Chemical and Microbiological Properties of Kefir Produced by Kefir Grains in Raw and Pasteurized Cow's Milk

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Abstract

The traditional production of kefir uses raw milk as a fermentation substrate which is not acceptable due to food safety issues. However, kefir grains were reported to have antimicrobial properties. Hence, this study determined if kefir grains inhibited certain spoilage and pathogenic microorganisms by comparing the chemical and microbiological properties of the initial substrates: raw milk (RM) and pasteurized milk (PM) to the final products: raw milk kefir (RMK) and pasteurized milk kefir (PMK). Both final products had a significant decrease in fat, moisture, and pH, and a significant increase in protein, total solids, and titratable acidity than RM and PM, respectively. Total solids, titratable acidity, pH, total alcohol, and ethanol were significantly higher in PMK than RMK. RMK had a significantly lower coliform count than RM while both RMK and PMK had significantly higher lactic acid bacteria, yeast and mold count than RM and PM, respectively. Kefir grains' microbial inhibiting activity was examined if plates were positive or negative against spoilage and pathogenic microorganisms. Escherichia coli-positive plates in Levine's eosin methylene blue agar decreased in RMK. Salmonella spp.-positive plates in bismuth sulphite agar (BSA), Hektoen enteric agar, and xylose lysine deoxycholate agar decreased in RMK and PMK except for RMK in BSA. Staphylococcus aureus-positive plates in Baird-Parker agar decreased in RMK. RMK and PMK attained the kefir's standard values for the chemical composition, lactic acid bacteria, yeast and mold count. Nonetheless, the mere presence of spoilage and pathogenic microorganisms in the final products made it unsafe for consumption.

Keywords*:* kefir, kefir grains, fermented milk, cow's milk, food safety

1. Introduction

Food fermentation has been one of the significant and well-known food preservation techniques. It is known that fermented foods have added

nutritional value and have a longer shelf life compared with unfermented foods (Farnworth, 2005a). Kefir is a viscous fermented dairy product that contains small quantities of alcohol and carbon dioxide produced by the action of yeasts embedded in kefir grains when inoculated in milk (Farnworth, 2005b). Moreover, lactic, acetic, butyric, hippuric, propionic and pyruvic acid, acetaldehyde, acetoin, diacetyl, and other by-products were generated during the fermentation process (Guzel-Seydim *et al*., 2011; Arslan, 2015). It is considered as a functional food due to its characteristics as a probiotic. Kefir was found to have antimicrobial, antiviral, antifungal, anticarcinogenic, and antimutagenic properties as well as β-galactosidase activity (Farnworth, 2005b; Arslan, 2015). Aside from the type of milk and kefir culture (kefir grains or starter cultures) used, the inoculation rate and incubation temperature and period affect the final chemical, microbiological, and sensory properties of kefir (Kok-Tas *et al*., 2012).

Kefir grains resemble cauliflower florets that are 3-35 mm in diameter. Lactic acid bacteria (lactobacilli, lactococci, leuconostocs, streptococci, bacilli, enterococci, and micrococci), yeasts (*Candida* sp., *Kluyveromyces* sp., *Saccharomyces* sp., *Torulaspora* sp., *Brettanomyces* sp., *Issatchenkia* sp., and *Zygosaccharomyce*s sp.), acetic acid bacteria (*Acetobacter* sp.), and even *Escherichia coli* were identified from kefir grains. These microorganisms are contained in a matrix of exopolysaccharides and proteins called kefiran which is a water-soluble branched glucogalactan (1:1 ratio of D-glucose and D-galactose) (Angulo *et al*., 1993; Farnworth, 2005b; Magalhães *et al*., 2011; Kok-Tas *et al*., 2012; Arslan, 2015).

Direct inoculation of kefir grains in raw milk commonly of sheep or goat origin is considered as a traditional process of kefir production (Otles and Cagindi, 2003; Bourrie *et al*., 2016). The microflora of raw milk usually contains spoilage and pathogenic microorganisms that when ingested may negatively affect the health of the consumer. This led to the prohibition by food safety authorities in using raw milk as a substrate for fermentation. However, it was found that microorganisms in kefir grains compete with naturally occurring microorganisms in raw milk resulting in lower counts of pathogenic microorganisms in kefir (Garrote *et al*., 2000). Hence, the objective of the present study was to determine if kefir grains under local conditions inhibited certain spoilage and pathogenic microorganisms by comparing the chemical and microbiological properties of the initial substrates: raw milk (RM) and pasteurized milk (PM) to the final products: raw milk kefir (RMK) and pasteurized milk kefir (PMK). In addition, the

study was conducted to evaluate the possible risk hazards of using raw milk in kefir production.

2. Methodology

2.1 Experimental Design

Two liters of kefir were each produced by inoculating kefir grains (2% w/v) in raw (RMK) and pasteurized (PMK) cow's milk and left to incubate in a partially closed sterile container at ≈20-25 °C for 20 h. Final kefir products were stored at 4 °C for chemical and microbiological analyses. In addition, 1 L each of raw (RM) and pasteurized (PM) cow's milk were tested for both analyses and served as the bases of comparison. The analyses used independent samples T-*t*est with three replications. Each replication had sample duplicates in every analysis.

2.2 Milk Collection

Fresh raw cow's milk was provided by the Dairy Training and Research Institute (DTRI), College of Agriculture and Food Science (CAFS), University of the Philippines Los Baños (UPLB), College, Laguna, Philippines every replication during the experiment. The time of lactation, storage temperature and duration were mid-lactation, 4 °C, and <4 h, respectively.

2.3 Production of Kefir from Kefir Grains

The methods of Kok-Tas *et al*. (2012) and Otles and Cagindi (2003) were carried out with modifications on the temperature and duration of heat treatment and cooling of cow's milk. In RMK, 2 L of raw cow's milk were warmed up to 30 °C to facilitate the growth of the microorganisms present in kefir grains when inoculated. In PMK, 2 L of raw cow's milk were pasteurized at 63 °C for 30 min then cooled down to 30 °C. After heat treatment and cooling, kefir grains were inoculated in raw and pasteurized cow's milk and left to incubate in a partially closed sterile container. The kefir grains were obtained from the University of the Philippines Diliman (UPD), Diliman, Quezon City. The inoculation rate, incubation temperature, and incubation period of kefir grains in cow's milk were 2% (w/v), room temperature (≈20-25 °C), and 20 h, respectively. These values were determined by a preliminary experiment in which the final kefir products attained the standard pH (≈4.60)

and titratable acidity (≈0.60%) (Food and Agriculture Organization [FAO], 2003). No standard alcohol percentage was indicated. After incubation, kefir grains were strained from kefir and transferred into milk then stored at 4 °C for future production. The final kefir products were stored at 4 °C for chemical and microbiological analyses.

2.4 Chemical Analysis

The initial substrates (RM and PM) and the final products (RMK and PMK) were tested for fat, protein, moisture and total solids, and titratable acidity (% lactic acid) using Gerber, Kjeldahl, oven, and titration methods, respectively (Association of Analytical Chemists [AOAC], 2006). All samples were tested for pH using a pH meter (Sartorius Basic pH Meter PB-11, Fisher Scientific Company LLC, USA). Sample preparation prior to measuring total alcohol and ethanol contents was done by collecting distillate from a mixture of each kefir sample and distilled water. Total alcohol content was determined using an alcoholmeter. Ethanol content was obtained using a gas chromatograph (Shimadzu GC-14B, Shimadzu Corporation, Japan) (AOAC, 2012).

2.5 Microbial Analysis

Serial dilutions from each sample were prepared in sterile phosphate buffer saline diluents (pH 7.2) (HiMedia®, Mumbai, India) for coliform, lactic acid bacteria, and yeast and mold counts. Coliform counts were enumerated on violet red bile agar (VRBA) (HiMedia®, Mumbai, India) using the pour plate method; plates were incubated for 24 h at 32 °C. To confirm that the colonies were coliforms, at least 10 representative colonies were each picked and transferred to a tube of brilliant green lactose bile broth (BGLBB) (HiMedia®, Mumbai, India). The BGLBB tubes were incubated at 35 °C for 24 and 48 h to examine gas production (Feng *et al*., 2018). For lactic acid bacteria counts, Lactobacillus de Man Rogosa Sharpe agar (MRSA) (HiMedia®, Mumbai, India) was used using the pour plate method; plates were incubated for 48 h at 37 °C (Frank and Yousef, 2004). Yeast and mold counts were enumerated on potato dextrose agar (PDA) (HiMedia®, Mumbai, India) by the spread plate method; plates were incubated for 5 d at 25 °C (Frank and Yousef, 2004).

The presumptive test for *E. coli* was performed by mixing each sample and Butterfield's phosphate-buffered water (pH 7.2) (HiMedia®, Mumbai, India) in a sterile high-speed blender jar. Serial dilutions from each sample were prepared in sterile Butterfield's phosphate-buffered diluents. Aliquots from each serial dilutions were inoculated in three lactose broth (LB) (HiMedia®,

Mumbai, India) tubes for a three-tube most probable number (MPN) analysis. The LB tubes were incubated at 35 °C for 24 and 48 h to examine gas production. From each gassing LB tube from the presumptive test, a loopful of each suspension was transferred to a tube of *E. coli* broth (ECB) (HiMedia®, Mumbai, India). The ECB tubes were incubated at 44.5 °C for 24 and 48 h to examine gas production. To perform the completed test for *E. coli*, each gassing ECB tubes were gently agitated before a loopful of each suspension were streaked onto Levine's eosin methylene blue agar (L-EMBA) (HiMedia®, Mumbai, India); plates were incubated at 35 °C for 24 h (Feng *et al*., 2018).

Salmonella spp. was determined by using selective enrichment of each sample in tetrathionate broth (TTB) (HiMedia®, Mumbai, India) and incubated for 24 h at 35 °C. After incubation, each enriched sample was streaked onto bismuth sulphite agar (BSA) (HiMedia®, Mumbai, India), Hektoen enteric agar (HEA) (HiMedia®, Mumbai, India), and xylose lysine deoxycholate agar (XLDA) (HiMedia®, Mumbai, India) and incubated for 24 h at 35 °C (Henning *et al*., 2004). For the presence of *Staphylococcus aureus*, each sample was spread using a sterile bent-glass rod on Baird-Parker agar (BPA) (HiMedia®, Mumbai, India) supplemented with concentrated egg yolk emulsion (HiMedia®, Mumbai, India) and egg yolk tellurite emulsion (HiMedia®, Mumbai, India); plates were incubated for 48 h at 35 °C (Henning *et al*., 2004).

2.6 Statistical Analysis

Chemical and microbiological data (coliform, lactic acid bacteria, and yeast and mold counts) of all samples were analyzed using independent samples T-*t*est carried out using the SAS® University Edition software version SAS Studio 3.8 and SAS 9.4M6 (SAS Institute Inc., USA).

3. Results and Discussion

3.1 Chemical Analysis

The chemical compositions of RM and RMK are presented in Table 1. Fat, moisture, and pH were significantly lower in RMK compared with RM while protein, total solids, and titratable acidity were significantly higher. Milk fat can be metabolized by some naturally occurring microorganisms in RM and microorganisms in kefir grains for their growth. Specifically, lactic acid

bacteria (LAB) in kefir grains can hydrolyze fat to increase free fatty acid (FFA) production. FFA can be converted to acetyl CoA through β-oxidation then acetyl CoA can be converted to acetone via ketogenesis. In anaerobic conditions, acetone can be converted to lactic acid, pyruvate, and ethanol (Vieira *et al*., 2015).

Table 1. Chemical compositions of RM and RMK

Components (%[1])	Treatments[2]		
	RM	RMK	*p*-value
Fat	4.05 ± 0.32[a]	3.63 ± 0.22[b]	0.0248
Protein	3.01 ± 0.10[b]	3.18 ± 0.02[a]	0.0489
Moisture	89.26 ± 0.78[a]	87.87 ± 1.09[b]	0.0298
Total solids	10.74 ± 0.78[b]	12.13 ± 1.09[a]	0.0298
Titratable acidity (% lactic acid)	0.1495 ± 0.02[b]	0.7857 ± 0.19[a]	<0.0001
pH	6.42 ± 0.16[a]	4.20 ± 0.44[b]	<0.0001
Total alcohol	-	1.33 ± 0.52	-
Ethanol	-	0.0200 ± 0.01	-

[1]Except pH values
[2(a,b)] means within rows having different superscripts are significantly different ($p < 0.05$)

Exopolysaccharides (EPS) such as kefiran are cell-surface polysaccharides and proteins produced by LAB that can be easily detached from kefir grains during fermentation. EPS together with the microbial biomass from kefir grains contributed to the increase in proteins and total solids as well as the decrease in moisture of RMK. The accumulation of organic acids specifically lactic acid during fermentation increased the titratable acidity and decreased the pH of kefir. Lactose in milk is converted to lactic acid by mostly LAB and lactose-fermenting yeasts present in the initial microflora of RM and microorganisms in kefir grains (Farnworth, 2005b).

The same trend from the results in Table 1 is observed in Table 2. Fat, moisture, and pH were significantly lower in PMK than in PM while protein, total solids, and titratable acidity were significantly higher. Milk fat globule membranes (MFGM) are composed of lipids and proteins that function as the protective covering of milk fat globules against coalescence and lipolytic enzymes. Pasteurization can induce structural changes in MFGM that may lead to significant losses of phospholipids and triacylglycerols (El-Loly, 2011). In addition, microorganisms in kefir grains can produce lipases which can decrease the content of milk fat (Magalhães *et al*., 2011). Kefir has anticarcinogenic and antimutagenic properties due to the high Δ^9-desaturase activity of kefir grains in milk resulting in higher monounsaturated fatty acids and lower saturated fatty acids (Vieira *et al*., 2015).

Table 2. Chemical compositions of PM and PMK

Components (%[1])	Treatments[2]		P-value
	PM	PMK	
Fat	3.67 ± 0.23^{a}	3.33 ± 0.08^{b}	0.0157
Protein	2.66 ± 0.05^{b}	2.86 ± 0.06^{a}	0.0476
Moisture	87.90 ± 0.84^{a}	86.27 ± 0.61^{b}	0.0031
Total solids	12.10 ± 0.84^{b}	13.73 ± 0.61^{a}	0.0031
Titratable acidity (% lactic acid)	0.0740 ± 0.01^{b}	0.8964 ± 0.13^{a}	<0.0001
pH	7.21 ± 0.07^{a}	3.96 ± 0.22^{b}	<0.0001
Total alcohol	-	2.33 ± 0.52	-
Ethanol	-	0.0433 ± 0.05	-

[1]Except pH values
[2(a,b)] means within rows having different superscripts are significantly different ($p < 0.05$)

The detachment of EPS from the kefir grains and the increase in microbial biomass may have caused the increase in proteins and total solids and the decrease in moisture of PMK (Vlahapoulou *et al*., 2001). In addition, loss of moisture can be attributed to pasteurization. The significant changes in titratable acidity and pH in PMK can be both due to pasteurization and fermentation. Pasteurization increases the precipitation of colloidal calcium phosphate in milk leading to a slight increase in titratable acidity and a decrease in pH (Fox *et al*., 2015). It can also damage or destroy naturally occurring microorganisms in raw milk that convert lactose to lactic acid. This facilitated a faster lactic acid production by kefir grains in milk due to the lack of competition in metabolizing substrates. Another reason for the increase in titratable acidity and the decrease in pH of PMK were the production of certain organic acids, ethanol, carbon dioxide, and other volatile compounds by the microorganisms in kefir grains (Magalhães *et al*., 2011).

The chemical compositions of RMK and PMK are presented in Table 3. All of the chemical properties were significantly different between treatments. Milk fat in RM and PM were both hydrolyzed by lipases from the microorganisms in kefir grains resulting in significantly lower fat in RMK and PMK, respectively. However, the significant reduction of fat in PMK can be explained by the destabilization of MFGM and fat globules in milk during pasteurization. Structural changes in MFGM can result in coalescence and oxidation (El-Loly, 2011). Both RMK and PMK had an increase in protein compared with their initial substrates due to the increase in EPS and microbial biomass produced by the microorganisms in kefir grains (Magalhães *et al*., 2011). However, protein was significantly lower in PMK than in RMK which can be a result of the denaturation of casein, whey, and MFGM proteins during heat treatment (Fox *et al*., 2015). A decrease in moisture and an increase in

total solids were observed in RMK and PMK compared with RM and PM, respectively. These can be attributed to the detachment of EPS from the kefir grains and the increase in microbial biomass. Magalhães *et al*. (2011) reported that *Lactobacillus kefiri* fixed on the grain surface might be easily freed from kefir grains into the milk which resulted in increased cell counts. However, PMK had significantly lower moisture and higher total solids than RMK due to pasteurization. Pasteurization can injure or kill naturally occurring microorganisms in raw milk resulting in faster production of microbial biomass by kefir grains in milk.

Table 3. Chemical compositions of RMK and PMK

Components (%[1])	Treatments[2]		
	RMK	PMK	P-value
Fat	3.63 ± 0.22^{a}	3.33 ± 0.08^{b}	0.0098
Protein	3.18 ± 0.02^{a}	2.86 ± 0.06^{b}	<0.0001
Moisture	87.87 ± 1.09^{a}	86.27 ± 0.61^{b}	0.0104
Total solids	12.13 ± 1.09^{b}	13.73 ± 0.61^{a}	0.0104
Titratable acidity (% lactic acid)	0.7857 ± 0.19^{b}	0.8964 ± 0.13^{a}	0.0426
pH	4.20 ± 0.44^{a}	3.96 ± 0.22^{b}	0.0360
Total alcohol	1.33 ± 0.52^{b}	2.33 ± 0.52^{a}	0.0073
Ethanol	0.0200 ± 0.01^{b}	0.0433 ± 0.05^{a}	0.0367

[1]Except pH values
[2(a,b)] means within rows having different superscripts are significantly different ($p < 0.05$)

Titratable acidity and pH were significantly different from the initial substrates to the final products. These results were due to the conversion of lactose to lactic acid during fermentation. However, PMK showed higher titratable acidity and lower pH than RMK due to pasteurization. The objective of pasteurization was to impair and eliminate spoilage and pathogenic microorganisms in milk. In line with this, pasteurization facilitated faster microbial activity of the inoculum due to the lack of competition between naturally occurring microorganisms in raw milk and microorganisms in kefir grains. Yeasts aside from bacteria in kefir grains can also alter the pH. Yeasts were reported to play a significant role in the production of fermented dairy products by producing alcohol and carbon dioxide, altering the pH, and providing essential growth nutrients such as amino acids and vitamins (Viljoen, 2001). Total alcohol and ethanol contents were significantly higher in PMK. The same principle of the effect of pasteurization on limiting the competition for substrates can be applied to explain the higher total alcohol and ethanol contents in PMK compared with RMK.

3.2 Microbial Analysis

The microbiological counts of RM and RMK are presented in Table 4. The coliform count was significantly lower in RMK compared with RM while lactic acid bacteria and yeast and mold counts were significantly higher. Both treatments exceeded the acceptable coliform count of 10-110 CFU/ml for fermented milk (Food and Drug Administration [FDA], 2013). This can be expected in RM due to the probable presence of spoilage and pathogenic microorganisms when milk is being collected. However, RMK had a significantly lower coliform count than RM. This can be attributed to the antimicrobial activity of kefir grains as well as kefir against *E. coli* (Leite *et al*., 2015; Prado *et al*., 2015; Rosa *et al*., 2017). The lactic acid bacteria and yeast and mold counts of RMK were within the standard for kefir which are a minimum of 107 and 104 CFU/ml, respectively (FAO, 2003). These results can be explained by the inoculation of kefir grains in RM. Kefir grains are comprised of a symbiotic community of LAB, yeasts, acetic acid bacteria, and often filamentous molds (Farnworth, 2005b).

Table 4. Microbiological counts of RM and RMK

Counts (Log_{10} CFU/ml)	Treatments[1]		
	RM	RMK	P-value
Coliform count	3.92^{a}	3.55^{b}	0.0391
Lactic acid bacteria count	4.00^{b}	7.53^{a}	0.0045
Yeast and mold count	3.43^{b}	4.76^{a}	0.0214

[1(a,b)] means within rows having different superscripts are significantly different ($p < 0.05$)

The same trend from the results in Table 4 is observed in Table 5. Coliform, lactic acid bacteria, and yeast and mold were not present in PM. The coliform counts of both PM and PMK were acceptable for fermented milk (FDA, 2013). However, PMK had a significantly higher coliform count than PM due to the presence of *E. coli*. *E. coli*, a member of the family *Enterobacteriaceae*, is a Gram-negative facultative anaerobe bacterium that was found to be embedded in kefir grains (Angulo *et al*., 1993; Dobson *et al*., 2011). However, most strains of *E. coli* are harmless and cannot induce foodborne illnesses (Centers for Disease Control and Prevention [CDC], 2018). The same with RMK, the lactic acid bacteria and yeast and mold counts were within the standard for kefir (FAO, 2003). These were due to the inclusion of kefir grains in PM which contains various LAB and yeasts (Farnworth, 2005b).

Table 5. Microbiological counts of PM and PMK

Counts (Log_{10} CFU/ml)	Treatments[1]		
	PM	PMK	P-value
Coliform count	0.00^{b}	0.60^{a}	0.0027
Lactic acid bacteria count	0.00^{b}	8.48^{a}	<0.0001
Yeast and mold count	0.00^{b}	5.49^{a}	<0.0001

[1(a,b)] means within rows having different superscripts are significantly different ($p < 0.05$)

Table 6 presents the microbiological counts of RMK and PMK. The coliform count was significantly lower in PMK compared with RMK while lactic acid bacteria and yeast and mold counts were significantly higher. PMK had a significantly lower coliform count than RMK. This can be explained by pasteurization which damages and eliminates naturally occurring microorganisms present in raw milk. The same principle of the effect of pasteurization on limiting the competition for substrates can be applied to explain the higher lactic acid bacteria and yeast and mold counts in PMK than the RMK.

Table 6. Microbiological counts of RMK and PMK

Counts (Log_{10} CFU/ml)	Treatments[1]		
	RMK	PMK	P-value
Coliform count	3.55^{a}	0.60^{b}	0.0180
Lactic acid bacteria count	7.53^{b}	8.48^{a}	0.0365
Yeast and mold count	4.76^{b}	5.49^{a}	0.0432

[1(a,b)] means within rows having different superscripts are significantly different ($p < 0.05$)

Yeasts primarily *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* are mainly responsible for the conversion of carbohydrates such as lactose to alcohol and carbon dioxide. Moreover, some *Lactobacillus* strains have alcohol dehydrogenase, which is an enzyme that converts acetaldehyde to ethanol (Magalhães *et al*., 2011). The result on the yeast and mold counts of RMK and PMK can be correlated to the significantly higher total alcohol and ethanol contents in PMK as shown in Table 3.

Figure 1 showed the percentages of plates positive for *E. coli*, *Salmonella* spp., and *S. aureus* in different selective media. The final products (RMK and PMK) had lower percentages of positive plates than the initial substrates (RM and PM), respectively. The results and discussion on the coliform counts in Tables 1 to 3 can be correlated to the percentages of *E. coli*-positive plates in L-EMBA. Kefir grains were reported to have antimicrobial activity against *E. coli* to a certain extent (Garrote *et al*., 2000; Kim *et al*., 2016; Prado *et al*., 2015; Rosa *et al*., 2017). Leite *et al*. (2015) reported that *Lactobacillus lactis*

and *Lactobacillus paracasei* isolates from kefir were capable of producing bacteriocin-like substances that were inhibitory to pathogenic microorganisms such as *E. coli*, *Salmonella enterica*, *S. aureus*, and *Listeria monocytogenes*.

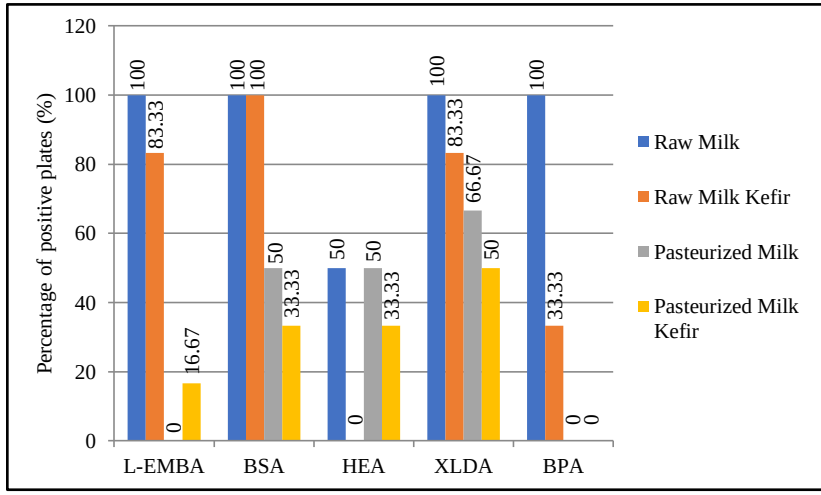

Figure 1. Percentages of *E. coli*-positive plates L-EMBA; *Salmonella* spp.-positive plates using BSA, HEA, and XLDA; and *S. aureus*-positive plates using BPA

However, Gulmez and Guven (2003) studied the antimicrobial effect of kefir in which foodborne bacterial pathogens such as *E. coli* O157:H7 were added at the start of kefir fermentation. They found out that kefir failed to produce antimicrobial compounds that could inhibit the growth of pathogens specifically *E. coli*. They postulated that this result was due to the slow acid development during kefir fermentation. In addition, pathogen suppression was found to be more effective with the combination of kefir and yogurt. *E. coli* should be absent in fermented milk products based on microbiological standards (FDA, 2013). In the case of some kefir grains which were found to have embedded *E. coli* on the grain surface, a microbial indicator should be set prior to kefir production. Aside from using kefir grains to produce kefir, bacteria and yeasts exhibiting beneficial and desirable properties can also be isolated to produce kefir starter cultures.

The presence of *Salmonella* spp. in the initial substrates (RM and PM) and the final products (RMK and PMK) was tested using three different selective media: BSA, HEA, and XLDA. Foodborne pathogens such as *Salmonella* spp. are naturally present in raw milk. However, a decrease in the percentages of

positive plates for *Salmonella* spp. was observed from RM and PM to RMK and PMK excluding RMK in BSA. These observations concur with the findings of Santos *et al*. (2003) wherein it was observed that some strains of *Lactobacillus* spp. isolated from kefir exhibited antimicrobial activity against certain strains of *Salmonella* spp. such as *Salmonella typhimurium*, *Salmonella enteritidis*, and *Salmonella flexneri*. Arslan (2015) also reported that certain strains of *L. kefiri* and their S-layer proteins inhibited the adhesion and/or invasion of *Salmonella enterica serovar* Enteritidis.

However, the presence of *Salmonella* spp. was evident in PM and PMK in all selective media. *Salmonella* spp. should be negative in 25 mL of fermented milk based on microbiological standards (FDA, 2013). Post-pasteurization contamination may be considered as one of the causes for these results. Nonetheless, a decrease in *Salmonella* spp.-positive plates in PMK from PM was observed in all selective media. Kefir grains produce a wide range of antimicrobial metabolites such as organic acids, ethanol, hydrogen peroxides, diacetyl, peptide, and possibly bacteriocins. These metabolites were proven to exhibit bacteriostatic and bactericidal activities resulting in a reduction of spoilage bacteria and foodborne pathogens. It can be suggested that these metabolites interact with each other to enhance their antimicrobial properties (Garrote *et al*., 2000; Farnworth, 2005b; Kim *et al*., 2016).

S. aureus is a Gram-positive bacterium commonly found in skin surfaces and mucous membranes. It is commonly found in raw milk from mastitic cows which can cause food poisoning through exotoxin production in food (Jayarao *et al*., 2004). In addition, this bacterium adequately survives in processing equipment of milk processing plants and acts as a source of contamination or recontamination (Cullor, 1997). A decrease in *S. aureus*-positive plates in RMK from RM was observed. The antimicrobial activity of kefir against Gram-negative and Gram-positive bacteria was studied by Cevikbas *et al*. (1994) and Garrote *et al*. (2000). Gram-positive bacteria were inhibited to a greater extent than Gram-negative bacteria. They reported that the production of organic acids such as lactic acid and acetic acid during the early stage of kefir fermentation has bacteriostatic properties against Gram-positive bacteria. Ulusoy *et al*. (2007) stated that the best antimicrobial activity of kefir was against *S. aureus*. Consistent results were obtained throughout the 7 d storage of kefir at 4 °C. On the other hand, there were no *S. aureus*-positive plates in PM and PMK. This result would be expected since *S. aureus* could not withstand proper pasteurization (Cullor, 1997).

4. Conclusion and Recommendation

Fat, moisture, and pH were significantly lower in the final products (RMK and PMK) compared with the initial substrates (RM and PM) while protein, total solids, and titratable acidity were significantly higher. PMK had significantly higher total solids, titratable acidity, pH, total alcohol, and ethanol compared with RMK. Both RMK and PMK had significantly lower coliform count and significantly higher lactic acid bacteria count and yeast and mold count than RM and PM, respectively. These results can be attributed to kefir fermentation and pasteurization. Plates positive for *E. coli* in L-EMBA decreased in RMK. *Salmonella* spp.-positive plates in BSA, HEA, and XLDA decreased from the initial substrates to the final products except for RMK in BSA. A decrease in positive plates for *S. aureus* in BPA was observed in RMK. RMK and PMK were able to attain the standard values for the chemical composition, lactic acid bacteria, yeast and mold count of kefir. Spoilage and pathogenic microorganisms decreased in the final products due to the inhibitory effect of the microorganisms in kefir grains. Nonetheless, the mere presence of these harmful microorganisms in the final products made it unsafe for consumption.

The authors would like to recommend the isolation and identification of specific microorganisms that exhibit antimicrobial activity against pathogenic microorganisms. Procedures such as agar well diffusion and deferred, or spot-on-lawn assays may be performed. Aside from using kefir grains as inoculum, bacterial and yeast isolates can be combined to produce kefir starter cultures.

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6. References

Angulo, L., Lopez, E., & Lema, C. (1993). Microflora present in kefir grains of the Galician region (north-west of Spain). Journal of Dairy Research, 60(2), 263-267. https://doi.org/10.1017/S002202990002759X

Arslan, S. (2015). A review: Chemical, microbiological and nutritional characteristics of kefir. CyTA – Journal of Food, 13(3), 340-345. http://dx.doi.org/10.1080/19476337.2014.981588

Association of Analytical Chemists (AOAC). (2006). Official methods of analysis of AOAC International (18th Ed.). USA: AOAC International.

Association of Analytical Chemists (AOAC). (2012). Official methods of analysis of AOAC International (19th Ed.). USA: AOAC International.

Bourrie, B.C.T., Willing, B.P., & Cotter, P.D. (2016). The microbiota and health promoting characteristics of the fermented beverage kefir. Frontiers in Microbiology, 7, 647. https://doi.org/10.3389/fmicb.2016.00647

Centers for Disease Control and Prevention (CDC). (2018). *E. coli* (*Escherichia coli*). Retrieved from https://www.cdc.gov/ecoli/index.html

Cevikbas, A., Yemni, E., Ezzedenn, F.W., Yardimici, T., Cevikbas, U., & Stohs, S.J. (1994). Antitumoural, antibacterial and antifungal activities of kefir and kefir grain. Phytotheraphy Research, 8(2), 78-82. https://doi.org/10.1002/ptr.2650080205

Cullor, J.S. (1997). Risk and prevention of contamination of dairy products. Revue Scientifique et Technique Office International des Epizooties, 16(2), 472-481. https://doi.org/10.20506/rst.16.2.1035

Dobson, A., O'Sullivan, O., Cotter, P.D., Ross, P., & Hill, C. (2011). High-throughput sequence-based analysis of the bacterial composition of kefir and an associated kefir grain. FEMS Microbiology Letters, 320, 56-62. https://doi.org/10.1111/j.1574-6968.2011.02290.x

El-Loly, M.M. (2011). Composition, properties and nutritional aspects of milk fat globule membrane – A review. Polish Journal of Food and Nutrition Science, 61(1), 7-32. https://doi.org/10.2478/v10222-011-0001-0

Farnworth, E.R. (2005a). The beneficial health effects of fermented foods – Potential probiotics around the world. Journal of Diet Supplements, 4(3-4), 93-117. https://doi.org/10.1300/J133v04n03_07

Farnworth, E.R. (2005b). Kefir – A complex probiotic. Food Science & Technology Bulletin: Functional Foods, 2(1), 1-17. https://doi.org/10.1616/1476-2137.13938

Feng, P., Weagant S.D., Grant M.A., & Burkhardt W. (2018). Enumeration of *Escherichia coli* and the coliform bacteria. Retrieved from https://www.fda.gov/food/laboratory-methods-food/bam-chapter-4-enumeration-escherichia-coli-and-coliform-bacteria

Food and Agriculture Organization (FAO). (2003). CODEX standard for fermented milk #243. Retrieved from http://www.fao.org

Food and Drug Administration (FDA). (2013). FDA Circular No. 2013-010: Revised guidelines for the assessment of microbiological quality of processed foods. Retrieved from https://ww2.fda.gov.ph/attachments/article/17218/FC2013-010.pdf

Fox, P.F., Uniacke-Lowe, T., McSweeney, P.L.H., & O'Mahony, J.A. (2015). Dairy chemistry and biochemistry (2nd Ed.). Switzerland: Springer.

Frank, J.F., & Yousef, A.E. (2004). Tests for groups of microorganisms. In: H.M. Wehr & J.F. Frank (Eds.), Standard methods for the examination of dairy products (pp. 227-248). USA: American Public Health Association (APHA).

Garrote, G.L., Abraham, A.G., & De Antoni, G.L. (2000). Inhibitory power of kefir: The role of organic acids. Journal of Food Protection, 63(3), 364-369. https://doi.org/10.4315/0362-028x-63.3.364

Gulmez, M., & Guven, A. (2003). Survival of *Escherichia coli* O157:H7, *Listeria monocytogenes* 4b and *Yersinia enterocolitica* 03 in different yoghurt and kefir combinations as prefermentation contaminant. Journal of Applied Microbiology, 95(3), 631-636. https://doi.org/10.1046/j.1365-2672.2003.02016.x

Guzel-Seydim, Z.B., Kok-Tas, T., Greene, A.K., & Seydim, A.C. (2011). Review: Functional properties of kefir. Critical Reviews in Food Science and Nutrition, 51(3), 261-268. https://doi.org/10.1080/10408390903579029

Henning, D.R., Flowers, R., Reiser, R., & Ryser, E.T. (2004). Pathogens in milk and milk products. In: H.M. Wehr & J.F. Frank (Eds.), Standard methods for the examination of dairy products (pp. 106-117; 132-135). USA: American Public Health Association (APHA).

Jayarao, B.M., Pillai, S.R., Sawant, A.A., Wolfgang, D.R., & Hedge, N.V. (2004). Guidelines for monitoring bulk tank milk somatic cell and bacterial counts. Journal of Dairy Science, 87(10), 3561-3573. https://doi.org/10.3168/jds.S0022-0302(04)73493-1

Kim, D., Jeong, D., Kim, H., Kang, I., Chon, J., Song, K., & Seo, K. (2016). Antimicrobial activity of kefir against various food pathogens and spoilage bacteria. Korean Journal for Food Science of Animal Resources 36(6), 787-790. https://doi.org/10.5851/kosfa.2016.36.6.787

Kok-Tas, T., Seydim, A.C., Ozer, B., & Guzel-Seydim Z.B. (2012). Effects of different fermentation parameters on quality characteristics of kefir. Journal of Dairy Science, 96(2), 780-790. https://doi.org/10.3168/jds.2012-5753

Leite, A., Miguel, M., Peixoto, R., Ruas-Madiedo, P., Paschoalin, V., Mayo, B., & Delgado, S. (2015). Probiotic potential of selected lactic acid bacteria strains isolated from Brazilian kefir grains. Journal of Dairy Science, 98(6), 3622-3632. https://doi.org/10.3168/jds.2014-9265

Magalhães, K.T., De Melo Pereira, G.V., Campos, C.R., Dragone, G., & Schwan, R.F. (2011). Brazilian kefir: Structure, microbial communities and chemical composition. Brazilian Journal of Microbiology, 42(2), 693-702. https://dx.doi.org/10.1590/S1517-838220110002000034

Otles, S., & Cagindi, O. (2003). Kefir: A probiotic dairy-composition, nutritional, and therapeutic aspects. Pakistan Journal of Nutrition, 2(2), 54-59. http://dx.doi.org/10.3923/pjn.2003.54.59

Prado, M.R., Blandón, L.M., Vandenberghe, L.P.S., Rodrigues, C., Castro, G.R., Thomaz-Soccol, V., & Soccol, C.R. (2015). Milk kefir: Composition, microbial cultures, biological activities, and related products. Frontiers in Microbiology, 6, 1117. https://doi.org/10.3389/fmicb.2015.01177

Rosa, D.D., Dias, M.M.S., Grzeskowiak, L.M., Reis, S.A., Conceição, L.L., & Peluzio, M.D.C.G. (2017). Milk kefir: Nutritional, microbiological and health benefits. Nutrition Research Reviews, 30, 82-96. https://doi.org/10.1017/S0954422416000275

Santos, A., San Mauro, M., Sanchez, A., Torres, J.M., & Marquina, D. (2003). The antimicrobial properties of different strains of *Lactobacillus* spp. isolated from kefir. Systematic and Applied Microbiology, 26(3), 434-437. https://doi.org/10.1078/072320203322497464

Ulusoy, B., Çolak, H., Hampikyan, H., & Erkan, M. (2007). An in vitro study on the antibacterial effect of kefir against some food-borne pathogens. Türk Mikrobiyoloji Cemiyeti Dergisi 37(2), 103-107.

Vieira, C.P., Alvares, T.S., Gomes, L.S., Torres, A.G., Paschoalin, V.M.F., & Conte-Junior, C.A. (2015). Kefir grains change fatty acid profile of milk during fermentation and storage. PLoS One, 10(10), e0139910. https://doi.org/10.1371/journal.pone.0139910

Viljoen, B.C. (2001). The interaction between yeasts and bacteria in dairy environments. International Journal of Food Microbiology, 69(1-2), 37-44. https://doi.org/10.1016/s0168-1605(01)00570-0

Vlahapoulou, I., Bell, A.E., & Wilbey, R.A. (2001). Effects of starter culture and its exopolysaccharides on the gelation of glucono-δ-lactone-acidified bovine and caprine milk. International Journal of Dairy Technology, 54(4), 135-140. https://doi.org/10.1046/j.1364-727x.2001.00023.x