, and *S. enterica*). According to the procedure, pure cultures of the organisms prepared above were swabbed uniformly on the individual Muller–Hinton agar plates using a sterile cotton swab. Wells of depth size 6 mm and diameter size 8 mm were made in the inoculated plates using gel puncture and 100 µl of the probiotic yogurt or kefir samples were transferred individually into the wells on all plates. Antibiotic ciprofloxacin (5 mcg) discs were used as control. The plates were incubated at 37 ± 0.5 ºC for 24 h and the zone of inhibition was measured [23]. The antibacterial activity was investigated on days 1, 5, 10, 15, and 20 of the storage period of the samples.

Statistical analysis

All the experiments were carried out three times. Statistical analyses of data were performed using the statistical

software package of SPSS (version 22.0). The results were analyzed by two-way repeated-measures analysis of variance (ANOVA) to determine the effect of starter culture and storage time on the pH, acidity, and antimicrobial activity. The significance level of 5% was used and data were shown as mean ± standard error of the mean.

Results and discussion

Chemical compositions

According to the chemical analysis (Table 1), protein and fat content decreased during fermentation in both probiotic yogurt and kefir samples ($p > 0.05$) that shows the slight proteolysis and lipolysis conducted by microorganisms. Also, the moisture content decreased in probiotic yogurt ($p < 0.05$) and kefir samples ($p > 0.05$). The amount of ash increased in all samples and ewe and camel yogurt had the highest ash content. The carbohydrate content of probiotic yogurt and kefir samples decreased significantly after the fermentation ($p < 0.05$) due to microbial consumption and degradation of carbohydrates by acid-producing microorganisms. It reduced 21.15%, 15.96%, 24.72%, 17.21%, 21.52%, 15.23%, 23.49%, and 14.65% for cow, camel, ewe, and goat kefir and probiotic yogurt, respectively. According to Gambe et al. carbohydrate concentration decreased in kefir of cow milk and the main sugar utilized by microorganisms was lactose [24]. The same results were observed by de Lima et al. that 18.77% decrease occurred in the total carbohydrate content of ewe milk after fermentation using kefir grains. Similarly in our study, the fermented ewe milk lactose content decreased significantly as kefir microorganisms used lactose as the main source of available carbohydrate to ferment the milk. Another factor that causes a decrease in carbohydrate content is hydrolysis and transformation of di- and oligosaccharides by kefir grain microorganisms [7].

pH value

The changes in pH values in kefir and probiotic yogurt samples during 20 days of storage period at 4 ºC are shown in Fig. 1. In dairy-fermented products, the variation of pH is a determining parameter that shows the fermenting potential of microorganisms of starter culture and has a great effect on the organoleptic quality and the shelf-life of the product. It is reported that the microbial growth rate and fermentation capacity of the starter culture depends considerably on the origin of milk, nutrient compounds of milk (protein, peptide, lactose, oligosaccharides, and micronutrients), temperature, and time-length of incubation [25]. In our study, at the endpoint of the fermentation of the yogurt samples pH of the products ranged from 4.60 to 4.69 (almost equal pH for all the samples). We found a decrease in pH values in all kefir and probiotic yogurt samples ($p < 0.05$) and the level of reduction varied depending on the milk type, carbohydrate content, and starter culture. Similar pH values (between 6.08 and 6.55) were obtained for all kefir and probiotic yogurt samples at the beginning phase of fermentation and then pH values decreased during the acidification period to reach the final pH. On day 1, no significant difference was detected between the pH of kefir samples and also between probiotic yogurt samples prepared from different types of milk ($p > 0.05$). At the end of the storage period (day 20) pH of

Table 1 Chemical composition of pasteurized milk, yogurt, and kefir samples after 24 h of fermentation

Composition	Moisture (%)	Ash (%)	Fat (%)	Protein (%)	Carbohydrate (%)
Pasteurized milk					
Cow	88.3 ± 0.25^{a}*	0.74 ± 0.08^{a}	3.30 ± 0.05^{c}	3.45 ± 0.70^{b}	5.01 ± 0.35^{a}
Camel	88.85 ± 0.51^{a}	0.78 ± 0.02^{a}	3.65 ± 0.07^{c}	2.98 ± 0.33^{c}	4.53 ± 0.50^{b}
Ewe	82.50 ± 0.43^{b}	0.78 ± 0.09^{a}	7.15 ± 0.03^{a}	6.40 ± 0.50^{a}	5.25 ± 0.42^{a}
Goat	88.55 ± 0.23^{a}	0.73 ± 0.05^{a}	4.05 ± 0.13^{b}	3.30 ± 0.45^{b}	4.64 ± 0.25^{b}
Kefir					
Cow	88.61 ± 0.50^{a}	0.76 ± 0.10^{a}	3.01 ± 0.09^{c}	3.17 ± 0.58^{b}	3.95 ± 0.26^{c}
Camel	88.97 ± 0.28^{a}	0.80 ± 0.05^{b}	3.61 ± 0.12^{c}	2.75 ± 0.23^{c}	3.41 ± 0.57^{c}
Ewe	82.67 ± 0.31^{b}	0.79 ± 0.06^{a}	7.11 ± 0.09^{a}	6.20 ± 0.44^{a}	4.12 ± 0.15^{b}
Goat	88.94 ± 0.19^{a}	0.75 ± 0.01^{a}	4.02 ± 0.08^{b}	3.14 ± 0.25^{b}	3.55 ± 0.38^{c}
Yogurt					
Cow	82.30 ± 0.68^{b}	0.81 ± 0.05^{b}	3.00 ± 0.07^{c}	3.34 ± 0.47^{b}	4.21 ± 0.39^{b}
Camel	82.39 ± 0.45^{b}	0.85 ± 0.04^{b}	3.62 ± 0.10^{c}	2.88 ± 0.35^{c}	3.75 ± 0.22^{c}
Ewe	81.74 ± 0.31^{c}	0.88 ± 0.03^{c}	7.14 ± 0.05^{a}	6.32 ± 0.29^{a}	4.45 ± 0.30^{b}
Goat	81.95 ± 0.34^{c}	0.82 ± 0.09^{b}	4.03 ± 0.11^{b}	3.20 ± 0.18^{b}	3.86 ± 0.55^{c}

*The data are the mean values of triplicate analyses ± standard error

[a–c] Different lowercase superscripts in a column express significant difference ($p < 0.05$)

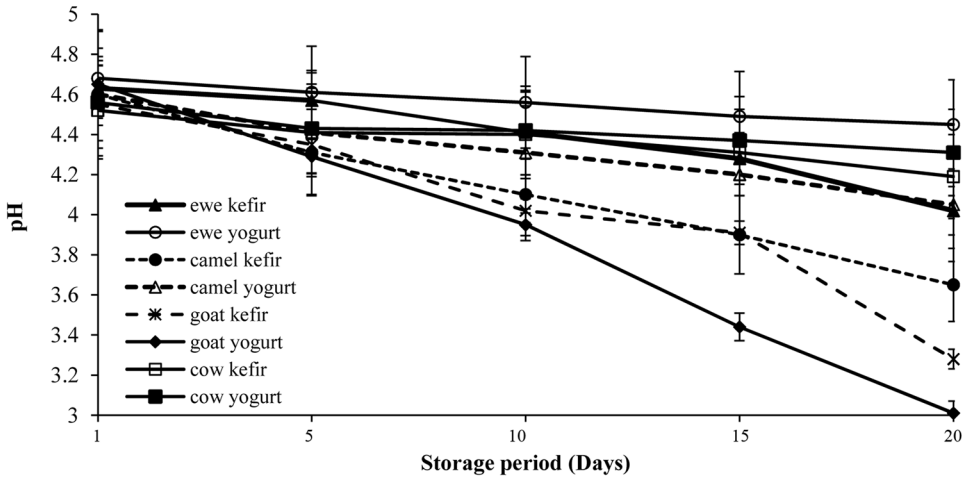


Fig. 1 Changes in pH of kefir and probiotic yogurt samples produced from cow, camel, ewe, and goat milk during storage at 4 °C

goat and camel kefir samples decreased to 3.65 ± 0.07 and 3.25 ± 0.05, respectively, while pH of ewe and cow kefirs reached to 4.02 ± 0.07 and 4.19 ± 0.03 ($p < 0.05$). Kim et al. reported the pH of different types of kefir fermented by different starter cultures between 3.64 and 4.05 [26]. Also, in another study, it was found that the pH of ewe milk kefir decreased from 4.50 to 3.70 during the 28-day of the storage period [7] which showed more pH variation than our results for ewe kefir; this difference in the present work may be due to shorter storage period or slower carbohydrate fermenting rate because of different microbial culture. Yilmaz-Ersan, Ozcan, Akpinar-Bayizit, and Sahin claimed that the pH of cow kefir was slightly higher than ewe kefir which was similar to our results [27].

Acidity

The titratable acidity of kefir and probiotic yogurt samples during 20 days of storage at 4 °C is shown in Fig. 2. The results showed that during the storage period the pH decreased and acidity increased in all samples. Similar founding has been reported for cow milk kefir [20], goat and cow milk kefir [28], and also Tibetan kefir [29]. Increasing the acidity of the product shows the rapid multiplication of the microbial populations of kefir grains or probiotic yogurt starter cultures such as yeasts and lactic acid bacteria and production of lactic acid, acetic acid, CO_2, alcohol, and volatile compounds. Metabolic activity of lactic acid bacteria and the production of lactic acid have a significant inhibitory effect on growth and metabolism of pathogenic and spoiling microorganisms [30, 31].

The acidity value of kefir samples was slightly higher than probiotic yogurt samples for the same milk type but there was no significant difference between the samples ($p > 0.05$), except for goat kefir and probiotic yogurt ($p < 0.05$). As seen in Fig. 2, the titratable acidity of goat kefir was higher than other samples; it may be due to the fatty acid composition of goat milk that is different from other milk types and characterized by the high content of medium-chain fatty acids and short-chain fatty acids, like caproic, caprylic and capric

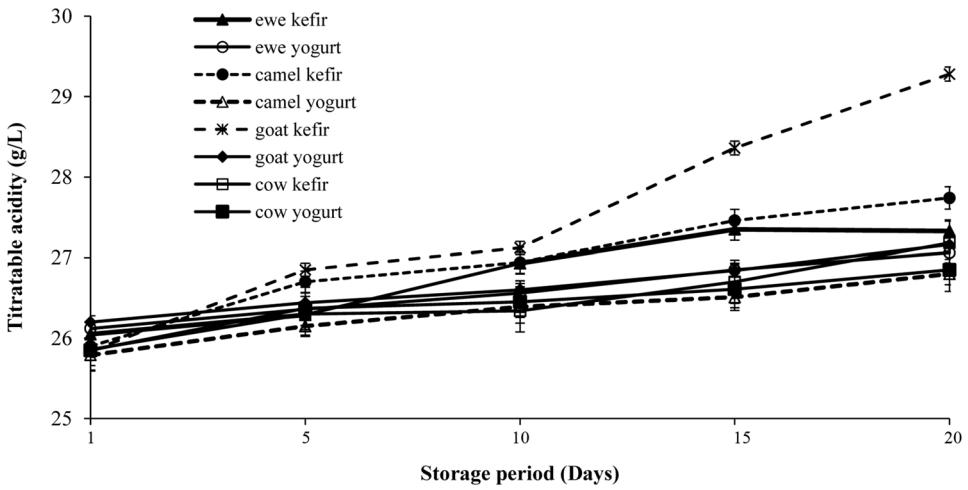


Fig. 2 Acidity variations of kefir and probiotic yogurt samples produced from cow, camel, ewe, and goat milk during storage at 4 °C

acid [10] which could increase the titratable acidity. In a study by de Lima et al. [7], the titratable acidity value of fermented ewe milk by kefir grains during 28 days of storage at 4 °C increased to approximately 27 g lactic acid/L which is similar to our results about ewe milk kefir and probiotic yogurt after 20 days of storage, 27.33 and 27.06 g lactic acid/L, respectively. It seems that the variety and the metabolic activity of microorganisms in kefir grains or starter culture have a great effect on carbohydrate fermentation rate, hydrolysis of proteins and fats, producing organic acids, and increasing the acidity [32].

Antifungal activity

The antifungal activity of kefir and probiotic yogurt samples are shown in Table 2 as the diameter of the inhibitory zone (mm). There was no significant difference between the antifungal activities of probiotic yogurt samples which had almost similar pH immediately after incubation period and the same results were found for kefir products (Day 0 of yogurt and kefir storage period, growth inhibitory diameter 4.3–4.4 mm for *A. niger*, 1.8–1.9 mm for *Penicillium* sp., and 1.1–1.3 mm for *Fusarium* sp.) ($p > 0.05$). Although a slow decrease of pH was observed in yogurt samples during 20-day storage period, a significant growth inhibitory effect obtained for all samples ($p < 0.05$). It seems that several parameters (such as metabolites and bioactive compounds produced after the end of incubation time and during storage) other than pH are effective on antimicrobial potential. For all types of milk, kefir samples expressed the strongest inhibitory potential in comparison to probiotic yogurt samples ($p < 0.05$). As it is obvious from the results, both kefir and probiotic yogurt samples inhibited the growth of *A. niger* more efficiently than other fungi ($p < 0.05$) and *Penicillium* sp. was the most resistant fungus. Ewe milk kefir showed the highest antifungal activity against all the tested fungi, for example, on day 5 of the storage period its inhibitory effect on *A. niger*, *Penicillium* sp., and *Fusarium* sp. was 85%, 39%, and 63%, respectively, followed by camel kefir, ewe probiotic yogurt, goat kefir, cow kefir, camel probiotic yogurt, goat probiotic yogurt, and cow probiotic yogurt. After 20 days of storage, the inhibition rate of ewe milk kefir reached 93.5%, 58%, and 79% for *A. niger*, *Penicillium* sp., and *Fusarium* sp., respectively. These results are in agreement with those claimed by Taheur et al. and Gamba et al., that the milk fermented by kefir grains inhibited the growth of *A. niger, A. carbonarius, A. parasiticus, A. fumigatus*, and *Rhizopus* sp. [20, 22]. There are several studies that have reported milk or whey fermented by kefir microorganisms had antifungal activity against *Penicillium s*pp., *A. flavus, A. fumigatus, Trichoderma longibrachiatum*, and *Rhizopus microsporus* [21, 33]. It has been demonstrated that even

Table 2 Antifungal activity of kefir and yogurt samples produced from cow, camel, ewe, and goat milk during storage at 4 °C

	Inhibition Zone (mm)							
	Cow kefir	Cow yogurt	Camel kefir	Camel yogurt	Ewe kefir	Ewe yogurt	Goat kefir	Goat yogurt
Aspergillus niger								
Day 1	12.5 ± 0.29^{aD}	7.6 ± 0.24^{aE}	18.8 ± 0.55^{aB}	12.1 ± 0.50^{aD}	22.5 ± 0.57^{aA}	14.9 ± 0.61^{aC}	14.2 ± 0.38^{aC}	7.5 ± 0.16^{aE}
Day 5	15.0 ± 0.53^{bE}	10.8 ± 0.35^{bF}	22.6 ± 0.30^{bB}	15.3 ± 0.46^{bE}	27.1 ± 0.43^{bA}	19.5 ± 0.65^{bC}	16.1 ± 0.43^{bD}	10.4 ± 0.62^{bF}
Day 10	17.5 ± 0.46^{cE}	12.5 ± 0.41^{cG}	27.7 ± 0.23^{cB}	17.0 ± 0.25^{cE}	32.6 ± 0.20^{cA}	22.8 ± 0.41^{cC}	19.0 ± 0.37^{cD}	14.1 ± 0.50^{cF}
Day 15	18.1 ± 0.20^{dF}	14.0 ± 0.23^{dH}	29.8 ± 0.71^{dB}	19.2 ± 0.37^{dE}	33.8 ± 0.17^{dA}	26.3 ± 0.58^{dC}	20.5 ± 0.28^{dD}	15.5 ± 0.42^{dG}
Day 20	19.5 ± 0.45^{eE}	15.8 ± 0.51^{eG}	30.5 ± 0.27^{eB}	20.1 ± 0.51^{eE}	35.5 ± 0.22^{eA}	27.3 ± 0.40^{eC}	21.4 ± 0.35^{eD}	16.8 ± 0.30^{eF}
Fusarium sp.								
Day 1	10.2 ± 0.38^{aE}	4.4 ± 0.51^{aH}	13.7 ± 0.23^{aB}	6.1 ± 0.14^{aG}	18.1 ± 0.33^{aA}	7.7 ± 0.19^{aF}	11.3 ± 0.20^{aD}	5.1 ± 0.24^{aH}
Day 5	13.5 ± 0.22^{bD}	5.2 ± 0.11^{bG}	17.5 ± 0.45^{bB}	7.5 ± 0.36^{bF}	21.5 ± 0.60^{bA}	10.4 ± 0.50^{bE}	14.6 ± 0.31^{bC}	7.3 ± 0.35^{bF}
Day 10	15.1 ± 0.47^{cE}	6.5 ± 0.20^{cH}	19.3 ± 0.27^{cB}	8.6 ± 0.55^{cG}	26.8 ± 0.22^{cA}	11.9 ± 0.43^{cF}	16.2 ± 0.54^{cD}	8.1 ± 0.58^{cG}
Day 15	17.8 ± 0.30^{dD}	7.9 ± 0.13^{dG}	20.8 ± 0.15^{dB}	9.2 ± 0.26^{cF}	28.1 ± 0.19^{dA}	12.2 ± 0.38^{cE}	19.7 ± 0.15^{dC}	9.2 ± 0.33^{dF}
Day 20	18.5 ± 0.55^{dD}	8.8 ± 0.65^{eG}	22.5 ± 0.70^{eB}	10.5 ± 0.48^{dF}	29.3 ± 0.40^{eA}	12.7 ± 0.20^{cE}	19.5 ± 0.36^{dC}	9.9 ± 0.75^{dF}
Penicillium sp.								
Day 1	8.9 ± 0.24^{aC}	3.1 ± 0.17^{aE}	9.9 ± 0.37^{aB}	4.8 ± 0.17^{aD}	15.0 ± 0.11^{aA}	5.2 ± 0.36^{aD}	9.0 ± 0.25^{aC}	3.5 ± 0.10^{aE}
Day 5	9.6 ± 0.18^{aC}	5.2 ± 0.40^{bE}	11.5 ± 0.14^{bB}	5.9 ± 0.26^{bE}	18.5 ± 0.46^{bA}	7.3 ± 0.51^{bD}	11.0 ± 0.45^{bB}	4.5 ± 0.32^{bF}
Day 10	11.8 ± 0.53^{bC}	5.9 ± 0.37^{bF}	12.7 ± 0.58^{cB}	6.8 ± 0.25^{cE}	20.7 ± 0.32^{cA}	8.2 ± 0.47^{cD}	11.9 ± 0.61^{cC}	5.7 ± 0.21^{cF}
Day 15	12.5 ± 0.44^{cC}	6.5 ± 0.21^{cE}	13.9 ± 0.70^{dB}	7.1 ± 0.39^{cE}	21.5 ± 0.40^{dA}	8.8 ± 0.65^{cD}	12.5 ± 0.58^{cC}	6.2 ± 0.37^{dE}
Day 20	12.8 ± 0.57^{cC}	7.0 ± 0.23^{cE}	14.5 ± 0.30^{dB}	7.4 ± 0.63^{cE}	22.1 ± 0.54^{dA}	9.5 ± 0.15^{dD}	13.3 ± 0.41^{dC}	6.9 ± 0.12^{De}

[a–g]Different lowercase superscripts in a column express significant difference between means for kefir and yogurt samples, for each microorganism ($p < 0.05$)

[A–E]Different uppercase superscripts in a row express significant difference between means during the storage period ($p < 0.05$)

water kefir inhibited the growth of fungi which reveals the potential of microorganisms of kefir grain to produce antifungal metabolites [34]. In our study, cow probiotic yogurt inhibited the growth of the tested fungi weakly (lower than 20%). Both ewe and camel milk kefir and probiotic yogurt have shown high values of acidity, antifungal activity, and significantly inhibited the diameter growth. As reported by Ismaiel et al. kefir has antifungal activity, as it inhibited the growth of *F. graminearum* and also kefir cell-free supernatant inhibited the growth of *A. flavus* completely [35]. It is deduced from the results (Table 2) that toward the end of the storage period the efficacy of the samples to inhibit the growth diameter has reduced. The same results are reported by Taheur et al. that the long storage period decreased the antifungal activity of whey permeate fermented by kefir grain [20]. Kefir grains mainly consist of polysaccharides, peptides, and proteins, which are the substrates for the microorganisms to produce organic acids and bioactive metabolites that inhibit the activity or growth of pathogenic microorganisms. As seen in the results there was no rapid and great reduction in pH values of the most of the samples but the antifungal activity improved during the storage time. It showed that in addition to the low pH, the antifungal activity of fermented products is attributed to the organic acids such as lactic, propionic, and acetic acids produced during the fermentation process that disrupt the proton transfer gradient in the intracellular membranes [36]. Also, Miao et al. reported that *Lactobacillus paracasei* found in Tibetan kefir grains produced bacteriocin F1, which had antifungal activity against *A. favus, A. niger*, and *P. glaucum* [37]. It seems that by the end of the storage period and reducing nutrients as the substrate for microorganisms, the production rate of the bioactive metabolites and organic acids decreased; therefore samples containing higher amounts of protein and carbohydrate (such as ewe and camel samples) showed higher antifungal activity in comparison with other samples.

Antibacterial activity

The results found for the antibacterial activity of kefir and probiotic yogurt samples during the storage period are presented in Table 3. No significant difference was observed between the antibacterial effects of probiotic yogurt ($p > 0.05$) and kefir ($p > 0.05$) samples immediately after incubation time (Day 0, growth inhibitory diameter 9.3–9.5 mm for *S. aureus*, 5.5–5.6 mm for *E. coli*, and 3.7–3.9 mm for *S. enterica*, and 6.8–7.0 mm for and *L. monocytogenes*). The antibacterial activity of kefir samples was higher than the probiotic yogurt samples ($p < 0.05$) against *S. aureus, E. coli, S. enterica*, and *L. monocytogenes*. The kefir samples produced from ewe milk showed the highest antibacterial activity and inhibited the growth of *S. aureus, E. coli, S. enterica*, and *L. monocytogenes* by 95%, 75.8%, 93.5%, and 94.4%, respectively, on the 20th day of the storage period, followed by the goat, camel, and cow kefir ($p < 0.05$). Among the probiotic yogurt samples, ewe probiotic yogurt presented the highest inhibitory potential against the growth of bacteria, followed by goat, camel, and cow milk probiotic yogurt ($p < 0.05$). The inhibitory effect of ewe milk probiotic yogurt was 70.5%, 32.1%, 68.1%, and 66.2% for *S. aureus, E. coli, S. enterica*, and *L. monocytogenes*, respectively, at the end of the storage period. The antimicrobial activity of the samples increased toward the end of the storage time ($p < 0.05$). It is obvious from the results that *S. aureus* and *L. monocytogenes* were the most sensitive bacteria and *E. coli* was the most resistant against the treatment by kefir and probiotic yogurt ($p < 0.5$). Totally, the inhibitory activity of the samples against the bacteria tested in this work is as follows in descending order: ewe kefir, goat kefir, camel kefir, cow kefir, ewe probiotic yogurt, goat probiotic yogurt and camel probiotic yogurt ($p > 0.05$), and cow probiotic yogurt.

Several researchers have studied the effect of different types of yogurt, kefir, and micro-flora on the inhibition of bacterial activity for a large variety of Gram-negative and Gram-positive bacteria. These studies demonstrated that the microorganisms isolated from fermented products inhibited the growth of *S. aureus, Shigella* spp., *Salmonella* spp., *E. coli*, *L. monocytogenes*, *Bacillus cereus, B. subtilis, Klebsiella pneumonia, Pseudomonas aeruginosa*, and *Enterococcus faecalis* [7, 14, 26, 38]. It is suggested that bioactive substances, organic acids, ethyl alcohol, hydrogen peroxide, diacetyl, peptides, possibly bacteriocins, and other inhibitory compounds were responsible for the growth inhibition of pathogenic bacteria.

In a study by Van et al. *L. acidophilus, Lactococcus* and the acetic acid bacteria isolated from kefir showed inhibitory potential toward a variety of Gram-positive and Gram-negative bacteria, and they inhibited the growth of *S. aureus* and *L. monocytogenes* significantly [39]. The results corroborate the hypothesis that the useful microorganisms and bioactive compounds are found in higher values and with more variety in kefir than yogurt, and is associated with stronger inhibitory activity of kefir against the pathogens.

It was found by Kim et al. and Chifiriuc et al., that kefirs from different origins showed different antimicrobial spectra which confirms our results [26, 40]. Also, the present data are consistent with previous studies reported that kefir inhibited the growth of *L. monocytogenes* significantly [26, 41].

As shown in Table 3, the rate of inhibitory activity decreases toward the end of the storage period. Considering that kefir and probiotic yogurt contain various metabolites and inhibitory substances as mentioned above, it could be assumed that these compounds might interact with each other to increase or inactivate their antimicrobial activity. For example, the inhibitory activities of bacteriocins could

Table 3 Antibacterial activity of kefir and yogurt samples produced from cow, camel, ewe, and goat milk during storage at 4 °C

	Inhibition Zone (mm)							
	Cow kefir	Cow yogurt	Camel kefir	Camel yogurt	Ewe kefir	Ewe yogurt	Goat kefir	Goat yogurt
S. aureus								
Day 1	19.3 ± 0.51^{aD}	17.9 ± 0.40^{aE}	26.9 ± 0.30^{aB}	25.3 ± 0.31^{aC}	29.4 ± 0.15^{aA}	27.1 ± 0.23^{aB}	28.5 ± 0.20^{aA}	24.7 ± 0.45^{aC}
Day 5	26.5 ± 0.72^{bE}	19.3 ± 0.55^{bF}	34.3 ± 0.50^{bC}	26.5 ± 0.17^{bE}	38.5 ± 0.44^{bA}	28.0 ± 0.21^{bD}	36.8 ± 0.23^{bB}	26.2 ± 0.39^{bE}
Day 10	33.1 ± 0.46^{cD}	20.4 ± 0.31^{cG}	43.8 ± 0.25^{cC}	27.9 ± 0.62^{cF}	46.0 ± 0.35^{cA}	29.8 ± 0.50^{cE}	44.7 ± 0.26^{cB}	27.8 ± 0.20^{cF}
Day 15	40.2 ± 0.17^{dC}	21.5 ± 0.25^{dF}	51.2 ± 0.41^{dB}	29.5 ± 0.23^{dE}	53.2 ± 0.18^{dA}	31.1 ± 0.35^{dD}	52.5 ± 0.17^{dA}	29.5 ± 0.48^{dE}
Day 20	48.0 ± 0.56^{eC}	22.9 ± 0.37^{eF}	59.1 ± 0.50^{eB}	30.7 ± 0.28^{eE}	61.5 ± 0.27^{eA}	37.7 ± 0.40^{eD}	60.0 ± 0.35^{eB}	31.1 ± 0.50^{eE}
E.coli								
Day 1	17.5 ± 0.30^{aF}	12.4 ± 0.35^{aG}	24.1 ± 0.26^{aC}	19.2 ± 0.63^{aE}	28.8 ± 0.10^{aA}	24.9 ± 0.54^{aC}	27.1 ± 0.45^{aB}	21.5 ± 0.50^{aD}
Day 5	18.1 ± 0.51^{bF}	12.9 ± 0.27^{aG}	25.3 ± 0.41^{bC}	20.0 ± 0.11^{bE}	30.1 ± 0.28^{bA}	25.8 ± 0.30^{bC}	28.0 ± 0.73^{bB}	22.7 ± 0.31^{bD}
Day 10	18.8 ± 0.23^{bF}	13.8 ± 0.25^{bG}	26.9 ± 0.55^{cC}	21.2 ± 0.36^{cE}	31.5 ± 0.65^{cA}	26.5 ± 0.11^{cC}	29.2 ± 0.40^{cB}	23.5 ± 0.67^{cD}
Day 15	19.5 ± 0.45^{cF}	14.6 ± 0.50^{cG}	27.8 ± 0.19^{dC}	22.7 ± 0.43^{cE}	33.0 ± 0.24^{dA}	27.0 ± 0.20^{cC}	30.5 ± 0.12^{dB}	24.4 ± 0.39^{dD}
Day 20	20.7 ± 0.63^{dF}	15.1 ± 0.29^{dG}	28.5 ± 0.33^{eC}	23.5 ± 0.50^{dE}	34.3 ± 0.60^{eA}	28.2 ± 0.41^{dC}	31.6 ± 0.27^{eB}	25.8 ± 0.32^{eD}
S. enterica								
Day 1	15.5 ± 0.21^{aE}	10.3 ± 0.27^{aF}	22.5 ± 0.14^{aB}	15.1 ± 0.30^{aE}	25.7 ± 0.21^{aA}	18.4 ± 0.29^{aC}	22.3 ± 0.17^{aB}	16.3 ± 0.22^{aD}
Day 5	22.3 ± 0.19^{bD}	15.0 ± 0.29^{bF}	29.6 ± 0.55^{bB}	20.2 ± 0.40^{bE}	32.3 ± 0.44^{bA}	23.7 ± 0.61^{bC}	29.9 ± 0.43^{bB}	21.5 ± 0.68^{bD}
Day 10	29.1 ± 0.38^{cC}	21.1 ± 0.46^{cE}	36.3 ± 0.17^{cB}	25.9 ± 0.54^{cD}	38.7 ± 0.15^{cA}	28.2 ± 0.37^{cD}	35.8 ± 0.27^{cB}	26.2 ± 0.40^{cD}
Day 15	37.3 ± 0.25^{dC}	25.0 ± 0.73^{dF}	44.5 ± 0.61^{dA}	32.0 ± 0.65^{dE}	45.5 ± 0.32^{dA}	33.4 ± 0.28^{dD}	43.5 ± 0.33^{dB}	32.9 ± 0.54^{dE}
Day 20	44.8 ± 0.35^{eC}	30.3 ± 0.59^{eE}	50.5 ± 0.38^{eB}	38.2 ± 0.11^{eD}	52.3 ± 0.71^{eA}	38.1 ± 0.57^{eD}	51.7 ± 0.25^{eA}	39.0 ± 0.66^{eD}
L. monocytogenes								
Day 1	18.2 ± 0.55^{aF}	15.0 ± 0.43^{aG}	26.4 ± 0.70^{aC}	24.9 ± 0.18^{aD}	29.1 ± 0.20^{aA}	20.5 ± 0.61^{aF}	28.0 ± 0.33^{aB}	23.2 ± 0.47^{aE}
Day 5	21.0 ± 0.19^{bF}	15.9 ± 0.31^{aG}	29.0 ± 0.55^{bC}	25.5 ± 0.33^{aD}	33.7 ± 0.45^{bA}	21.4 ± 0.28^{aD}	32.1 ± 0.65^{bB}	24.3 ± 0.15^{bE}
Day 10	26.1 ± 0.58^{cD}	17.5 ± 0.28^{bF}	33.8 ± 0.52^{cC}	26.4 ± 0.15^{bD}	38.5 ± 0.14^{bA}	23.5 ± 0.10^{bE}	35.5 ± 0.73^{cB}	25.6 ± 0.30^{cD}
Day 15	30.3 ± 0.27^{dD}	18.6 ± 0.45^{cG}	36.3 ± 0.21^{dC}	27.1 ± 0.30^{bE}	43.1 ± 0.86^{cA}	25.0 ± 0.17^{cF}	38.0 ± 0.12^{dB}	27.2 ± 0.61^{dE}
Day 20	34.0 ± 0.69^{eD}	20.4 ± 0.36^{dF}	39.2 ± 0.45^{eC}	28.9 ± 0.46^{cE}	48.5 ± 0.26^{dA}	28.9 ± 0.25^{dD}	40.8 ± 0.51^{eB}	28.5 ± 0.75^{eE}

$^{a-g}$Different lowercase superscripts in a column express significant difference between means for kefir and yogurt samples, for each microorganism ($p < 0.05$)

$^{A-E}$Different uppercase superscripts in a row express significant difference between means during the storage period ($p < 0.05$)

be antagonized by organic acids or enzymatic degradation [19].

Conclusions

In the present study, we investigated the inhibitory activity of kefir and probiotic yogurt produced from cow, camel, ewe, and goat milk on the most common foodborne pathogenic bacteria and fungi. The results revealed that kefir samples had stronger antifungal and antibacterial effect than probiotic yogurt samples. Also, the storage period had a significant effect on the antimicrobial potential of both kefir and probiotic yogurt samples. Among kefir samples, the ewe and cow milk kefir expressed the highest and the lowest antifungal and antibacterial activity, respectively. The same results were observed for probiotic yogurt samples. The difference between the antimicrobial activity found in kefir and probiotic yogurt samples produced from different types of milk could be due to multiple parameters like the fatty acids composition, lactose content that has a considerable effect on final pH and acidity of the product, types of peptides and other chemicals' composition that play role in the production of bioactive compounds, type and population of microorganisms, and variety of enzymes of the kefir grains and starter culture. Further researches are being performed by our group to evaluate the antimicrobial activity of kefir and probiotic yogurt components.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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