

Inhibitory Effect of Different Types of Fermented Milk on *Candida albicans*



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Abstract

Background: *Candida albicans* (*C. albicans*) is known as an opportunistic fungal pathogen, and although it is a normal flora of the gastrointestinal tract, it has the ability to colonize every human tissue, causing serious and invasive infections.

Objective: This study focused on the antifungal activity of the produced yogurts and kefir samples from cow, camel, sheep, and goat milk against *C. albicans* during the storage period at 4 °C.

Materials and Methods: The pH, titratable acidity, the content of some organic acids, and anti-*Candida* activity of yogurt and kefir samples were evaluated based on the aim of the study.

Results: The titratable acidity of the samples significantly increased after the fermentation, along with pH reductions. The concentration of lactic and pyruvic acids increased during the fermentation while citric, uric, and hippuric acid content demonstrated a decrease. The results revealed that kefir samples had a stronger anti-*Candida* effect compared to yogurt samples. On the first day of the storage period, the growth inhibitory effect of sheep milk kefir was 90.20%, followed by camel kefir (78.37%), goat kefir (74.52%), cow kefir (73.23%), sheep yogurt (48.56%), camel yogurt (37.51%), cow yogurt (34.42%), and goat yogurt (30.32%). Eventually, the inhibition rate of sheep milk kefir reached 96.50% after 20 days of storage.

Conclusion: It seems that kefir may be used as a nutraceutical and functional food against *C. albicans* infections.

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Background

Probiotic food products contain live probiotic microorganisms that have beneficial effects on the intestinal microflora and the health of the host ¹. Kefir and yogurt are the most popular probiotic fermented dairy products in the world. Yogurt is prepared using a bacterial starter culture (consisting of lactic acid bacteria) to ferment the milk. Kefir drink originated from the Caucasus, Eastern Europe, and Russia is fermented milk produced by kefir grains. These grains consist of a symbiotic mixture of lactic acid bacteria (*Streptococcus* spp., *Lactobacillus*, *Lactococcus*, and *Leuconostoc*), acetic acid bacteria (*Acetobacter*), yeasts (*Saccharomyces* spp., *Candida*, *Torula*, and *Kluyveromyces*), and mycelial fungi aggregated in a glucogalactan matrix named kefiran. Kefiran is a gelatinous irregularly mass with white or light yellow color ². The microbial diversity may vary based on the origin of kefir, the applied substrate composition in the fermentation, and the maintenance method of the starter culture. The microorganisms of the yogurt starter

culture and kefiran produce bioactive compounds which have been reported to possess antibacterial and antifungal activity, have the potential for improving the function of the immune system and digestive organs, and help the treatment of blood hypertension, allergies, metabolic defects, and antitumour activity ³.

It has been reported that kefir acts against some pathogenic bacteria (e.g., *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella*, *Shigella*, *Escherichia coli*, *Listeria monocytogenes*, *Enterobacter aerogenes*, *Streptococcus pyogenes*, *Proteus vulgaris*, and *Micrococcus luteus*), and fungi (e.g., *Aspergillus flavus*, *Fusarium graminearum*, and *Candida albicans*) ^{4,5}. *C. albicans* is introduced as an opportunistic fungal pathogen. Although this pathogen is a normal flora of the gastrointestinal tract, it has the ability to colonize every human tissue, causing serious and invasive infections ⁶. The treatment of candidiasis is usually drug-based but using anti-*candida* drugs has resulted in appearing resistant *Candida* species and the failure of subsequent drug treatments. Therefore, research

should be conducted toward new therapeutic methods⁷. It seems that fermented dairy products such as kefir are natural safe food preservatives offering protection against microbial pathogens⁴.

The publication is scarce on the antifungal potential of kefir and yogurt produced from camel, sheep, and goat milk probably because of the restricted and low availability of the milk of these species on the market. It is assumed that fermented dairy products possess different antimicrobial activities based on their milk sources, starter culture, and shelf-life. Hence, the aim of the present work was to determine the antifungal activity of the produced yogurts and kefir from cow, camel, sheep, and goat milk against *C. albicans*.

Materials and Methods

Materials

All the applied culture media, chemicals, and reagents were purchased from Merck Company (Darmstadt, Germany). Raw cow, sheep, 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).

Inoculum Preparation

Yogurt commercial starter culture (*L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) as the direct vat set culture was purchased from Danisco (Denmark). The traditional kefir grains (kefir) were collected from the rural kefir producers on the outskirts of Semnan (Semnan, Iran). The recovery of active kefir grains was performed by transferring the grains into the heated (90 °C) and cooled (25-30 °C) low-fat cow milk (approximately 0.45% fat content) and incubated at 25 ± 1 °C for about 24 hours. This step was repeated for 7 consecutive days⁸. Then, to separate the milk curds, the kefir grains were filtered and washed with sterile distilled water 3 times. Finally, the grains were inoculated into pasteurized cow milk and stored at 25 ± 1 °C until its use.

Fermentation

The milk samples were pasteurized in the hot water bath (90 ± 1 °C for 10 minutes) and cooled to 43 ± 1 °C and 25 ± 1 °C for yogurt and kefir production, respectively. Then, yogurt starter culture (2% v/v) and activated kefir grains (5% v/v) were inoculated to each milk, and the samples were incubated (Mettler Incubator 400, Switzerland) at 43-45 °C (for 8 hours) and 25 ± 1 °C (for 20 hours) to produce kefir and yogurt, respectively, until obtaining the desired pH of the product (4.5-4.6 for both kefir and yogurt). At the end of the fermentation period, the kefir samples were filtered through a sterile 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.

Preparation of the Samples

To prepare samples for the chemical assays, 2 g of each sample was mixed with 20 mL of the extracting solvent (methanol/water, 70:30 v/v) using a magnetic stirrer (model RSM-03-10K, Phoenix, Germany) for 4 hours at 20 ± 1 °C in a dark place. Then, the mixture was centrifuged (model Z206A, Hermle, Germany) at 3000 rpm (12 minutes) 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 acidity⁹.

To prepare samples for anti-*Candida* experiments, the fermented products were filtered through a sieve of 1.2-mm² mesh size, and the filtrate was centrifuged at 13500 ×g for 15 minute 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 using.

pH Measurement

The pH of the kefir and 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 and Ethanol Content

The acidity and ethanol content of the samples were determined according to the AOAC (2002, 2016) procedure and reported as grams of lactic acid per liter of the product¹⁰.

Determination of the Organic Acids in the Samples

The composition and concentration of organic acids in kefir and yogurt samples on the 1st day of the storage period were determined according to the method used by GuKzel-Seydim et al (2000). Briefly, 4 g of each sample was mixed with 25 mL H₂SO₄ (0.01 N), vortexed for 1 minutes to obtain the extract of the sample, and kept at 4 °C until HPLC analysis. Sulphuric acid-extracted samples were then passed through a 0.45-µm filter and injected into a PerkinElmer Altus HPLC system, equipped with the A-10 Solvent/Sample Manager, the A-10 column heater, and the A-10 UV detector. In addition, a PerkinElmer Brownlee Validated Aqueous C18 5 µm, 4.6 x 250 mm column was applied for all the analyses (PerkinElmer, Shelton, CT, USA). The injection volume for both samples and standards was 10 µL. Degassed HPLC-grade H₂SO₄ (0.009 N) was used as the mobile phase. The organic acids, lactic, citric, pyruvic, uric, and hippuric were detected using UV detection at a wavelength of 275 nm. The applied external standards for quantifying the organic acids were prepared in distilled deionized water (ddH₂O) and passed through a 0.45-µm filter. Standard curves were plotted based on peak height for each organic acid covering a wide range of concentrations.

Anti-Candida Activity

The antifungal activity of kefir and yogurt samples against *C. albicans* was evaluated according to the method described by Eddine et al (2020) with some modifications. *C. albicans* (ATCC 76615) was kindly donated by the Department of Mycology of Faculty of Veterinary Medicine, University of Tehran (Tehran, Iran). *C. albicans* was grown in Yeast Potato Dextrose (containing 1% yeast extract, 2% peptone, and 2% glucose/dextrose) broth until reaching an optical density of 0.5 of McFarland scale. The final population of *C. albicans* was adjusted at 5×10^5 conidia/mL. The antifungal assay was performed on Petri dishes and the solid medium consisted of the malt extract (1% w/v), yeast extract (2% w/v), and agar (2% w/v). The culture medium was sterilized at 121 °C for 15 minutes, and after cooling to 45 °C, 20 mL of the medium was transferred into the Petri dishes (90 mm diameter). Then, 1 mL of the yeast suspension was uniformly spread on the plates using a sterile cotton swab. The wells with a depth of 6 mm and a diameter of 8 mm were formed in the inoculated plates using a sterile glass tube and a vacuum pump. Next, 100 µL of the yogurt or kefir extracts were individually transferred into the wells on all plates. Ketoconazole (50 µg/mL) was used as a control antifungal compound. The diameter of the inhibition zone was measured after 48 hours of incubation at 37 °C. The antifungal 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. The obtained data were statistically analyzed using the statistical software package of SPSS (version 22.0). The results were analyzed by two-way repeated-measures analysis of variance to determine the effect of starter culture and storage time on the pH, acidity, and anti-*Candida* activity. The significance level of 5% and 1% was used, and data were shown as the mean \pm standard error of the mean.

Results and Discussion

pH

Table 1 provides the variations in the pH value in kefir and yogurt samples during 20 days of the storage period at 4 °C. It should be noted that in dairy products produced through fermentation, the change in the pH value is a determining factor that expresses the fermenting ability of the microorganisms of the starter culture and has a considerable effect on the sensory properties and the quality of the product during the shelf-life period. It is demonstrated that the microbial growth rate and the fermentation potential of the starter culture significantly depends on the origin of milk, the nutrient compositions of milk (i.e., protein, peptide, lactose, oligosaccharides, and micronutrients), storage temperature, and the length of the incubation period¹¹. In the present work, a decrease was observed in pH values in all kefir and yogurt samples ($P < 0.01$) and the level of reductions varied relying on the milk source and the starter culture. Similar pH values (6.08-6.55) were found for all kefir and yogurt samples at the very beginning phase of the fermentation process, and then pH values decreased during the acidification period to reach the final pH. The pH values of kefir samples were lower than those of the yogurt samples, revealing that the microorganisms of the kefir grain had higher fermentation capacity compared to the yogurt starter culture. On day 1, the pH of kefir and yogurt samples was 4.52 ± 0.03 - 4.63 ± 0.1 and 4.56 ± 0.1 - 4.68 ± 0.05 , respectively. No significant difference was detected between the pH values of the products prepared from different types of milk ($P > 0.01$). At the end of the storage period (day 20), the pH of goat and camel kefir samples decreased to 3.65 ± 0.07 and 3.25 ± 0.05 , respectively, while that of sheep and cow kefirs reached 4.02 ± 0.07 and 4.19 ± 0.03 ($P < 0.01$). It is reported that the pH of sheep milk kefir decreased from 4.50 to 3.70 during the 28-day storage period¹², which showed more pH changes compared to our results for sheep kefir. Yilmaz-Ersan et al found that the pH of cow kefir was slightly higher in comparison to sheep kefir¹³, which is similar to our results. The significant decrease

Table 1. Changes in the pH of Kefir and Yogurt Samples Produced From Sheep, Camel, Goat, and Cow Milk During Storage at 4 °C

| | Storage Period (day) | | | | |
|--------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| | 1 | 5 | 10 | 15 | 20 |
| Sheep kefir | 4.63 \pm 0.01 ^{aA} | 4.57 \pm 0.07 ^{aA} | 4.41 \pm 0.01 ^{bB} | 4.28 \pm 0.05 ^{bB} | 4.02 \pm 0.09 ^{bD} |
| Sheep yogurt | 4.68 \pm 0.03 ^{aA} | 4.61 \pm 0.09 ^{aA} | 4.56 \pm 0.00 ^{bA} | 4.49 \pm 0.06 ^{bA} | 4.45 \pm 0.01 ^{bA} |
| Camel kefir | 4.6 \pm 0.01 ^{aA} | 4.31 \pm 0.00 ^{bB} | 4.1 \pm 0.01 ^{cD} | 3.9 \pm 0.05 ^{dD} | 3.65 \pm 0.03 ^{eE} |
| Camel yogurt | 4.6 \pm 0.04 ^{aA} | 4.41 \pm 0.01 ^{bB} | 4.31 \pm 0.02 ^{cC} | 4.2 \pm 0.07 ^{dC} | 4.05 \pm 0.00 ^{eD} |
| Goat kefir | 4.55 \pm 0.00 ^{bB} | 4.35 \pm 0.07 ^{bB} | 4.02 \pm 0.03 ^{cE} | 3.91 \pm 0.05 ^{dD} | 3.28 \pm 0.10 ^{eF} |
| Goat yogurt | 4.65 \pm 0.01 ^{aA} | 4.29 \pm 0.05 ^{bC} | 3.95 \pm 0.03 ^{cE} | 3.44 \pm 0.06 ^{dE} | 3.01 \pm 0.04 ^{eG} |
| Cow kefir | 4.52 \pm 0.07 ^{bB} | 4.41 \pm 0.03 ^{bB} | 4.4 \pm 0.00 ^{bB} | 4.31 \pm 0.11 ^{bB} | 4.19 \pm 0.08 ^{cC} |
| Cow yogurt | 4.56 \pm 0.10 ^{bB} | 4.43 \pm 0.09 ^{bB} | 4.42 \pm 0.02 ^{bB} | 4.37 \pm 0.05 ^{bB} | 4.31 \pm 0.00 ^{eB} |

Note. ^{a-g}Different lowercase superscripts in a row express a significant difference between the means during the storage period ($P < 0.01$). ^{A-F}Different uppercase superscripts in a column indicate a significant difference between the means for kefir and yogurt samples ($P < 0.01$).

in the pH of kefir samples revealed the higher metabolic activity of kefir compared to the commercial yogurt starter culture. Furthermore, it seems that differences in the pH values of kefir and yogurt samples during the storage period are related to the buffering properties of the types of the milk and the fermentation potential of the applied microbial populations.

2 Titratable Acidity

The ethanol content of samples measured on the 1st day of the storage period was obtained as 1.7%, 1.2%, 1.08%, 1.03%, 0.011%, 0.009%, 0.006%, and 0.005% for cow kefir, camel kefir, goat kefir, sheep kefir, cow yogurt, camel yogurt, goat yogurt, and sheep yogurt, respectively. The results of measuring the titratable acidity of kefir and yogurt samples during storage at 4 °C are shown in Table 2. During the storage period, the acidity value increased in all the samples. Similar observations have been reported for cow milk kefir¹⁴, goat and cow milk kefir¹⁵, and Tibetan kefir¹⁶. The rapid multiplication of the microorganisms of kefir and yogurt starter cultures such as yeasts and lactic acid bacteria and thus the production of lactic acid, acetic acid, CO₂, alcohol, and volatile compounds resulted in increasing the acidity of the product. Accordingly to several studies, the metabolic activity of lactic acid bacteria and the production of lactic acid and bacteriocins have a great inhibitory effect on the growth and metabolism of pathogens and spoiling microorganisms^{17,18}.

In our study, the acidity of yogurt samples was lower than that of kefir samples for the same milk type although there was no significant difference between the samples ($P > 0.01$) except for goat kefir and yogurt ($P < 0.01$). According to Table 2, the highest titratable acidity belonged to goat kefir, which may be due to the fatty acid composition of goat milk and the high content of medium and short-chain fatty acids (e.g., caproic, caprylic, and capric acids)¹⁹. Our findings of sheep milk kefir and yogurt are consistent with the results of de Lima et al, representing an increase of up to 27 g lactic acid/L in the

titratable acidity of sheep kefir during 28 days of storage at 4 °C. Several factors such as the variety and the metabolic activity of microorganisms, the fermentation period, and the fermentation rate affect the production of organic acids and increasing the acidity²⁰.

Organic Acid Composition

Organic acids are produced in dairy foods as a result of the hydrolysis of fatty acids, biochemical reactions, and bacterial or fungal metabolism. Lactic acid is the common final product of bacterial and yeast fermentation. The concentrations of lactic acid in milk, kefir, and yogurt samples (Table 3) showed that lactate production increased during the fermentation ($P < 0.01$). It is reported that lactose is degraded to glucose and galactose by microorganisms and, glucose is metabolized to pyruvate during the homofermentative pathway. Therefore, the concentration of pyruvic acid represents an increase. The lactic acid content in cow milk kefir samples was reported 8.76 g/L²¹ and 6.40 g/L²² in previous studies. In this work, yogurt starter culture produced 10.45, 5.50, 7.33, and 9.43 g/L lactic acid in the sheep, camel, goat, and cow milk yogurt, respectively, upon one-day of fermentation. Thus, kefir samples had lower lactic acid (i.e., 9.05, 4.81, 5.80, and 8.19 g/L lactic acid in the sheep, camel, goat, and cow milk kefir, respectively) content compared to yogurt samples. This could be due to the use of a heterofermentative pathway with a resultant production of CO₂ which leads to the over-acidification problem in yogurt, this problem rarely occurs in kefir. Concurrent with lactic acid production, pyruvic acid content increased during fermentation ($P < 0.01$). *Streptococcus lactis*, as a common component of the kefir, produces pyruvate, which is converted to lactate, acetaldehyde, and diacetyl²².

The citric acid concentrations of sheep, camel, goat, and cow milk samples were 1.84, 0.35, 0.65, and 1.80 g/L, respectively. As shown in Table 3, the citrate content decreased by the completion of fermentation ($P < 0.01$). It may be because citrate is the preferred substrate for

Table 2. Acidity Variations of Kefir and Yogurt Samples Produced From Sheep, Camel, Goat, and Cow Milk During Storage at 4 °C

| | Storage Period (day) | | | | |
|--------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| | 1 | 5 | 10 | 15 | 20 |
| Sheep kefir | 26.05±0.15 ^{aA} | 26.29±0.40 ^{aB} | 26.93±0.37 ^{bA} | 27.35±0.43 ^{cB} | 27.33±0.19 ^{bC} |
| Sheep yogurt | 26.12±0.55 ^{aA} | 26.36±0.21 ^{aA} | 26.55±0.30 ^{bB} | 26.85±0.46 ^{bC} | 27.06±0.25 ^{cC} |
| Camel kefir | 25.90±0.41 ^{aA} | 26.71±0.57 ^{bA} | 26.94±0.32 ^{bA} | 27.46±0.11 ^{cB} | 27.74±0.24 ^{cB} |
| Camel yogurt | 25.79±0.28 ^{aB} | 26.15±0.35 ^{bB} | 26.39±0.63 ^{bC} | 26.51±0.40 ^{bC} | 26.80±0.17 ^{cD} |
| Goat kefir | 25.84±0.71 ^{aB} | 26.75±0.15 ^{aA} | 27.12±0.38 ^{bA} | 28.36±0.50 ^{cA} | 29.28±0.43 ^{dA} |
| Goat yogurt | 26.20±0.64 ^{aA} | 26.44±0.70 ^{aA} | 26.60±0.92 ^{bB} | 26.84±0.60 ^{bC} | 27.16±0.21 ^{cC} |
| Cow kefir | 25.86±0.35 ^{aB} | 26.30±0.82 ^{bB} | 26.34±0.50 ^{bC} | 26.70±0.33 ^{cC} | 27.18±0.49 ^{bC} |
| Cow yogurt | 25.65±0.58 ^{aB} | 26.37±0.46 ^{bB} | 26.45±0.23 ^{bC} | 26.61±0.65 ^{bC} | 26.85±0.31 ^{cD} |

Note. ^{a-e}Different lowercase superscripts in a row express a significant difference between the means during the storage period ($P < 0.01$). ^{A-E}Different uppercase superscripts in a column represent a significant difference between the means for kefir and yogurt samples ($P < 0.01$).

Table 3. Organic Acid Composition of Milk, Kefir, and Yogurt Samples at the 1st Day of the Storage Period

| | Organic Acid Composition (g/L) | | | | |
|--------------|--------------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| | Lactic Acid | Citric Acid | Pyruvic Acid | Uric Acid | Hippuric Acid |
| Sheep milk | 0.071±0.005 ^C | 1.84±0.230 ^{AA} | ND ^{AC} | 0.20±0.000 ^{AA} | 0.041±0.009 ^{AB} |
| Sheep kefir | 9.05±0.711 ^{CB} | 0.740±0.151 ^{BC} | 0.021±0.001 ^{BA} | 0.08±0.002 ^{BB} | ND ^{BD} |
| Sheep yogurt | 10.45±0.520 ^{BA} | 0.618±0.035 ^{CD} | 0.027±0.005 ^{BA} | 0.1±0.072 ^{BB} | ND ^{BD} |
| Camel milk | 0.013±0.000 ^{AH} | 0.35±0.060 ^{AF} | ND ^{AC} | 0.10±0.028 ^{AB} | 0.18±0.056 ^{AA} |
| Camel kefir | 4.81±0.300 ^{CF} | 0.30±0.059 ^{AF} | 0.011±0.001 ^{BB} | 0.04±0.003 ^{BC} | ND ^{BD} |
| Camel yogurt | 5.50±0.149 ^{BE} | 0.28±0.075 ^{AG} | 0.017±0.000 ^{BB} | 0.05±0.000 ^{BC} | ND ^{BD} |
| Goat milk | 0.06±0.001 ^{AG} | 0.65±0.030 ^D | ND ^{AC} | 0.012±0.000 ^{AC} | 0.010±0.003 ^{AC} |
| Goat kefir | 5.80±0.02 ^{CE} | 0.515±0.08 ^{BE} | 0.019±0.001 ^{BB} | ND ^{BD} | ND ^{BD} |
| Goat yogurt | 7.33±0.91 ^{BD} | 0.545±0.005 ^{BE} | 0.023±0.007 ^{BA} | ND ^{BD} | ND ^{BD} |
| Cow milk | 0.07±0.000 ^{AG} | 1.80±0.031 ^{AA} | ND ^{AC} | 0.062±0.001 ^B | 0.014±0.005 ^{AB} |
| Cow kefir | 8.19±0.331 ^{CC} | 1.20±0.055 ^{BB} | 0.015±0.001 ^{BB} | ND ^{BD} | ND ^{BD} |
| Cow yogurt | 9.43±0.110 ^{BB} | 0.635±0.082 ^{CD} | 0.016±0.000 ^{BB} | 0.030±0.005 ^{CC} | ND ^{BD} |

Note. ND: Not detected; ^{A-E}Different lowercase superscripts express a significant difference between the means of the organic acid content of milk, kefir, and yogurt for each species ($P < 0.01$). ^{A-E}Different uppercase superscripts in a column denote a significant difference between the means for milk, kefir, and yogurt samples ($P < 0.01$).

Table 4. Anti-*Candida* Activity of Kefir and Yogurt Samples Produced From Cow, Camel, Sheep, and Goat Milk Against *Candida albicans* During Storage at 4 °C

| | Inhibition Zone (mm)* | | | | | | | |
|--------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| | Cow Kefir | Cow Yogurt | Camel Kefir | Camel Yogurt | Sheep Kefir | Sheep Yogurt | Goat Kefir | Goat Yogurt |
| Day 1 | 23.5±0.35 ^{AC} | 8.4±0.29 ^{AE} | 25.5±0.50 ^{AB} | 9.6±0.37 ^{AD} | 30.1±0.42 ^{AA} | 13.9±0.21 ^{AC} | 24.0±0.63 ^{AC} | 6.8±0.25 ^{AF} |
| Day 5 | 25.0±0.64 ^{BD} | 11.5±0.70 ^{BE} | 28.7±0.61 ^{BB} | 11.1±0.23 ^{BE} | 33.5±0.27 ^{BA} | 15.1±0.33 ^{BC} | 26.5±0.72 ^{BC} | 10.3±0.65 ^{BF} |
| Day 10 | 26.3±0.20 ^{CD} | 15.8±0.61 ^{CE} | 29.5±0.92 ^{BB} | 13.5±0.54 ^{CF} | 35.3±0.51 ^{CA} | 16.0±0.15 ^{CC} | 27.9±0.57 ^{CC} | 13.5±0.23 ^{CF} |
| Day 15 | 28.0±0.82 ^{DD} | 19.1±0.55 ^{DE} | 33.0±0.51 ^{CB} | 14.1±0.50 ^{CG} | 36.5±0.70 ^{DA} | 17.5±0.81 ^{DF} | 30.0±0.56 ^{CC} | 17.6±0.37 ^{DF} |
| Day 20 | 28.7±0.45 ^{DD} | 22.5±0.38 ^{EE} | 34.5±0.24 ^{DB} | 15.5±0.33 ^{CG} | 37.8±0.65 ^{EA} | 20.3±0.46 ^{EF} | 31.8±0.64 ^{CC} | 19.5±0.81 ^{EF} |

Note. * The inhibition zone diameter of Ketoconazole was 21.1±0.58 mm. ^{A-E}Different lowercase superscripts in a column express a significant difference between the means for kefir and yogurt samples for each microorganism ($P < 0.05$). ^{A-E}Different uppercase superscripts in a row indicate a significant difference between the means during the storage period ($P < 0.05$).

diacetyl and acetoin production by lactic acid bacteria. Moreover, hippuric and uric acids decreased (Table 3) after one-day fermentation ($P < 0.01$) to a non-detectable level. Similar reductions in hippuric acid content were observed during yogurt and kefir fermentation by other researchers ^{22,23}. Considering that hippuric acid is a precursor in the synthesis of benzoic acid by lactic acid bacteria, it probably decreased as a result of the metabolic activity of lactic acid bacteria to form benzoic acid ^{22,23}.

Antifungal Activity

The antifungal activity of kefir and yogurt samples against *C. albicans* was determined using an *in vitro* culture method. The diameter of the growth inhibitory zone was measured as well (Table 4). Totally, kefir samples showed the highest inhibitory activity against *C. albicans* in comparison to yogurt samples ($P < 0.05$). Based on the results, sheep milk kefir demonstrated the highest antifungal activity against *C. albicans*. For example, its inhibitory effect was 90.20% on the first day of the storage period, followed by camel kefir (78.37%), goat kefir (74.52%), cow kefir (73.23%), sheep yogurt (48.56%), camel yogurt (37.51%), cow yogurt (34.42%),

and goat yogurt (30.32%). After 20 days of storage, the inhibition rate of sheep milk kefir reached 96.50%. It should be noted that kefir samples had higher inhibitory potential compared to ketoconazole. These results are in agreement with those found by Rodrigues et al, indicating the growth inhibitory zone diameter of kefir and kefir against *C. albicans* was 23.2 and 28.0 mm, respectively, and significantly higher compared to ketoconazole ²⁴. It has been demonstrated that kefir inhibited the growth of fungi and the microorganisms of kefir grain possessed the potential of producing antifungal metabolites ²⁵. In our study, cow yogurt had lower acidity in comparison with other samples and inhibited the growth of the tested fungus weakly although both sheep and camel milk kefir and yogurt showed higher acidity, stronger anti-*Candida* activity, and significantly inhibited the fungal growth. Based on the results (Table 4) that the efficacy of the samples for inhibiting the growth diameter reduced toward the end of the storage period. The same results were reported by Taheur et al representing that the long storage period decreased the antifungal activity of whey permeates fermented by kefir grains ¹⁴. Kefir consists of polysaccharides, peptides, and proteins, which are the

substrates for the microorganisms regarding produce several organic acids and bioactive compounds that inhibit the activity or the growth of pathogenic microorganisms. In addition to the low pH, the antifungal activity of fermented products is attributed to the organic acids such as lactic, citric, pyruvic, propionic, and acetic acids formed during the fermentation period disrupting the proton transfer gradient in the intracellular membranes²⁶. Given that organic acids do not completely dissociate in water, they are considered as weak acids. It should be noted that the dissociation degree widely depends on pH²⁷. The decreasing of pH results in a higher concentration of protons and enhancing acid diffusion across the cell membrane and into the cytoplasm. Therefore, a lower level of the pH of the product leads to a higher growth inhibitory effect.

In addition to intracellular acidification, the presence of weak organic acids leads to internal aggregation of the negatively charged counter-ion resulting in multiple deleterious effects on yeast cells including oxidative stress, increases in turgor pressure, and finally, the depletion of ribosomal RNA and relevant cofactors²⁸. Several studies have demonstrated the above-mentioned effects in the model yeast *Saccharomyces cerevisiae*, *C. glabrata*, and *C. albicans*²⁹⁻³¹. In another study, lactic and acetic acids produced in the fermented foods were shown as fungal inhibitors³². Furthermore, Segun investigated the inhibitory potential of lactic acid bacteria (LAB) isolated from several types of yogurt (home-made and commercially available yogurts) against *C. albicans*. The produced inhibition zone by LAB ranged from 5.8 mm to 13.3 mm, which is lower than our results. Additionally, Segun claimed that drinking natural yogurt and its direct application to an infected area helps in curing or preventing candidiasis³³. The mechanism by which LAB inhibits the growth of pathogens is the production of organic acids such as lactic acid, antimicrobial metabolites, and antibiotic-like compounds which have antagonistic actions against pathogens³⁴. It was reported that bacteriocins such as acidophilin by *L. acidophilus* and bulgarican by *L. bulgaricus* effectively inhibited the growth of *C. albicans*³⁵. An antimicrobial compound named bacteriocin F1, produced by *L. paracasei* in Tibetan kefir grains, had antifungal activity³⁶. Similarly, it was found that metabolites produced by *L. plantarum* inhibited the growth of *C. albicans*³⁷. In the present study, the anti-*Candida* activity of kefir samples was stronger than that of yogurt samples, which may be due to the diversity of fermenting microorganisms and antimicrobial substances produced in kefir. It is shown that the production rate of the bioactive metabolites and organic acids decreased by the end of the storage period and reductions in nutrients and substrates for microorganisms. Accordingly, milk containing higher amounts of protein and carbohydrates (e.g., sheep and camel milk) provides enough nutrients for the microorganisms, and the produced yogurt and

kefir have stronger antifungal activity compared to other types of milk. Totally, the inhibitory capability of the available probiotics in yogurts and kefirs against *C. albicans* emphasizes the health benefits resulted from the consumption of these products.

Conclusion

In general, this study investigated the anti-*Candida* potential of kefir and yogurt produced from cow, camel, sheep, and goat milk. The results revealed the higher antifungal activity of kefir samples compared to yogurt samples. The sheep and cow milk kefir expressed the highest and the lowest anti-*Candida* activity, respectively. It seems that kefir may be used as a nutraceutical and functional food against *C. albicans* infections and in-vivo studies are needed to understand its anti-*Candida* mechanism.

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Authors' Contributions

MA: Supervision, validation, original draft writing, writing-reviewing and editing preparation, investigation, and software running; P.E.J. S.: Advising in terms of conception and design of the study; M. B.: Conceptualization, methodology, data analysis.

Conflict of Interests Disclosure

The authors declare that they have no conflict of interests.

Ethical Approval

Not applicable.

References

1. Sfakianakis P, Tzia C. Conventional and innovative processing of milk for yogurt manufacture; development of texture and flavor: A review. *Foods*. 2014;3(1):176-193. doi: 10.3390/foods3010176
2. Yilmaz L, Ozcan Yilsay T, Akpınar Bayazit A. The sensory characteristics of berry-flavoured kefir. *Czech Journal of food Sciences*. 2006;24(1):26. doi: 10.17221/3290-CJFS
3. Cenesiz S, Devrim A, Kamber U, Sozmen M. The effect of kefir on glutathione (GSH), malondialdehyde (MDA) and nitric oxide (NO) levels in mice with colonic abnormal crypt formation (ACF) induced by azoxymethane (AOM). *DTW. Deutsche tierärztliche Wochenschrift*. 2008;115(1):15-19.
4. Ismaiel AA, Ghaly MF, El-Naggar AK. Milk kefir: ultrastructure, antimicrobial activity and efficacy on aflatoxin B1 production by *Aspergillus flavus*. *Current Microbiology*. 2011;62(5):1602-1609. doi: 10.1007/s00284-011-9901-9
5. Prado MR, Blandón LM, Vandenberghe LP, et al. Milk kefir: composition, microbial cultures, biological activities, and related products. *Frontiers in Microbiology*. 2015;6:1177. doi: 10.3389/fmicb.2015.01177
6. Weissman J, Guthrie C, Fink GR. *Guide to yeast genetics: functional genomics, proteomics, and other systems analysis*: Academic Press; 2010.
7. Dovigo LN, Carmello JC, de Souza Costa CA, et al. Curcumin-mediated photodynamic inactivation of *Candida albicans* in a murine model of oral candidiasis. *Sabouraudia*. 2013;51(3):243-251. doi: 10.3109/13693786.2012.714081

8. Puerari C, Magalhães KT, Schwan RF. New cocoa pulp-based kefir beverages: Microbiological, chemical composition and sensory analysis. *Food Research International*. 2012;48(2):634-640. doi:10.1016/j.foodres.2012.06.005
9. Yilmaz-Ersan L, Ozcan T, Akpinar-Bayazit A, Sahin S. The antioxidative capacity of kefir produced from goat milk. *International Journal of Chemical Engineering and Applications*. 2016;7(1):22-26. doi: 10.7763/IJCEA.2016
10. AOAC. Official methods of analysis of the Association of Official Analytical Chemists. AOAC International, Washington. 2002 and 2016.
11. Matar C, LeBlanc JG, Martin L, Perdigon G. Biologically active peptides released in fermented milk: role and functions. *Handbook of fermented functional foods*: CRC Press; 2003:193-218.
12. de Lima MdSF, da Silva RA, da Silva MF, et al. Brazilian Kefir-Fermented Sheep's Milk, a Source of Antimicrobial and Antioxidant Peptides. *Probiotics and Antimicrobial Proteins*. 2018;10(3):446-455. doi: 10.1007/s12602-017-9365-8
13. Yilmaz-Ersan L, Ozcan T, Akpinar-Bayazit A, Sahin S. Comparison of antioxidant capacity of cow and ewe milk kefirs. *Journal of Dairy Science*. 2018;101(5):3788-3798. doi: 10.3168/jds.2017-13871
14. Taheur FB, Mansour C, Chaieb K. Inhibitory effect of kefir on *Aspergillus* growth and mycotoxin production. *Euro-Mediterranean Journal for Environmental Integration*. 2020;5(1):5. doi:10.1007/s41207-020-0141-x
15. Satir G, Guzel-Seydim ZB. Influence of Kefir fermentation on the bioactive substances of different breed goat milks. *LWT-Food Science and Technology*. 2015;63(2):852-858.
16. Gao W, Zhang L, Feng Z, et al. Microbial diversity and stability during primary cultivation and subcultivation processes of Tibetan kefir. *International Journal of Food Science & Technology*. 2015;50(6):1468-1476. doi: 10.1111/ijfs.12801
17. Montanuci F, Garcia S, Prudencio S. Sensory characterization and acceptance of sweetened full fat and low fat kefir with inulin. *Brazilian Journal of Food Technology*. 2010;8:79-90. doi: 10.1016/j.tifs.2014.01.002
18. Magalhães KT, Pereira GVdM, Campos CR, Dragone G, Schwan RF. Brazilian kefir: structure, microbial communities and chemical composition. *Brazilian Journal of Microbiology*. 2011;42(2):693-702. doi: 10.1590/S1517-83822011000200034
19. BoyCheVa S, Dimitrov T, NayDeNoVa N, MihayloVa G. Quality characteristics of yogurt from goat's milk, supplemented with fruit juice. *Czech Journal of Food Sciences*. 2011;29(1):24-30. doi: 10.17221/171/2008-CJFS
20. Miguel MGdCP, Cardoso PG, de Assis Lago L, Schwan RF. Diversity of bacteria present in milk kefir grains using culture-dependent and culture-independent methods. *Food Research International*. 2010;43(5):1523-1528. doi: 10.1016/j.foodres.2010.04.031
21. Fernandez-Garcia E, McGregor JU. Determination of organic acids during the fermentation and cold storage of yogurt. *Journal of Dairy Science*. 1994;77(10):2934-2939. doi: 10.3168/jds
22. Güzel-Seydim Z, Seydim A, Greene A, Bodine A. Determination of organic acids and volatile flavor substances in kefir during fermentation. *Journal of Food Composition and Analysis*. 2000;13(1):35-43. doi: 10.1006/jfca.1999.0842
23. da Costa MP, da Silva Frasco B, da Costa Lima BRC, Rodrigues BL, Junior CAC. Simultaneous analysis of carbohydrates and organic acids by HPLC-DAD-RI for monitoring goat's milk yogurts fermentation. *Talanta*. 2016;152:162-170. doi: 10.1016/j.talanta.2016.01.061
24. Rodrigues KL, Caputo LRG, Carvalho JCT, Evangelista J, Schneedorf JM. Antimicrobial and healing activity of kefir and kefir extract. *International Journal of Antimicrobial Agents*. 2005;25(5):404-408. doi:10.1016/j.ijantimicag.2004.09.020
25. Caro Velez CA, Leon Pelaez ÁM. Fungal growth inhibition of *Aspergillus ochraceus* with "panela" fermented with water kefir grains. *Vitae*. 2014;21(3):191-200.
26. Gamba RR, Moure MC, Diosma G, Giannuzzi L, De Antoni GL, León Peláez AM. Application of Whey Permeate Fermented with Kefir Grains for the Shelf-Life Improvement of Food and Feed. 2016; 6(9):650-661. doi: 10.4236/aim.2016.69064
27. Vermeulen A, Dang TDT, Geeraerd A, et al. Modelling the unexpected effect of acetic and lactic acid in combination with pH and aw on the growth/no growth interface of *Zygosaccharomyces bailii*. *International Journal of Food Microbiology*. 2008;124(1):79-90. doi: 10.1016/j.ijfoodmicro.2008.02.020
28. Mira NP, Teixeira MC, Sá-Correia I. Adaptive response and tolerance to weak acids in *Saccharomyces cerevisiae*: a genome-wide view. *Omic: A Journal of Integrative Biology*. 2010;14(5):525-540. doi: 10.1089/omi.2010.0072
29. Cunha DV, Salazar SB, Lopes MM, Mira NP. Mechanistic insights underlying tolerance to acetic acid stress in vaginal *Candida glabrata* clinical isolates. *Frontiers in Microbiology*. 2017;8:259. doi: 10.3389/fmicb.2017.00259
30. Bernardo RT, Cunha DV, Wang C, et al. The CgHaa1-regulon mediates response and tolerance to acetic acid stress in the human pathogen *Candida glabrata*. *G3: Genes, Genomes, Genetics*. 2017;7(1):1-18. doi: 10.1534/g3.116.034660
31. Cottier F, Tan ASM, Yurieva M, et al. The transcriptional response of *Candida albicans* to weak organic acids, carbon source, and MIG1 inactivation unveils a role for HGT16 in mediating the fungistatic effect of acetic acid. *G3: Genes, Genomes, Genetics*. 2017;7(11):3597-3604. doi: 10.1534/g3.117.300238
32. Hassan R, El-Kadi S, Sand M. Effect of some organic acids on some fungal growth and their toxins production. *International Journal of Advanced Biology*. 2015;2(1):1-11. doi: 10.1.1.651.8322
33. Segun AA. Antimicrobial Activity of Bacteriocin-Producing Lactic Acid Bacteria Isolated from Yogurts Against *Candida albicans*. *International Journal of Microbiology and Application*. 2015;2(5):84.
34. Tadesse G, Ephraim E, Ashenafi M. Assessment of the antimicrobial activity of lactic acid bacteria isolated from Borde and Shamita, traditional Ethiopian fermented beverages, on some foodborne pathogens and effect of growth medium on the inhibitory activity. *Internet Journal of Food Safety*. 2005;5:13-20.
35. Piard Jc, Desmazeaud M. Inhibiting factors produced by lactic acid bacteria. 2. Bacteriocins and other antibacterial substances. *Le Lait*, INRA Editions. 1992, 72 (2):113-142.
36. Miao J, Guo H, Ou Y, et al. Purification and characterization of bacteriocin F1, a novel bacteriocin produced by *Lactobacillus paracasei* subsp. *tolerans* FX-6 from Tibetan kefir, a traditional fermented milk from Tibet, China. *Food Control*. 2014;42:48-53. doi: 10.1016/j.foodcont.2014.01.041
37. Okpara A, Okolo B, Ugwuanyi J. Antimicrobial activities of lactic acid bacteria isolated from akamu and kunun-zaki (cereal based non-alcoholic beverages) in Nigeria. *African Journal of Biotechnology*. 2014;13(29):2977-2984. doi: 10.5897/AJB12.376