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Antibacterial activity of cow's milk and almond milk fermented by kefir grains

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ABSTRACT

Kefir is a probiotic beverage produced mainly from cow's milk fermentation by kefir grains. Some persons do not eat cow's milk. Plant milk can be a replacement for animal milk. This study aims to investigate the antibacterial effect of kefir produced with cow's milk and almond milk. We added kefir grains to cow's milk (full-fat and low-fat) and almond milk (with and without sucrose) and the fermentation was done at 25 and 37°C. We extracted kefir and the antibacterial effect of kefir extract against 8 bacteria was evaluated by the well method. The temperature significantly affected the antibacterial activity of kefir prepared with cow's milk against all tested bacteria except for *E. faecalis* ($P<0.05$) so kefir samples prepared at 37°C compared to 25°C showed more antibacterial activity. The type of cow's milk (full-fat and low-fat) did not significantly affect the antibacterial activity against the tested bacteria, except for *S. aureus*, *S. typhimurium*, and *B. cereus*. Furthermore, *E. faecalis* and S. *typhimurium* were the most sensitive and resistant bacteria to kefir prepared with cow's milk. Fermentation temperature had no significant effect on the antibacterial activity of kefir samples prepared with almond milk, but adding of sucrose showed a significant effect on the antibacterial activity against *E. faecalis*, *S. dysenteriae*, *B. cereus,* and *P. aeruginosa* ($P<0.05$). Overall, we recommended a fermentation temperature of 37°C for cow's milk and adding sucrose to almond milk to achieve the most antibacterial effect against *E. faecalis*, *S. dysenteriae*, *B. cereus*, and *P. aeruginosa*.

1. Introduction

Milk and dairy products have the fourth rank in the food pyramid. Many studies have been done about their nutritional value and useful effects on health [1]. However, many people cannot consume animal milk. They may be lactose intolerant or allergic to milk protein. In addition, vegetarians prefer not to drink animal milk. The trend towards plant-based milk instead of traditional milk can solve these problems. On the other hand, plant milks have no cholesterol and are low-calorie [2]. In addition, there are frequent reports based on the consumption of plant milk with sources rich in antioxidants had therapeutic effects on diseases such as cancer, arteriosclerosis, and inflammatory diseases [3].

Plant-based milk or non-dairy milk is a watery liquid that is mainly produced from various sources such as cereals, legumes, nuts, seeds, and pseudo-cereals, and unlike milk of animal origin, it has little saturated fat and contains a lot of calcium and nutrients so that can be alternative for animal milk [4].

Human beings have used living microorganisms in food, especially lactic acid-producing bacteria, to maintain and improve health for a long time in history. Probiotics are useful alive microorganisms for human health when it is used in enough amounts [5].

Fermentation is one of the traditional ways to preservation of food. Through fermentation, the sensory characteristics of the end products as well as the nutritional quality improve by break down of sugars and increasing the level of B family vitamins such as thiamin (B1), niacin (B3), and amino acids such as lysine [6,7]. The fermentation of plant milk leads to the production of organic acids and antimicrobial components such as acetaldehyde and diacetyl [1]. Also, fermentation by producing amino acids and vitamins causes betterness of the nutritional value of milk as well as therapeutic effects such as antimicrobial, anti-tumor, and immune-regulating [1,8,9].

Lactic acid bacteria (LAB) are the most important probiotic microorganisms that are naturally present in dairy fermented products, including yogurt, buttermilk, kefir, etc., which regulate the intestinal microbiota population and limit pathogenic species [10]. Notably, the minimum level of probiotic bacteria in plant milk fermented products must be between 10^6 and 10^7 CFU/ml by the expiration date [1,11,12].

Kefir is a natural probiotic beverage and one of the most ancient fermented milk products, that is obtained from milk fermentation with kefir grains. Kefir regulates the body's immune system, and blood pressure, treats digestive diseases, and decreases serum cholesterol, and also has antibacterial, antifungal, and antitumor effects [13,14,15].

Kefir grains are jelly-like and look like small cauliflower. They are 1 to 3 cm long, irregularly shaped, white to yellow, and have a slimy but firm texture [13]. The microflora of kefir grains varies depending on the grain origin. Grains contain a group of specific microbes that exist in a complex symbiotic relationship, including species of yeasts, lactic acid bacteria (Lactobacillus and Lactococcus), and acetic acid bacteria [14,15].

The biological effects of kefir are related to the intermediate compounds and end products resulting from microorganisms' metabolism and the fermentation conditions such as fermentation time, temperature, type of milk, and grains inoculation ratio [14,15,16,17,18].

Cow's milk is the raw material of kefir. However, as mentioned, some people are vegetarians because can not tolerate lactose or have an allergy to casein. As a result, these people should not drink fermented drinks such as kefir, so they can replace it with plant-based milk.

In the meantime, almond milk is one of the most popular vegetable milks, the trend towards its consumption is increasing day by day and it can have benefits for the consumer. Almonds contain many nutrients, phytochemicals, and unsaturated fatty acids that greatly decrease cardiovascular diseases. Consumption of saturated fatty acids is associated with an increase in LDL cholesterol. Almond milk does not contain lactose, cholesterol, or saturated fat and has very few calories. It also has more calcium than cow's milk and other plant-based milks. Also, almond milk includes iron, magnesium, phosphorus, zinc, and potassium along with various vitamins. Almond milk acts as an antioxidant, anti-inflammatory, anti-fatty blood, and anti-tumor decreases platelet aggregation or breaks blood clots, and boosts the immune system [1,4].

In addition to the mentioned cases, it has been reported that the milk prepared from almonds supports the growth of lactic acid bacteria and the survival of these microorganisms under conditions of storage in the refrigerator for long periods and is a suitable substrate for carrying probiotics [19,20].

This study aims to investigate the antibacterial effect of prepared kefir samples with cow's milk and almond milk against 8 pathogenic bacteria, including *Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis*, *Shigella dysenteriae*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli*, and *Klebsiella pneumonia*. Also, we evaluated the sensory characteristics of prepared kefir samples.

2. Materials and Methods

Preparation of almond milk

To prepare almond milk, first, almond kernels were soaked in water overnight, to remove almond brown skin. We mixed almond and water in a ratio of 1:5 (1 part almond and 5 parts water) in a blender for 15 minutes and then filtered with double-layer sterile gauze. These filtered almond milk were thermally treated for 20 minutes at 95°C in a water bath and used to inoculate kefir grains and preparation of kefir beverages. Almond milk containing 5% sucrose was also prepared by adding sucrose syrup [19,21,22].

Preparation of kefir

Kefir grains were obtained from the prebiotic store, and the grains regeneration and fermentation process was carried out (Ajam and Kohsari 2020). Briefly, grains were subculture in fresh milk for 4 days at 25°C. The milk was changed every 24 hours. After regeneration, milk passed from the sieve, and kefir grains were separated and washed with sterile distilled water. Then, 5 grams of grains were inoculated into 50 ml of cow's milk (full-fat and low-fat) and almond milk (with and without sucrose) and incubated at 25°C and 37°C. After 48 hours, kefir grains were separated from the fermented product. Kefir extracts were kept in the refrigerator until antibacterial tests [18].

bacterial strains

The bacteria strains were *Escherichia coli* (PTCC1338), *Shigella dysenteriae* (PTCC 1188), *Salmonella typhimurium* (PTCC 1596), *Pseudomonas aeruginosa* (PTCC 1811) and *Klebsiella pneumoniae* (PTCC 1290) and *Staphylococcus aureus* (PTCC 1112), *Bacillus cereus* (PTCC 1154), and *Enterococcus faecalis* (PTCC 1778). They were procured from the Iranian Research Organization for Science and Technology (IROST) in a lyophilized form. They recovered in the brain heart infusion medium (Merck) for 24 hours at 37°C and then inoculated into a Nutrient Broth culture medium (Merck). We used bacterial suspension with turbidity equal to 0.5 McFarland = 1.5×10^8CFU/ml [23].

Well method

We assayed the antibacterial effect of kefir by the well method. We cultured a suspension equal to 0.5 McFarland (1.5×10^8 CFU/ml) of bacteria with a sterile swab on Mueller Hinton agar, then, wells were dug in the culture media using a sterile cork borer, kefir extracts were added into the wells, and plates were incubated at 37°C for 24-48 hours. After incubation, the diameter of the inhibition zone around the wells was measured [23].

Sensory evaluation

Sensory characteristics of prepared kefir samples were evaluated by 10 judges from volunteers in terms of sensory characteristics, including general appearance, color, aroma, taste, and mouthfeel (texture) tests. The panel members determined their standard of sensory evaluation of prepared kefir samples using a 9-point sensory scale. On this scale, score 9 is very excellent, score 8 is excellent, score 7 is good, score 6 is relatively good, score 5 is neither good nor bad, score 4 is relatively bad, score 3 is bad, score 2 is very bad, and finally score 1 is extremely bad [24].

Statistical analysis

The independent variables of the research include the type of kefir (kefir samples prepared from full-fat and low-fat cow's milk and kefir samples prepared with almond milk with and without sucrose, temperature (25 and 37°C) and 8 species of bacteria and the dependent variables of the research are antibacterial activity (diameter of inhibition zone of each bacteria) and the sensory characteristics of the prepared kefir samples. All data were expressed as mean ± standard deviation (n=3) with ANOVA. Also, data was analysis Duncan's Test by SPSS software.

Results and Discussion

Antibacterial activity of kefir prepared with cow's milk

Table 1 and Figure 1 show the mean diameter of the inhibition zone of tested bacteria **in kefir** prepared with cow's milk (full-fat and low-fat) at temperatures of 25 and 37°C.

Table 1- The mean diameter of the inhibition zone of tested bacteria in kefir prepared with Cow milk

Kefir / Bacteria	Cow milk Full Fat/37°C	Cow milk Full Fat/25°C	Cow milk Low Fat/37°C	Cow milk Low Fat/25°C
E. coli	18.33 ± 1.52^{bcA}	15.33 ± 1.52^{bB}	18.66 ± 0.57^{bA}	14.00 ± 1.00^{cdB}
E. faecalis	22.33 ± 1.52^{aA}	20.66 ± 1.15^{aA}	22.00 ± 2.64^{aA}	20.00 ± 1.00^{aA}
S. typhimurium	16.66 ± 0.57^{cB}	13.00 ± 0.00^{cC}	17.66 ± 0.57^{bA}	12.66 ± 0.57^{dC}
S. dysenteriae	20.33 ± 2.08^{bA}	13.33 ± 0.57^{cB}	20.00 ± 1.15^{abA}	14.00 ± 0.00^{cdB}
S. aureus	17.00 ± 0.00^{cB}	14.66 ± 0.57^{bcC}	20.00 ± 0.00^{abA}	15.00 ± 0.00^{bcC}
K. pneumoniae	18.00 ± 0.00^{cA}	15.33 ± 0.57^{bB}	18.00 ± 1.00^{bA}	15.33 ± 0.57^{bcB}
B. cereus	17.00 ± 0.00^{cA}	14.33 ± 0.57^{bcB}	17.33 ± 1.15^{bA}	16.00 ± 1.73^{bAB}

P. aeruginosa	18.33±0.57^{bcA}	14.33±0.57^{bcB}	19.00±1.00^{bA}	14.33±1.15^{bcdB}

Mean ± standard deviation. Different lowercase letters in each column indicate significant differences at the 5% probability level (P≤0.05).
Mean ± standard deviation. Different uppercase letters in each row indicate significant differences at the 5% probability level (P≤0.05).

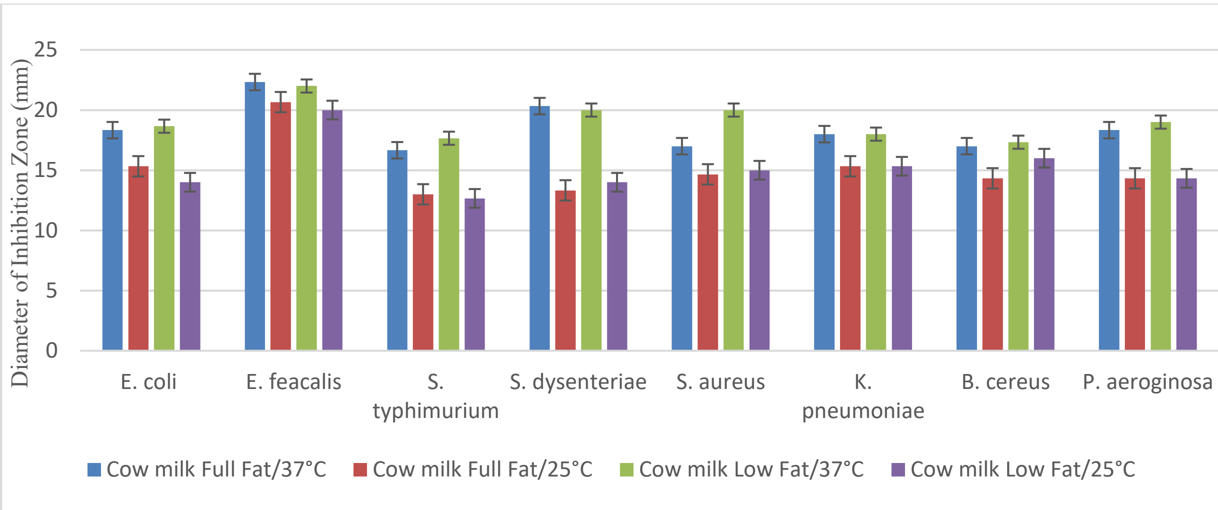

Figure 1- The mean diameter of the inhibition zone of bacteria in the presence of kefir prepared with Cow milk

The results indicate significant antibacterial activity of kefir samples prepared with cow's milk. Many studies have reported the antimicrobial activity of kefir prepared from cow's milk [15,25,26,27,28]. These studies show that kefir is an antimicrobial beverage and this antimicrobial activity is related to the production of organic acids (lactic acid and acetic acid), peptides (bacteriocins), carbon dioxide, hydrogen peroxide, ethanol, and diacetyl [14,15]. In addition to, kefiran produced by kefir grains has antibacterial effects [29,30]. These compounds are not only effective in antimicrobial activity against gastrointestinal pathogens during beverage production and storage but are also effective in the treatment and prevention of gastrointestinal infections, gastroenteritis, and vaginal infections [13,14,15].

Microorganisms in kefir grains produce organic acids, such as lactic acid, and acetic acid. Lactic acid penetrates through the cytoplasmic membrane, acidifying the cytoplasm and inhibiting the activity of enzymes. At intracellular pH, most acids lead to the production of hydrogen ions, which interfere with important metabolic processes such as oxidative phosphorylation and inhibit aerobic species. Furthermore, it was shown that lactic acid and acetic acid when produced together in kefir extract have an excellent synergistic inhibitory effect, and this effect is related to the enhancement of acetic acid in reducing pH by lactic acid [13].
Bacteriocins change the membrane potential by corrupting potassium ions and ATP and make cells unable to balance the intracellular pH [31].
A protein matrix and also polysaccharide matrix called kefiran surround microorganisms in kefir grains. Kefiran is a branched glucogalactan

soluble in water, which consists of equal amounts of D-galactose and D-glucose. The production of kefir is mostly done by *Lactobacillus kefiranofaciens* and *Lactobacillus kefiri* species. The antibacterial, antifungal, and antitumor activities of kefir can be attributed to kefiran. Kefiran production can be increased by cultivation conditions and intermediate compositions, also adding of kefir grains and mineral resources [32,33,34].
The biological roles of kefir are related to intermediate compounds and the end products. The difference in the amount and type of these microorganisms and intermediate compounds is related to fermentation conditions such as time and temperature of fermentation, type of milk, grains inoculation ratio as well as pH and the sensitivities of different bacterial strains [14,15,16,17].

In Table 1, the effect of the type of milk and the fermentation temperature on the diameter of the inhibition zone of bacteria and the antibacterial activity of kefir samples prepared with cow's milk can be seen. Fermentation temperature significantly affected the antibacterial activity of kefir samples prepared with cow's milk in all tested bacteria except *E. faecalis* (P<0.05). The diameter of the inhibition zone of the tested bacteria in the presence of kefir samples prepared at 37°C was greater compared to kefir samples prepared at 25°C, which shows that the tested bacteria are more sensitive to kefir samples prepared at 37°C (Table 1).

In the meantime, *S. dysenteriae* showed the mean diameter of the inhibition zone of 20.33 and 20 mm respectively in the presence of kefir samples prepared with full-fat and low-fat milk at the fermentation temperature of 37°C while this gram-negative bacterium showed the mean diameter of inhibition zone of 13.33 and 14 mm respectively when exposed to samples prepared with full-fat and low-fat milk at fermentation temperature of 25°C. The results show a significant difference between treatments (P<0.05) and indicate the effect of fermentation temperature on the antibacterial activity of kefir samples (Figure 2).

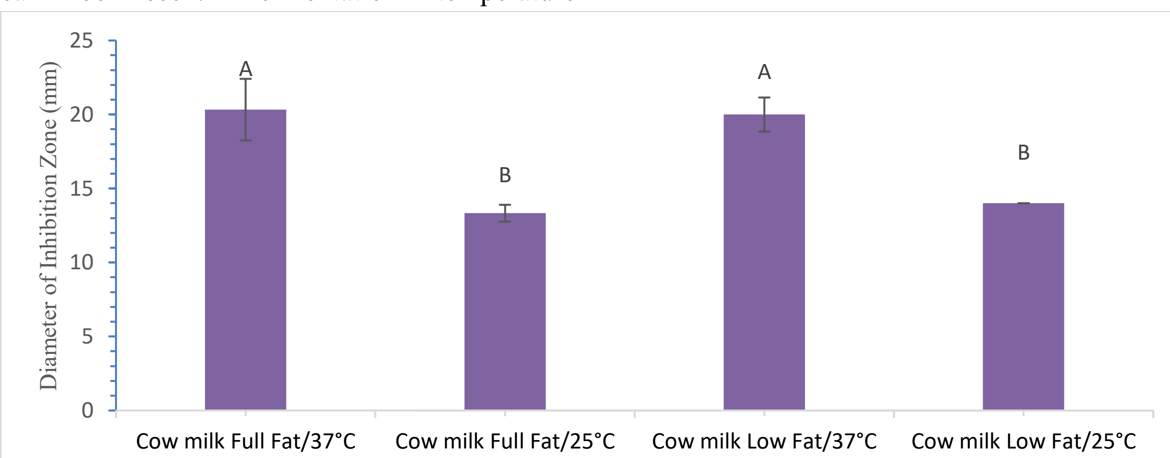

Figure 2. The effect of kefir samples prepared from low-fat and full-fat cow's milk at 25 and 37°C on *S. dysenteriae*

(Different uppercase letters at the top of the columns indicate significant differences between treatments (P≤0.05).)

The findings related to *S. aureus* also showed a significant difference between the treatments (P<0.05). So kefir samples prepared at 37°C showed more antibacterial activity than samples prepared at 25°C, which indicates the effect of fermentation temperature on the antibacterial activity of kefir samples. *S. aureus* showed the mean diameter of the inhibition zone of 20 and 17 mm respectively in the presence of kefir samples prepared with low-fat and full-fat milk at the fermentation temperature of 37°C while this gram-positive bacterium showed the mean diameter of the inhibition zone of 15 and 14.66 mm respectively when exposed to samples prepared with low-fat and full-fat milk at fermentation temperature of 25°C (Figure 3).

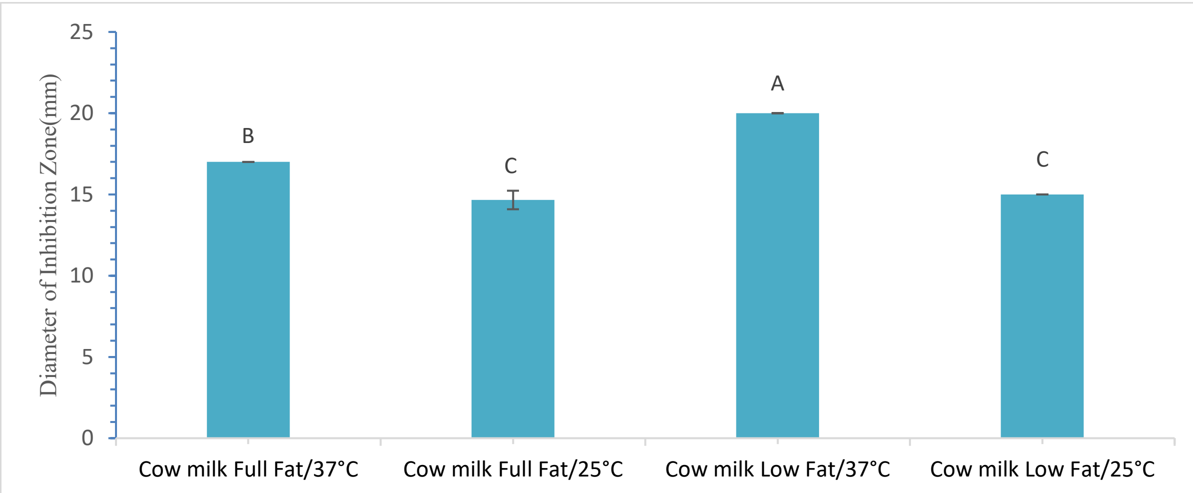

Figure 3. The effect of kefir samples prepared from low-fat and full-fat cow's milk at 25 and 37°C on *S. aureus*

As mentioned, other tested bacteria showed a similar behavior and their mean diameter of inhibition in the presence of kefir samples prepared at a fermentation temperature of 37°C was higher than at a fermentation temperature of 25°C, and the results indicated a significant difference and the effect of temperature on the antibacterial activity of kefir samples. Of course, regarding *E. faecalis*, fermentation temperature did not show a significant difference in the antibacterial activity of kefir samples prepared with cow's milk (Table 1 and Figure 1).

In the study of the effect of different fermentation conditions on the antibacterial activity of cow's milk fermented by kefir grains against four bacteria *S. aureus*, *E. coli*, *B. cereus,* and *S. dysenteriae*, the results of the study by Ajam and Koohsari (2020) showed that fermentation temperature had a significant effect on the antibacterial activity of kefir samples against all tested bacteria except S. dysenteriae, and considering all the factors and their interactions, the highest antibacterial activity against the tested bacteria was observed in kefir samples fermented at 37°C [18].

The growth of kefir grains in different culture environments depends on the presence and amount of substrates needed for the growth of microorganisms in kefir grains, including protein and lactose, and the amounts of the main components of milk can vary significantly between cows, different breeds, and between cows of the same breed and it varies depending on the type of feed [35].

On the other hand, the difference in the results of the present study with other studies may be due to the difference in the origin of the kefir grains. Differences in the antibacterial activity of kefir samples with different origins have been reported in other studies [36,37].

Regional differences and fermentation conditions can change the microbial diversity in kefir grains, and the microbial diversity is responsible for the biological activities and physicochemical characteristics of kefir [38,39,40,41].

On the other hand, the type of cow's milk used in the preparation of kefir samples in terms of fat content (low-fat and full-fat), did not show a significant effect on the antibacterial activity against the tested bacteria, except *S. aureus*, *S. typhimurium*, and *B. cereus* (Table 1). That is, kefir samples prepared with full-fat and low-fat milk have shown similar antibacterial activity.

Of course, as mentioned, the effect of the type of milk on the antibacterial activity of the kefir samples against *S. aureus*, *S. typhimurium*, and *B. cereus* was significant and samples prepared with low-fat milk at a fermentation temperature of 37°C compared to samples prepared with full-fat milk at temperature of 37°C showed more antibacterial activity against *S. aureus* and *S. typhimurium*. Also, samples prepared with low-fat milk at a fermentation temperature of 25°C showed more antibacterial activity against *B. cereus* compared to samples prepared with full-fat milk at a temperature of 25°C (Table 1).

S. aureus showed the highest inhibition zone with a mean of 20 mm in the presence of a kefir sample prepared with low-fat milk and a fermentation temperature of 37°C, and it showed the lowest sensitivity to the kefir sample prepared with full-fat milk and fermentation temperature of 25°C with the mean diameter of inhibition zone 14.66 mm (Table 1 and Figure 3).

Many parameters can affect the final antimicrobial activity of kefir. One of the most important parameters is the type of substrate and milk used in the preparation of this fermented beverage [18]. Fatty acid composition and lactose content can affect the final pH and acid content of the product, in addition to the types of peptides and other bioactive ingredients. Also, the antimicrobial activity observed in kefir depends on the type of microorganism, microbial population, and different enzymes in the grains [42].

In addition, kefir produced from low-fat and plant-based milk, such as rice and soy milk, generally shows less antimicrobial activity than kefir produced using full-fat, animal-based milk such as cow's milk [22,43,44,45].

In general, the type of milk used-full-fat or low-fat-can influence the antibacterial activity of kefir, though the effect is generally modest compared to other factors like fermentation time and the microbiota present in the kefir grains. Studies indicate that milk type (including fat content) affects the antibacterial activity of kefir, with significant differences observed in some cases [18]. However, most research emphasizes that the main contributors to kefir's antimicrobial properties are the bioactive compounds (such as organic acids, peptides, and bacteriocins) produced during fermentation and the specific microbial composition, rather than the fat content alone [46,47].

Among the tested bacteria, Gram-positive *E. faecalis* was the most sensitive bacterium to kefir samples prepared with cow's milk. The mean diameter of the inhibition zone of this bacterium in the presence of kefir samples prepared with full-fat and low-fat milk at a fermentation temperature of 37°C was 22.33 and 22 mm, respectively, and in the presence of samples prepared with full-fat and low-fat milk at a temperature of 25°C was 20.66 and 20 mm, respectively.

The most resistant bacteria to kefir samples was *S. typhimurium*. The mean diameter of the inhibition zone of this bacterium for kefir samples prepared with low-fat and full-fat milk at fermentation temperature of 25°C was 12.66 and 13 mm, respectively, and for samples prepared with full-fat and low-fat milk at temperature of 37°C was 16.66 and 17.66 mm, respectively (Table 1).

In general, kefir shows antimicrobial effects on gram-negative bacteria, but it is more effective against gram-positive bacteria [14,15,48]. Shafie et al reported the highest antibacterial activity of kefir samples prepared with cow's milk, almond milk, and their mixture against *S. aureus* compared to *E. coli* and *S. typhi* [22] which was also supported by the study of Ulusoy et al [49]. Of course, contradictory and different results are also reported. For example, Gamba et al reported that cow's milk and soy milk kefirs showed antibacterial activity against *S. aureus*, *E. coli,* and *S. typhi*. This study also showed that *S. aureus* was least sensitive to 25% cow's milk kefir solution and 75% soy milk kefir solution [50]. The difference in the result may be due to the difference in the origin of the grains and their microbial population and fermentation conditions, which produce different antimicrobial activity [42].

The difference in the sensitivity of gram-positive and gram-negative bacteria to kefir samples can be attributed to the differences in structure and the composition of the cell wall. The main composition of the cell wall in gram-positive bacteria is peptidoglycan, while the amount of peptidoglycan in gram-negative bacteria is very low, most of the cell wall is composed of the outer membrane containing lipid compounds, and this structure is not seen in the cell wall of gram-positive bacteria [51,52].

To be antibacterial, a compound must cross the outer membrane. The resistance of Gram-negative bacteria, including *S. typhimurium*, *E. coli,* and *P. aeruginosa*, can be due to the lower permeability of the outer membrane of these bacteria, which limits the entry of antimicrobial agents into the bacterial cell [53].

Despite all the above, the sensitivity and resistance of Gram-positive and Gram-negative bacteria depends on the type of mechanism of antibacterial activity. For example, one of the most important mechanisms of kefir's antimicrobial activity against Gram-positive and Gram-negative bacteria is the reduction of pH through the production of organic acids such as lactic acid and acetic acid [44]. Although most studies point to the role of organic acids in the antibacterial activity of kefir against Gram-negative bacteria, some studies have shown that pH is not the only factor determines the antimicrobial activity of kefir, and the antimicrobial activity against Gram-positive and Gram-negative bacteria are also related on others special compounds such as bacteriocins and kefiran exopolysaccharide [22,45,54].

However, the inhibitory effects of the kefir microbiota can’t be attributed to only one of the compounds. In addition, as reported by Silva et al. (2009), different carbon source concentrations and different fermentation times of kefir grains affect the inhibition of pathogenic microorganisms [28]. Furthermore, as reported by Kim et al. (2016), different inhibitory compounds can be present under different fermentation conditions [45].

Therefore, considering that kefir beverages contain different inhibitory compounds, it is assumed that these compounds interact with each other to enhance or counteract their antibacterial effects. An example of this mechanism can be the inactivation of bacteriocins by organic acids or enzymatic degradation during fermentation [54].

In general, fermentation conditions significantly affect the quantity and type of microorganisms and chemical compounds produced and thus the biological activities of this fermented beverage. So, in the study of Ajam and Kohsari (2020), to achieve the best antibacterial activity against *E. coli*, *S. aureus*, *B. cereus,* and *S. dysenteriae* fermentation times of 48, 72, 120, and 120 hours were recommended respectively [18].

Antibacterial effect of kefir prepared with almond milk

One of the raw materials that has dominated the plant-based milk market in recent years is almonds. The almond-based milk was primarily introduced and marketed as a milk substitute for children and adults suffering from conditions such as cow's milk allergy and lactose intolerance [55]. Also, milk prepared from almonds is reported to be a suitable matrix for the fermentation process [19,20,56].

Investigation the antibacterial activity of kefir samples prepared with almond milk (with and without sucrose) at fermentation temperatures of 25 and 37°C against each of the tested bacteria showed that despite the lack of effect of fermentation temperature on the antibacterial activity of kefir samples, but the presence of sucrose has a significant effect on the antibacterial activity of kefir samples prepared with almond milk against *E. faecalis*, *S. dysenteriae*, *B. cereus* and *P. aeruginosa* (P<0.05).

Table 2- The mean diameter of the inhibition zone of bacteria from kefir samples prepared with Almond milk

Kefir / Bacteria	Almond milk without sucrose/37°C	Almond milk without sucrose/25°C	Almond milk with sucrose/37°C	Almond milk with sucrose/25°C
E. coli	14.00 ± 1.00^{abA}	-	14.00 ± 1.00^{cA}	14.00 ± 1.00^{cA}
E. faecalis	13.00 ± 1.00^{bB}	13.00 ± 1.00^{bB}	16.00 ± 1.00^{bA}	16.00 ± 1.00^{bA}
S. typhimurium	13.00 ± 1.00^{bA}	-	13.00 ± 1.00^{cdA}	13.00 ± 1.00^{cA}
S. dysenteriae	13.00 ± 1.00^{bB}	-	14.66 ± 0.57^{bcA}	14.00 ± 1.00^{cAB}
S. aureus	13.00 ± 1.00^{bA}	-	14.00 ± 1.00^{cA}	14.00 ± 1.00^{cA}
K. pneumoniae	14.00 ± 1.00^{abA}	15.00 ± 1.00^{aA}	14.00 ± 1.00^{cA}	14.00 ± 1.00^{cA}
B. cereus	15.00 ± 1.00^{aB}	14.00 ± 1.00^{abB}	21.00 ± 1.00^{aA}	21.00 ± 1.00^{aA}
P. aeruginosa	13.00 ± 1.0^{bAB}	-	12.00 ± 1.00^{dB}	14.33 ± 0.57^{cA}

Mean ± standard deviation. Different lowercase letters in each column indicate significant differences at the 5% probability level (P≤0.05).

Mean ± standard deviation. Different uppercase letters in each row indicate significant differences at the 5% probability level (P≤0.05).

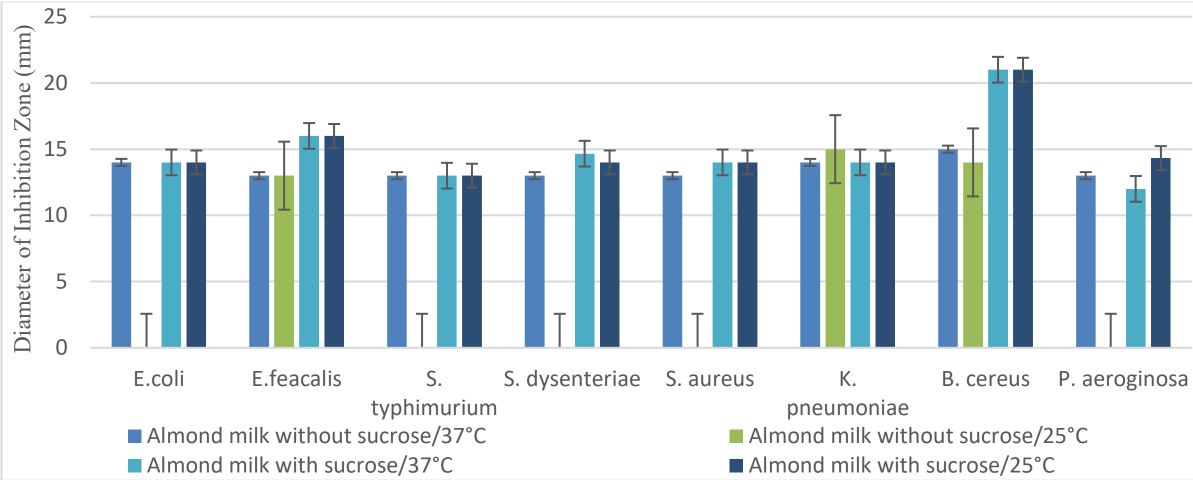

Figure 4- The mean diameter of the inhibition zone of bacteria from kefir samples with almond milk

The sensitivity of *B. cereus* to kefir samples prepared with almond milk, especially samples containing sucrose, was interesting. So that this bacterium showed a mean diameter of the inhibition zone of 21 mm in the presence of kefir samples prepared with almond milk with sucrose (Table 2). The effect of adding sucrose on the antibacterial activity of kefir samples prepared with almond milk against this gram-positive gastrointestinal pathogenic bacterium was noticeable (Figure 5).

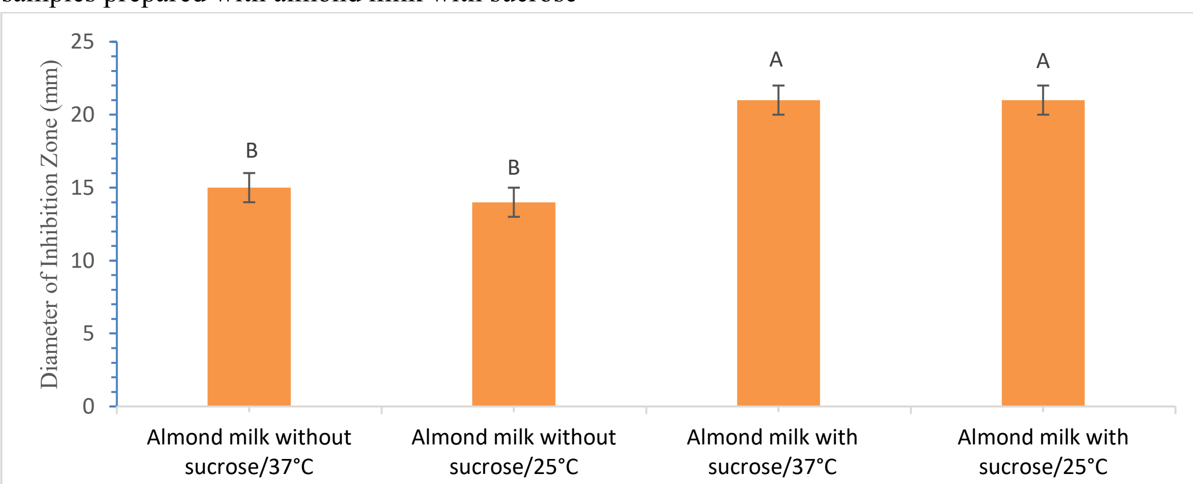

Figure 5. The effect of fermentation temperature and the presence of sucrose on antibacterial activity against *B. cereus* in kefir samples prepared with almond milk

Similar behavior with *B. cereus* was observed in the Gram-positive bacterium of *E. faecalis* so a significant difference was observed between samples prepared with almond milk containing sucrose and samples without sucrose. This bacterium, which was one of the most sensitive bacteria studied in kefir samples prepared with cow's milk, showed more sensitivity in the presence of samples containing sucrose compared to kefir samples prepared from almond milk without sucrose. The inhibition zone of *E. faecalis* in the presence of kefir samples prepared with almond milk with sucrose and without sucrose was observed to be 16 and 13 mm, respectively (Table 2 and Figure 6).

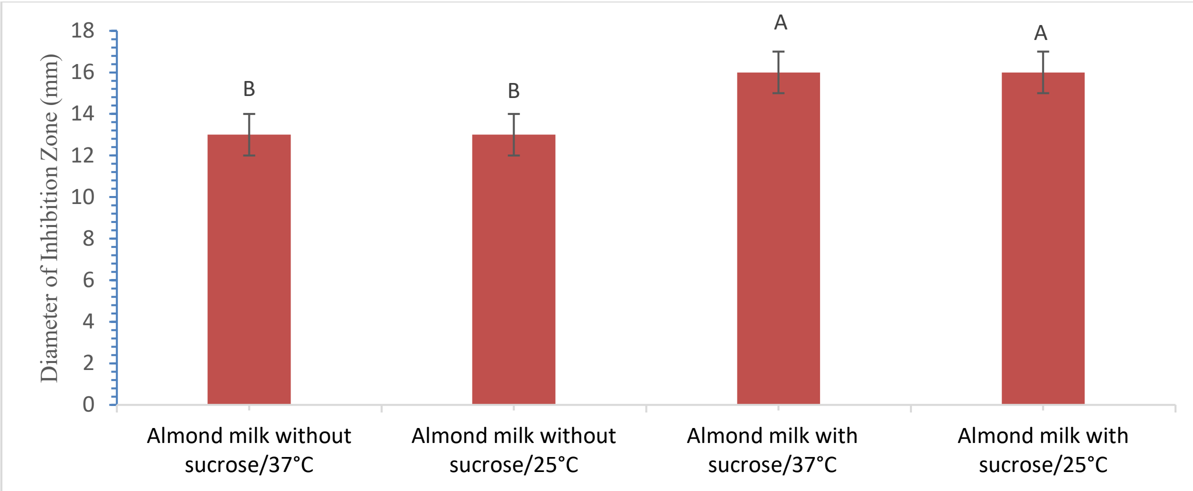

Figure 6. The effect of fermentation temperature and sucrose on antibacterial activity against *E. faecalis* in kefir samples prepared with almond milk

The results related to the gram-negative bacterium of *P. aeruginosa* indicate a significant difference between kefir treatments prepared with almond milk containing sucrose at a fermentation temperature of 25°C with samples with and without sucrose at a temperature of 37°C, which indicates the effect of temperature and the presence of sucrose on the antibacterial activity of kefir samples prepared with almond milk against this bacterium (Figure 7).

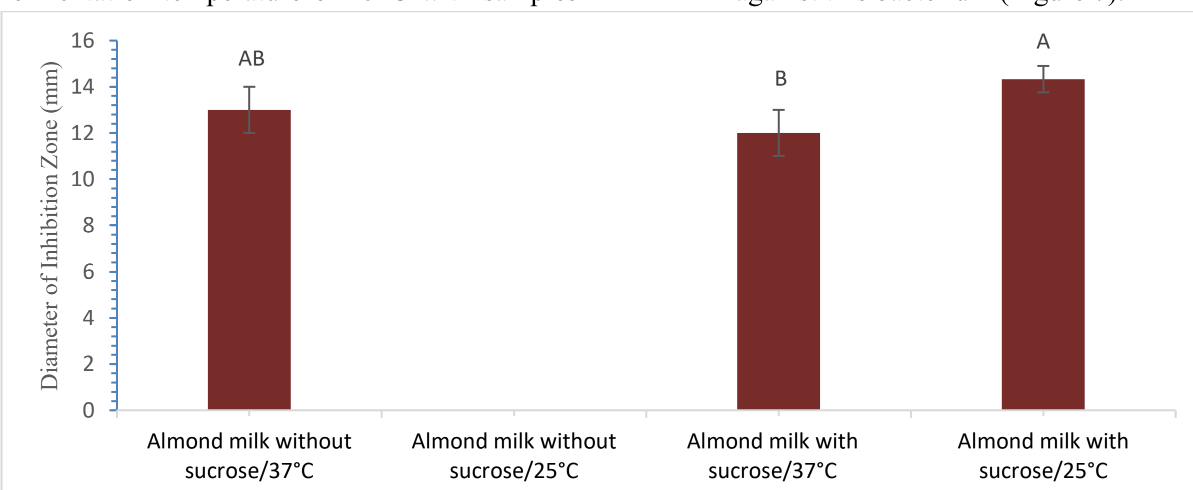

Figure 7. The effect of fermentation temperature and the presence of sucrose on antibacterial activity against *P. aeruginosa* in kefir samples prepared with almond milk

Also, the results related to the antibacterial activity of kefir samples prepared with almond milk against *S. dysenteriae* showed significant difference between the samples prepared with almond milk with and without sucrose at 37°C, so that the mean diameter of inhibition zone of this bacterium in the samples with and without sucrose were observed 14.66 and 13 mm, respectively (Table 2 and Figure 8), which indicates the positive effect of the presence of sucrose on antibacterial activity.

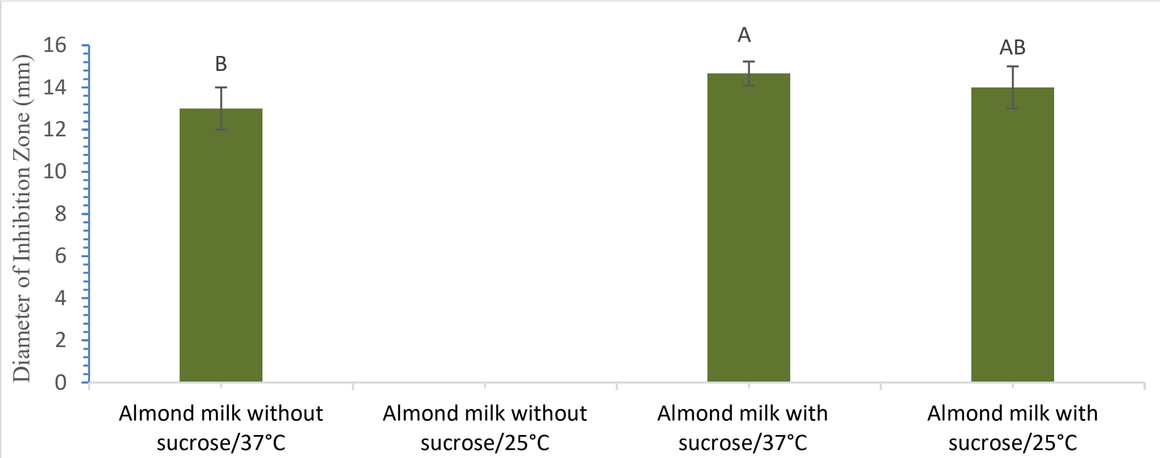

Figure 8. The effect of fermentation temperature and the presence of sucrose on antibacterial activity against *S. dysenteriae* in kefir samples prepared with almond milk

Also, the findings related to antibacterial activity in kefir samples prepared with almond milk with and without sucrose in *E. coli*, *S. typhimurium*, *S. aureus,* and *K. pneumoniae* bacteria indicate no significant difference between the treatments (P>0.05) (Table 2).

Of the notable points, was the lack of antibacterial activity of kefir samples prepared with almond milk without sucrose at a fermentation temperature of 25°C against *E. coli*, *S. typhimurium*, *S. dysenteriae*, *S. aureus,* and *P. aeruginosa* which introduces this type of kefir as a sample of kefir (Among the kefir samples prepared with almond milk) with the lowest antibacterial activity against the tested bacteria (Table 2).

In a study, Shafie et al. investigated the antibacterial activity of kefir samples produced from cow's milk, almond milk, and their mixture against three bacteria, *E. coli*, *S. aureus,* and *S. typhi*. The results of their study showed that the mixture of cow's milk and almonds compared to pure cow's milk and pure almond milk, in addition to better nutritional characteristics, showed more antibacterial activity against the tested pathogenic bacteria. As a result, almond milk may be a suitable substrate for kefir, but it was not an effective inhibitor for all bacteria, and the antibacterial activity of kefir samples prepared with pure almond milk was reported to be lower than samples prepared with cow's milk. In general, the use of a mixture of almond and cow's milk as an alternative substrate for the production of kefir-fermented beverages and potential use as a source of probiotics was suggested [22].

In addition, some studies have indicated that kefir produced from low-fat and plant-based milk, such as rice and soy milk, generally shows less antimicrobial activity than kefir produced using full-fat, animal-based milk such as cow's milk [43,44,45].

Among nuts, milk prepared from almond has been reported as a suitable matrix for the fermentation process. Because they supported the growth of lactic acid bacteria and the survival of these microorganisms in the conditions of storage in the refrigerator for long periods, and as a result, a suitable substrate for carrying probiotics was reported [19,20,56].

After fermentation of almond beverages with lactic acid bacteria, the number of bacteria reaches between 8.97 to 9.12 log CFU/mL after 24 hours [20]. High viability of probiotic bacteria was also recorded during storage in almond beverages fermented with *Lactobacillus reuteri* and *Streptococcus thermophilus*. In all 31 samples, viable bacterial cells exceeded 7 log CFU/mL after 28 days of storage in the refrigerator [19].

A study has shown that almond milk fermented by lactic acid bacteria has antioxidant properties due to its phenolic content, which may minimize oxidative stress-related diseases such as atherosclerosis, coronary heart disease, and cancer. The growth of lactic acid bacteria after 24 hours in this plant-based milk was evaluated as good and reached more than 10^9 CFU/ml [19].

Sensory characteristics of kefir samples produced from cow's milk and almond milk

In addition to the growth of microorganisms in plant milk tissue and their proper survival during storage and refrigeration, one of the most important challenges in the field of production of fermented products from plant substitutes is the desirability of sensory characteristics of products with acceptable texture and taste [57].

Few studies are comparing the sensory analysis of fermented plant milk in the literature. However, a study by Jesle et al. (2018) compared the sensory properties of a variety of plant-based milks, including almond, barley, rice, soy, hemp, and lentil. Consumers ranked appearance, aroma, taste, mouthfeel, and overall feel as the most important sensory attributes. The variety of plant-based milk can be effective along with the industrial process used to improve the organoleptic quality of plant-based milk [6].

The findings related to sensory evaluation of kefir prepared from cow's milk and almond milk kefir are shown in Figure 9. To comprehensively evaluate; The characteristics of color, general appearance, aroma, taste and mouthfeel, and total score were measured. The findings indicated that there was no significant difference between the treatments (P>0.05) however, despite this, kefir samples prepared with almond milk obtained a lower overall score compared to kefir samples prepared from cow's milk, and kefir samples prepared from cow's milk had a higher level of acceptability in most of the indicators.

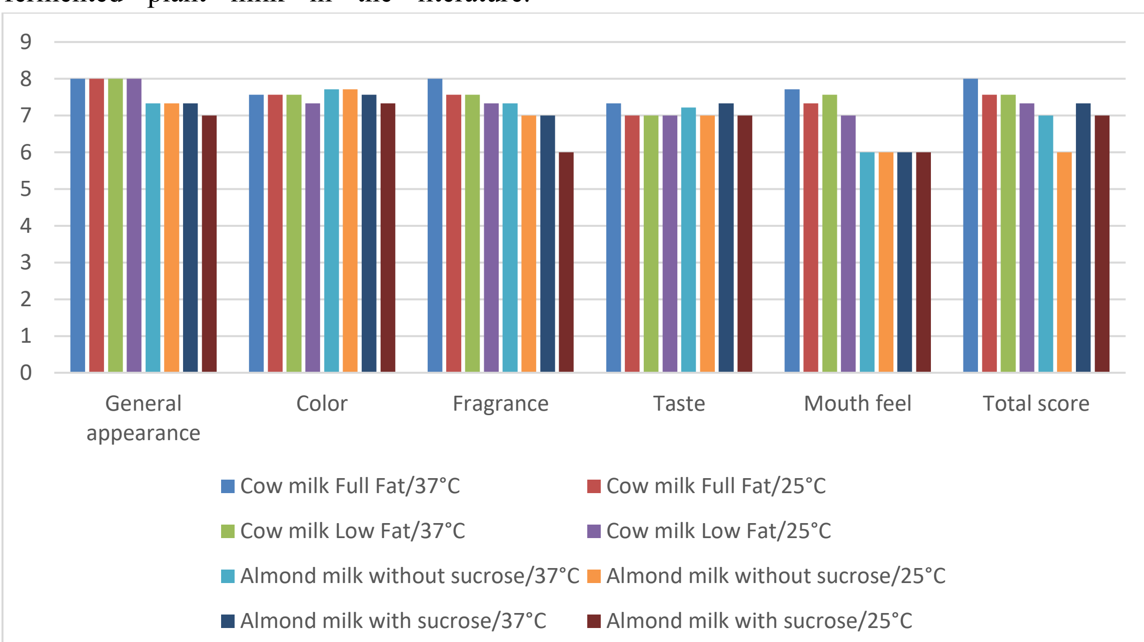

Figure 9. Sensory characteristics of kefir samples prepared with cow's milk and almond milk

In general, the lack of desirability of the sensory characteristics of plant-based milk compared to cow's milk has been reported in studies. Consistent with the results of the present study, Sajjadi et al. (2024) reported the acceptability of kefir produced with cow's milk compared to soy milk kefir in most indicators [8].

In the study of Silva et al. (2018), the sensory evaluation of kefir samples prepared with soy milk showed that samples with a higher percentage of soy milk (40%) were the least acceptable [56]. Products with a higher percentage of soy are often less accepted by consumers due to their specific taste known as "beany" [59].

The ideal formula in the probiotic herbal milk drink produced by Kempka et al. (2008) was soy milk (30.1%), milk serum (33.3%), and cow's milk (36.6%), which provided good sensory acceptance and higher viable cell counts [60].

In most fermented products, physical texture instability may occur due to the coagulation of proteins and the separation of components at the beginning or during the storage period [3].

During the fermentation process, organic acids (lactic and acetic) are the main products, and acetic acid is strongly associated with the unpleasant taste of vinegar. The presence of lactic acid is more desirable in fermented beverages because it provides the appropriate "mild sour" flavor [61].

One of the reasons for resistance to the acceptance of plant-based milk is the existence of unpleasant "bean" flavors and undesirable textures. These off-flavors can be largely attributed to aldehydes, primarily hexanal also 3-Z-hexenal, as well as alcohols such as n-hexanol, n-pentanol, and n-heptanol, ketones such as ethyl vinyl ketone and furans [62]. It has been reported that the fermentation of plant-based milk by lactic acid bacteria can remove hexanal and reduce the concentration of other volatile organic compounds contributing to soy milk [63].

There are also anti-nutritional factors (ANFs) in plant-based milk that negatively affect the sensory characteristics as well as the bioavailability of macro and micronutrients and protein quality [64]. ANFs are mainly oligosaccharides from the raffinose family, protease inhibitors, saponins, and phytates. Off-flavors of "beany" originate from lipoxygenase activity [2]. However, the fermentation of plant-based milks improves the sensory characteristics, because it reduces the Off-flavors of "beany" of plant materials and provides favorable flavors [65]. Fermentation with lactic acid bacteria leads to the improvement of the nutritional profile and sensory characteristics due to the release of amino acids and bioactive compounds, the reduction of ANF activity, and the increase of protein digestibility [64,66].

4. Conclusion

Fermentation temperature significantly affected the antibacterial activity of kefir samples prepared with cow's milk, and kefir samples prepared at 37°C showed more antibacterial activity compared to 25°C. However, the type of cow's milk used in the preparation of kefir samples in terms of fat content (low fat and high fat) did not show a significant effect on the antibacterial activity against the tested bacteria except *S. aureus*, *S. typhimurium,* and *B. cereus*. Despite the lack of effect of fermentation temperature on the antibacterial activity of kefir samples prepared with almond milk against the tested bacteria, the presence of sucrose showed a significant effect on the antibacterial activity against *E. faecalis*, *S. dysenteriae*, *B. cereus,* and *P. aeruginosa* which indicates the positive effect of the presence of sucrose on antibacterial activity. Achieving desirable sensory characteristics is one of the main challenges in the production of fermented plant-based milk. It is recommended to carry out the fermentation process in different conditions such as temperature and fermentation time and add different compounds such as flavorings, emulsifiers, different sugars, vitamins, colors, and minerals to almond milk to achieve samples with better biological and sensory characteristics.

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مقاله علمی_پژوهشی

فعالیت ضدباکتریایی شیر گاو و شیر بادام تخمیرشده بوسیله دانه‌های کفیر

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چکیده

نوشیدنی کفیر یک پروبیوتیک پیچیده از یک مجتمع همزیستی میکروبی است که عمدتاً از تخمیر شیر گاو توسط دانه‌های کفیر به دست می‌آید. برخی از افراد شیر گاو مصرف نمی کنند. شیر گیاهی می تواند جایگزین شیر حیوانی باشد. هدف از این مطالعه بررسی فعالیت ضد باکتریایی نمونه‌های کفیر تهیه شده با شیر گاو و شیر بادام می باشد. دانه‌های کفیر فعال‌شده به شیر گاو (پرچرب و کم چرب) و شیر بادام (با و بدون ساکارز) اضافه شد و فرآیند تخمیر در دمای ۲۵ و ۳۷ درجه سانتیگراد انجام شد. دانه‌ها از عصاره کفیر جدا شدند و فعالیت ضدباکتریایی عصاره کفیر در برابر ۸ باکتری با روش چاهک مورد بررسی قرار گرفت. دمای تخمیر بر فعالیت ضدباکتریایی نمونه‌های کفیر تهیه‌شده با شیر گاو در تمام باکتری‌های مورد آزمایش به جز انتروکوکوس فکالیس تأثیر معنی‌داری داشت (P<۰/۰۵) و نمونه‌های کفیر تهیه‌شده در دمای ۳۷ درجه سانتیگراد نسبت به ۲۵ درجه سانتیگراد فعالیت ضد باکتریایی بیشتری نشان دادند. نوع شیر گاو (پرچرب و کم چرب) به استثنای استافیلوکوکوس اورئوس، سالمونلا تیفی‌موریوم و باسیلوس سرئوس بر فعالیت ضدباکتریایی در برابر باکتری‌های مورد آزمایش اثر معنی‌داری نشان نداد. انتروکوکوس فکالیس و سالمونلا تیفی موریوم به ترتیب حساس‌ترین و مقاوم‌ترین باکتری‌ها به نمونه‌های کفیر تهیه شده با شیر گاو بودند. علیرغم عدم تأثیر دمای تخمیر بر فعالیت ضدباکتریایی نمونه‌های کفیر تهیه‌شده با شیر بادام، وجود ساکارز تأثیر قابل‌توجهی بر فعالیت ضدباکتریایی علیه انتروکوکوس فکالیس، شیگلا دیسانتری، باسیلوس سرئوس و سودوموناس آئروژینوزا نشان داد ($P < 0.05$) ، که نشان‌دهنده تأثیر مثبت حضور ساکارز بر فعالیت ضدباکتریایی است. به طور کلی تخمیر در دمای ۳۷ درجه سانتی‌گراد برای دستیابی به بالاترین فعالیت ضدباکتریایی نمونه‌های کفیر تهیه شده با شیر گاو و همچنین افزودن ساکارز به شیر بادام برای دستیابی به بالاترین فعالیت ضدباکتریایی در برابر انتروکوکوس فکالیس، شیگلا دیسانتری، باسیلوس سرئوس و سودوموناس آئروژینوزا توصیه می‌شود.