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RESEARCH

Potential use of whey in kefir production: evaluation of fermentation kinetics and microbiota

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Abstract

This study was carried out on the effect of different amounts of whey on the kefir production process and the bacterial microbiota of the beverages. Fermentation was carried out by mixing commercial kefir grains with cow, sheep, and goat milk with their whey at concentrations of 0% (control group), 25%, 50%, and 75%. The bacterial microbiota in the produced beverages and the relative abundances of seven microorganisms (*Lactobacillus kefiranofaciens*, *Lactobacillus kefiri*, *Lactobacillus delbrueckii*, *Lactococcus lactis*, *Streptococcus thermophilus*, *Leuconostoc mesenteroides*, and acetic acid bacteria) were determined using metagenomic analysis targeting the V3-V4 region of 16 S rRNA gene, and quantitative polymerase chain reaction (qPCR), respectively. *Lactococcus lactis* and *Leuconostoc mesenteroides* were detected in higher abundance in whey-containing goat samples. While *Lactobacillus* was dominant in the cow milk samples containing 0%, 25%, and 50% whey, *Enterococcus* was dominant in the samples containing 75% whey. In sheep milk samples, *Lactobacillus* was dominant in samples with 75% and 50% whey, *Lactococcus* was dominant in samples with 25% whey, and *Enterococcus* was dominant in samples with no (0%) whey. *Lactobacillus* was dominant in all goat milk samples. In conclusion, high levels of lactic acid bacteria were shown to preserve their viability in the experimentally produced beverages with the addition of whey at different concentrations, and the whey could be used to produce kefir.

Keywords Kefir · Whey · Microbiota · Metagenomic analysis · QPCR

Introduction

Kefir is a globally popular fermented milk beverage that originated in Eastern Europe, the Balkans, and the Caucasus (Mendes et al., 2021). Traditionally, homemade kefir is made using kefir grains (Yilmaz et al., 2022), which are white to yellow and range in size from 0.3 to 3.5 cm in diameter. While cow's milk is the most common base for kefir, it can also be prepared using milk from goats, sheep, camels, and buffalos (Rosa et al., 2017). The choice of milk influences the beverage's organic acid levels, amino acid profile, and overall sensory and microbiological attributes. Kefir production typically starts with the addition of grains to milk, followed by a fermentation period that lasts from 10 to 40 h (most commonly 18 to 24 h) at temperatures between 8 °C and 25 °C in a partially sealed container (Rosa et al., 2017; Pothuraju et al., 2018; Alves et al., 2021). The final physicochemical, nutritional, and microbiological properties of kefir are affected by several factors, including the geographical origin of the grains, the milk-to-grain ratio, the type of milk, fermentation duration and temperature, methods of separating the grains after fermentation, the equipment used, agitation during fermentation, the cleaning procedures for the grains, and the storage conditions (Garrote et al., 1998; Vieira et al., 2015; Hecer et al., 2019).

Kefir and kefir grains each contain a rich and diverse microbiota, though their microbial compositions differ. Various bacterial and yeast genera have been identified in both kefir beverages and grains (Vardjan et al., 2013; Bourrie et al., 2016). To date, over 80 bacterial and yeast strains have been isolated from kefir, with lactic acid bacteria being the predominant microbial group (Düven et al.,

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2021; Ganatsios et al., 2021). The most commonly reported bacterial species in kefir grains include *Lactobacillus kefiranofaciens*, *Lactobacillus acidophilus*, *Lacticaseibacillus paracasei*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Lactiplantibacillus plantarum* (Azizi et al., 2021). In contrast, *Lactococcus lactis* subsp. *lactis* and *Streptococcus thermophilus* tend to be found in greater abundance in kefir beverages than in grains (Bourrie et al., 2016; Biçer et al., 2021). Accurately identifying the microbial composition of kefir is crucial for understanding its functional role and potential applications in the food industry. To achieve this, both culture-dependent and culture-independent analytical techniques have been used in recent years. Next-generation sequencing (NGS) has proven to be a valuable tool for characterizing kefir's microbial communities; however, it has certain limitations, such as challenges in consistently identifying microbes at the species level (Winand et al., 2020). Misclassification may also lead to the incorrect identification of bacteria and yeasts from the same genus. However, these techniques have provided further detailed information regarding the species present in low abundances in the microbial composition of kefir (Zamberi et al., 2016; Gao & Zhang, 2019). The currently used identification techniques have not been adequate for the study of kefir in aspects other than its pure microbial composition, such as grain formation and population changes due to environmental conditions. Therefore, researchers propose developing a species-specific analytical approach to investigate the formation, structural integrity, and functionality of kefir grains, along with the microbial species isolated from them. In this context, quantitative polymerase chain reaction (qPCR) is considered a suitable tool for detecting a specific bacterium in a microbial matrix, with great specificity and selectivity (Smith & Osborn, 2009).

Whey is the green-yellow fraction that remains after the milk proteins have been coagulated with rennet or organic acid, and the curd, the leading cheese component, has been removed. Whey is often discarded as a waste byproduct in cheese production (Jeličić et al., 2008). However, whey accounts for approximately 85%–90% of the milk and contains approximately 55% of the milk's dry matter, including lactose, serum proteins, lipids, and mineral salts as the most abundant nutrients (Dragone et al., 2009; Cabizza et al., 2021). Kefir microorganisms grow in various medium, such as cow, sheep, and goat milk (Otles & Cagindi, 2003) and also whey (Rimada & Abraham, 2001). Whey proteins are important nutritional sources that are resistant to digestion by pepsin and offer good nutritional content and beneficial functional qualities.

Several studies have reported the fermentation of whey through the microbiological approach using lactic acid bacteria (Lagoa-Costa et al., 2020; Mazorra-Manzano et al., 2020; Zotta et al., 2020) The food industry could benefit hugely through whey fermentation, which allows for obtaining high-value products and lowers the biological oxygen demand (Panchal et al., 2020). Nevertheless, studies exploring the preparation of whey from goat, sheep, and cow cheese are limited.

This study investigated the potential utilization of whey derived from cow, sheep, and goat cheese production in kefir manufacturing. To achieve this, milk and whey were combined in varying ratios, and the fermentation kinetics, microbiota composition, and the relative abundance of the six species and the acetic acid bacteria were analyzed.

Material and method

Milk samples and whey preparation

The cow milk used in this study was sourced from Holstein cattle raised at the Veterinary Faculty Farm of Selçuk University. Sheep milk was collected from a private farm specializing in Akkaraman sheep breeding, while goat milk was obtained from a farm that raises Hair goats. Permission was granted by the farm owners to collect milk samples from each species. The kefir grains used in the study were purchased from a commercial supplier (Oğuzhan Yalçınkaya Kefirikafkas, Ankara, Turkey, kefirikafkas.com). Cheese production was carried out using approximately 10 l of milk, following the method described by García-Gómez et al. (2019). In summary, the milk was first heated to 90 °C for 5 min before being cooled to 35 °C. A microbial protease (Intermak, Konya, Turkey) at a concentration of 220 IMCU/mL, diluted tenfold, was then added, allowing coagulation to occur within approximately 40 min. The curd obtained after this enzymatic coagulation was cut into pieces approximately 1 cm^3 in size and left undisturbed for 15–20 min, while the whey was filtered and placed into a clean container and stored+4 °C till whey-based kefir beverages preparation. Whey obtained from cow, sheep and goat milk was mixed with their own type of milk.

Kefir production

Kefir grains were initially activated in cow, sheep, and goat milk at room temperature for 12 h, with this process being repeated three times. Following activation, the grains were filtered, rinsed with sterile distilled water, and then evenly distributed for use in different experimental groups. The milk samples from each animal species were then subjected to heat treatment at 90 °C for 5 min. After heating, milk from each species was blended with its corresponding whey

at varying concentrations of 0%, 25%, 50%, and 75%, using sterile, air-permeable glass jars. Once mixed, the samples were cooled to approximately 25 °C, and kefir grains were introduced at a ratio of 1% (5 g of kefir grains per 500 mL of the milk-whey mixture). Fermentation was carried out for 24 h. The specific milk-to-whey ratios and corresponding sample codes are detailed in Table 1.

Determination of fermentation kinetics

The fermentation kinetics of all samples were tracked and recorded throughout the entire fermentation process. Three key parameters were analyzed: the time required to reach an acidity level of 0.6% lactic acid ($T_{\%0.6\,l.a.}$), the point at which the highest acidification rate occurred (T_{Vmax}), and the maximum acidification rate itself (V_{max}). These parameters were determined following the methodology outlined by Sözeri Atik et al. (2021). The V_{max} value (expressed as 10^{-3} upH min^{-1}) was calculated using the formula ΔpH/120*1000. pH levels were measured with a digital pH meter (Inolab WTW, 720), and acidity was assessed every two hours over a 24-hour period, expressed as a percentage of lactic acid. To determine acidity, 0.1 mL of phenolphthalein was added to 10 mL of the kefir sample, and the mixture was titrated with 0.1 N NaOH until a pink endpoint was observed, following the protocols described by Sulmiyati et al. (2019) and Biçer et al. (2024).

Microbiological analysis

MRS agar (Merck, VM771160 706) and M17 agar (Merck, VM773608 709) were used to determine the viable lactobacilli and lactococci-streptococci counts. Serial dilutions (10^{-1} -10^{-6}) were prepared using sterile Ringer's solution (Merck, TP717725 425). These diluted samples were then spread onto MRS agar plates, which were incubated anaerobically in anaerobic jars (Anaerocult System: Merck 16275, Merck KGaA) at 30 °C for 72 h. Meanwhile, M17 agar plates were incubated aerobically at 30 °C for 48 h. The methodology followed was based on the approach described by Kesmen et al. (2012). After incubation, the developed colonies on each medium were counted to determine microbial populations.

The quantification of specific microorganisms using qPCR

Metagenomic analysis is limited at the species level due to incomplete databases for assigning taxonomic and functional annotations. Thus, in the present study, the metagenomic analysis results were determined at the genus level. The kefir samples were evaluated for the presence of major bacteria species in kefir consortia such as *Lactobacillus kefiranofaciens*, *Lb. kefiri*, *Lb. delbrueckii*, *Lactococcus lactis*, *Streptococcus thermophilus*, *Leuconostoc mesenteroides*, and acetic acid bacteria using species-specific primers, which are listed in Table 2. The PCR conditions were determined by optimizing the reported primer pairs.

DNA was extracted from the kefir beverages obtained after 24 h of fermentation using a commercial kit (Magrev Magnetic Bead Manual Isolation Kit, Anatolia Geneworks, Istanbul, Turkey). Magrev Kits are based on the binding and elution of nucleic acids onto magnetic beads coated with silica. The extraction process included lysis (release of DNA from cell and membrane structures, and denaturation of proteins), binding (DNA/RNA binding to a surface), washing (removal of salt and protein), and elution (recovery of bound DNA/RNA).

The DNA concentrations were adjusted to 100 ng/µL using nuclease-free water. A Light Cycler Nano (Roche Diagnostics, Germany) was employed for the qPCR reactions. The qPCR reaction mix comprised 2 µL of DNA, 50 pM each of the forward and reverse primers, and 5 µL of

Table 1 The milk and Whey ratios of the samples and their sample codes

Sample codes	Cow milk	Cow whey	Sheep milk	Sheep whey	Goat milk	Goat whey
C100	100	-	-	-	-	-
C75	75	25	-	-	-	-
C50	50	50	-	-	-	-
C25	25	75	-	-	-	-
S100	-	-	100	-	-	-
S75	-	-	75	25	-	-
S50	-	-	50	50	-	-
S25	-	-	25	75	-	-
G100	-	-	-	-	100	-
G75	-	-	-	-	75	25
G50	-	-	-	-	50	50
G25	-	-	-	-	25	75

Table 2 The sequences of the primers and the annealing temperatures

Target species	Primer sequences (5'−3')	Annealing temperature	Amplicon length (bp)	References
Lb. kefiranofaciens	GCAACAACCAAAGTATTGTA TAGCCGAAGAGGATCTAA	60 °C	118	Nejati et al. (2020)
Lb. kefiri	TCGCTTTCAAGCATTGAA CGAACTTCCCATTATCCATA	60 °C	134	Nejati et al. (2020)
Lb. delbrueckii	GAACTTGATGTTGTTGAAGGGATGCAATTCG GAGCGGCCTTGTTGCACGATTTC	64 °C	181	Herbel et al. (2013)
Str. thermophilus	TTCGACAGTTGAGGGTGGATCCATACATGCAAAGCC TCCT	60 °C	179	Rossi et al. (2013)
Lc. lactis	TGAAGAATTGATGGAACTCG CATTGTGGTTCACCGTTC	60 °C	126	Achilleos and Berthier (2013)
Leu. mesenteroides	CGAACCAACAACTTATCTATG CCTCATGTAGATCCTACCTTA	60 °C	107	Nejati et al. (2020)
Acetic acid bacteria	AAGGGGGCTAGCGTTGCTCG ACCGCCTACACGCCCTTTACG	62 °C	52	Kim et al. (2019)

iTaq Universal SYBR Green qPCR (BioRad, USA) mix. The thermal program for the PCR was as follows: a 300-second initial denaturation at 98 °C followed by 45 cycles of 15 s at 95 °C, 30 s at the annealing temperature (Table 2), and 30 s at 72 °C. The reactions concluded with a melting program, where the temperature was increased from 60 °C to 95 °C at a rate of 0.1 °C/10 s. The specificity of the amplicons was additionally verified using electrophoresis by analyzing the band size on a 1% agarose gel. All reactions for each sample were conducted in triplicate.

Metagenomic analysis

To analyze the microbial diversity in the kefir samples, the V3-V4 regions of the 16S rRNA gene were amplified using MiSeq341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGC GCAG-3') and MiSeq805R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGAGAGAGGACTACHVGGGT TCTAATCC-3') primers, as described by Yong et al. (2017). The amplification process was carried out using the KAPA HiFi HotStart ReadyMix PCR Kit (KAPA BioSystems, USA). The PCR conditions included an initial denaturation at 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension step at 72 °C for 5 min. To ensure purity, all PCR products were cleaned using the Agencourt AMPure XP kit (Beckman Coulter, Brea, CA, USA). Index barcodes were attached to the samples to enable differentiation during next-generation sequencing. Adapter sequences were incorporated to facilitate binding to oligo-binding sites on the sequencing platform. The Nextera XT index kit was used for PCR, with an initial denaturation at 95 °C for 3 min, followed by 8 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. The resulting PCR products underwent another purification step. The libraries were quantified using qPCR to determine their exact concentrations. This process was performed using the Eco Real-Time PCR System with the KAPA Library Quantification Kit (KAPA Biosystems). Sequencing was conducted on the Illumina NovaSeq System (Illumina, USA) with paired-end reads of 2 × 250 bp. The sequencing reads were processed using Novaseq Control Software, and base calling was carried out with Real Time Analysis v1.18. The output data were converted into FASTQ format using the bcl2fastq (v1.8.4) program. For bioinformatics analysis, Geneious Prime software was utilized.

Statistical analysis

The log CFU/mL values of culturable microorganisms were statistically analyzed by performing ANOVA and Tukey's mean comparison tests ($P \leq 0.05$) using SPSS (Version 22.0) to determine the significant differences between the evaluated responses. The delta ct values according to the cow milk samples (C100, C50, C75, and C25) were used as controls to determine the relative fold changes in the qPCR analysis.

Results

Fermentation parameters [pH and titratable acidity (%)

The pH values of the samples were determined to be in the range of 4.98–6.90 at 0 h. The lowest and highest pH values were noted for G25 and S100, respectively. The titratable acidity was in the range of 0.1230–0.5623%l.a. at 0 h. The lowest acidity was observed for C25, and the highest for

Table 3 The parameters of the kinetics of acidification for all Kefir samples

Samples	V_{max} 10^{-3} upH min^{-1} ± SD*	T_{Vmax} (h)	$T_{\%\ 0.6\ la}$ (h)
C100	2.44 ± 0.38	12	24
C75	3.00 ± 0.43	8	24
C50	3.22 ± 0.21	6	24
C25	3.03 ± 0.38	6	24
S100	2.31 ± 0.21	24	16
S75	2.31 ± 0.17	24	18
S50	2.00 ± 0.17	24	12
S25	1.03 ± 0.35	18	4
G100	2.25 ± 0.80	24	22
G75	2.58 ± 0.42	24	12
G50	1.31 ± 0.10	24	22
G25	0.94 ± 0.13	16	12

V_{max}: maximum acidification rate; T_{Vmax}: time to reach V_{max}; $T_{\%la\ 0.6}$: time to obtain % 0.6 l.a. Results are expressed as the mean ± standard deviation (n = 3). *: (ΔpH/120)*1000 **C100**: %100 cow milk, **C75**: %75 cow milk %25 cow whey, **C50**: %50 cow milk %50 cow whey, **C25**: %25 cow milk %75 cow whey, **S100**: %100 sheep milk, **S75**: %75 sheep milk %25 sheep whey, **S50**: %50 sheep milk %50 sheep whey, **S25**: %25 sheep milk %75 sheep whey, **G100**: %100 goat milk, **G75**: %75 goat milk %25 goat whey, **G50**: %50 goat milk %50 goat whey, **G25**: %25 goat milk %75 goat whey

S25. After 24 h, the pH values were between 4.39 and 5.21, with the lowest value noted for G25 and the highest value noted for S100. The titratable acidity values were determined to be in the range of 0.61–0.91%l.a., with the lowest acidity value noted for C50 and the highest value noted for S75.

The acidification kinetics of each kefir sample was assessed based on three parameters; the time necessary to reach 0.6% l.a. ($T_{\%0.6\ l.a.}$), the maximum acidification rate (V_{max}), and the time at which the maximum acidification rate is reached (T_{Vmax}). The results are presented in Table 3. The $T_{\%0.6\ l.a.}$, T_{Vmax}, and V_{max} values ranged from 4 to 24 h, 6 to 24 h, and 0.94 to 3.22×10^{-3} pH units per minute, respectively. The change in pH and titratable acidity during the fermentation period in all samples is presented in Figs. 1 and 2, respectively.

Microbiological analysis

After 24 h of fermentation, the bacterial counts growing on MRS and M17 agar mediums were determined. The results are presented in Table 4. The lowest counts were observed for G25, while the highest were recorded for S100 on both media. Bacterial counts ranged from 6.52 to 8.39 log CFU/mL on MRS agar and from 5.65 to 8.54 log CFU/mL on M17 agar.

Quantification of selected microorganisms using qPCR

The relative levels of *Lb. kefiranofaciens*, *Lb. delbrueckii*, *Lb. kefiri*, *L. lactis*, *Leu. mesenteroides*, *Str. thermophilus*, and acetic acid bacteria were determined using qPCR. Cow milk samples without whey addition were used as controls.

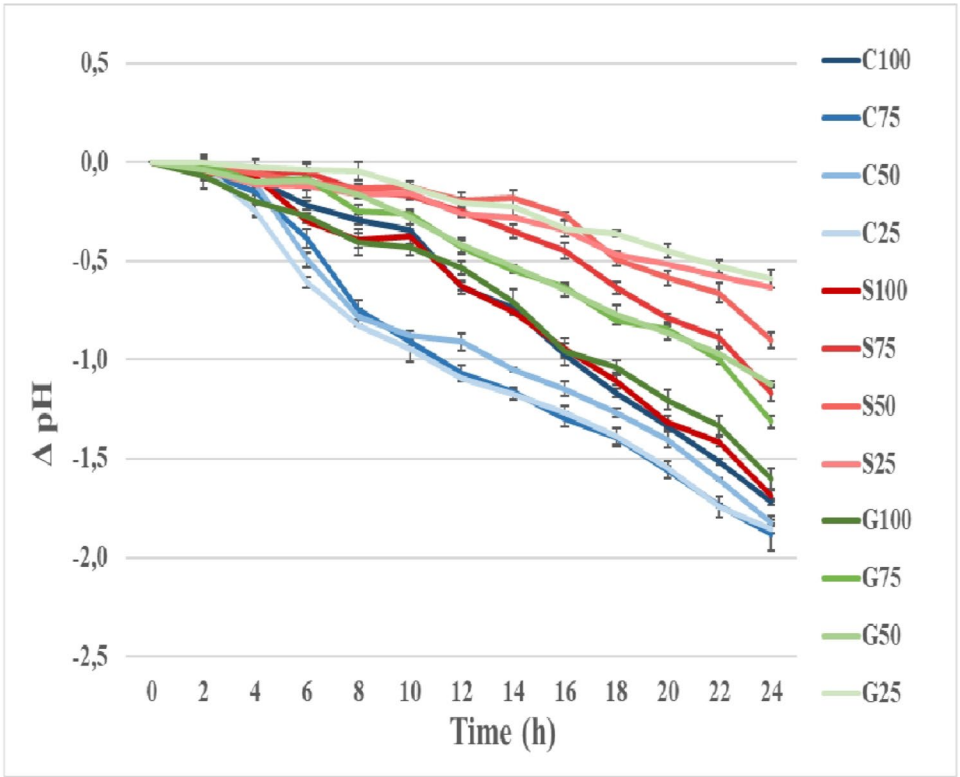
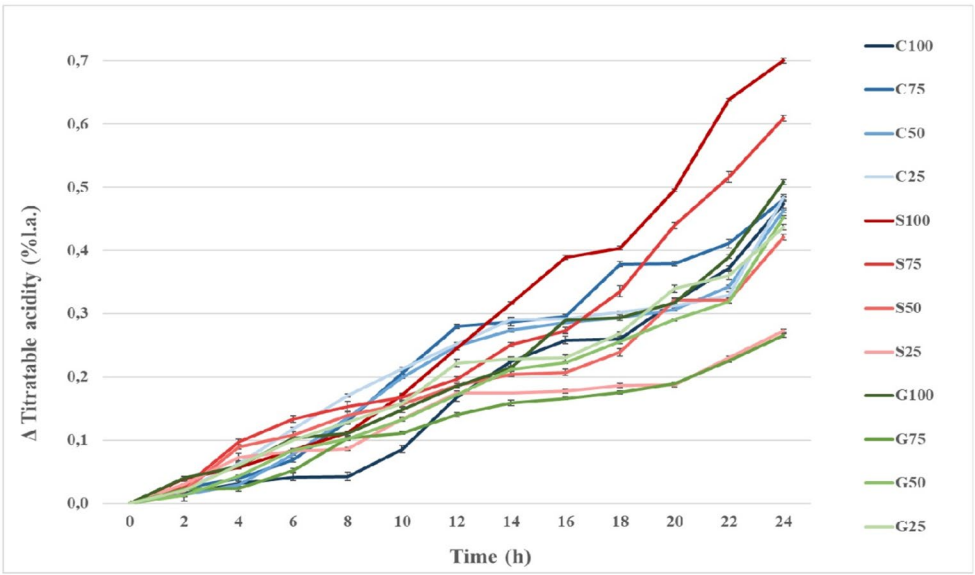


Fig. 1 The pH changes (ΔpH) in samples over 24 h. *The error bars represent the standard deviation of means of 3 replicates.



2. Titratable acidity changes (Δtitratable acidity) of all samples over 24 h
*The error bars represent the standard deviation of means of 3 replicates.

Fig. 2 Titratable acidity changes (Δtitratable acidity) of all samples over 24 h. *The error bars represent the standard deviation of means of 3 replicates.

Table 4 Bacterial counts obtained on MRS and M17 agar media at the end of fermentation (log CFU/mL±standard deviation)

Media		
Samples	MRS	M17
C100	7.78 ± 0.54^{abc}	8.50 ± 0.21^{a}
C75	7.85 ± 0.01^{abc}	8.52 ± 0.11^{a}
C50	7.99 ± 0.04^{ab}	8.53 ± 0.11^{a}
C25	7.62 ± 0.10^{bc}	8.45 ± 0.03^{a}
S100	8.39 ± 0.05^{a}	8.54 ± 0.31^{a}
S75	8.28 ± 0.09^{ab}	8.40 ± 0.06^{a}
S50	7.13 ± 0.02^{cd}	6.89 ± 0.58^{b}
S25	6.80 ± 0.14^{d}	7.09 ± 0.55^{b}
G100	6.57 ± 0.05^{d}	8.67 ± 0.11^{a}
G75	6.80 ± 0.21^{d}	7.78 ± 0.18^{ab}
G50	6.68 ± 0.11^{d}	7.87 ± 0.12^{ab}
G25	6.52 ± 0.25^{d}	5.65 ± 0.49^{c}

C100: %100 cow milk, **C75:** %75 cow milk %25 cow whey, **C50:** %50 cow milk %50 cow whey, **C25:** %25 cow milk %75 cow whey, **S100:** %100 sheep milk, **S75:** %75 sheep milk %25 sheep whey, **S50:** %50 sheep milk %50 sheep whey, **S25:** %25 sheep milk %75 sheep whey, **G100:** %100 goat milk, **G75:** %75 goat milk %25 goat whey, **G50:** %50 goat milk %50 goat whey, **G25:** %25 goat milk %75 goat whey. Different letters in the same column show statistical difference ($P<0.05$)

The relative changes in the levels of the selected microorganisms are presented in Figs. 3, 4 and 5. *Lb. kefiranofaciens* was detected at the highest level in C100 and C75, followed by those in goat's milk samples. In the other two rate groups, the levels of *Lb. kefiranofaciens* in cow's milk were lower than that in sheep and goat milk samples. When all samples were evaluated, the highest level was noted in G25. The highest level of *Lb. kefiri* was detected in C100, followed by S100 and G100. In all other samples, sheep's milk had the highest level of this species, followed by cow's and goat's milk, respectively. When all samples were compared, the highest level was noted in S50. The levels of *Lb. delbrueckii* and *L. lactis* were the highest in C100, followed by goat's and sheep's milk samples, respectively. However, the goat samples in the other three whey-containing groups contained significantly higher levels of *L. lactis*. Among all samples, the highest *Lb. delbrueckii* level was noted in C25, while the highest level of *L. lactis* was noted in G25. *Str. thermophilus* was detected at higher levels in cow's milk in all samples. The highest *Str. thermophilus* level was noted in C50. *Leu. mesenteroides* was significantly higher in the goat samples at all rates, with the highest level noted in G25. The acetic acid bacteria were detected at the highest level in C100, followed by S100 and G100, respectively. While its levels were higher in S75 and S50, goat milk contained significantly higher levels of acetic acid bacteria in the 25% milk group. Among all samples, the highest level of these bacteria was noted in G25.

Microbiota diversity in the samples

The V3–V4 region of the 16s rRNA gene was sequenced to determine the microbiota in the samples. The results revealed that Bacillota and Pseudomonadota were dominant in all samples. Bacillota was dominant in ten samples, while Pseudomonadota was dominant in the C25 and G100 samples. The distribution of samples at the phylum level is presented in Fig. 6. *Lactobacillus* was the dominant species in the cow samples containing high amounts of milk. *Enterococcus* was dominant in C25. In sheep samples, *Lactobacillus* was dominant in S25 and S50, *Lactococcus* was

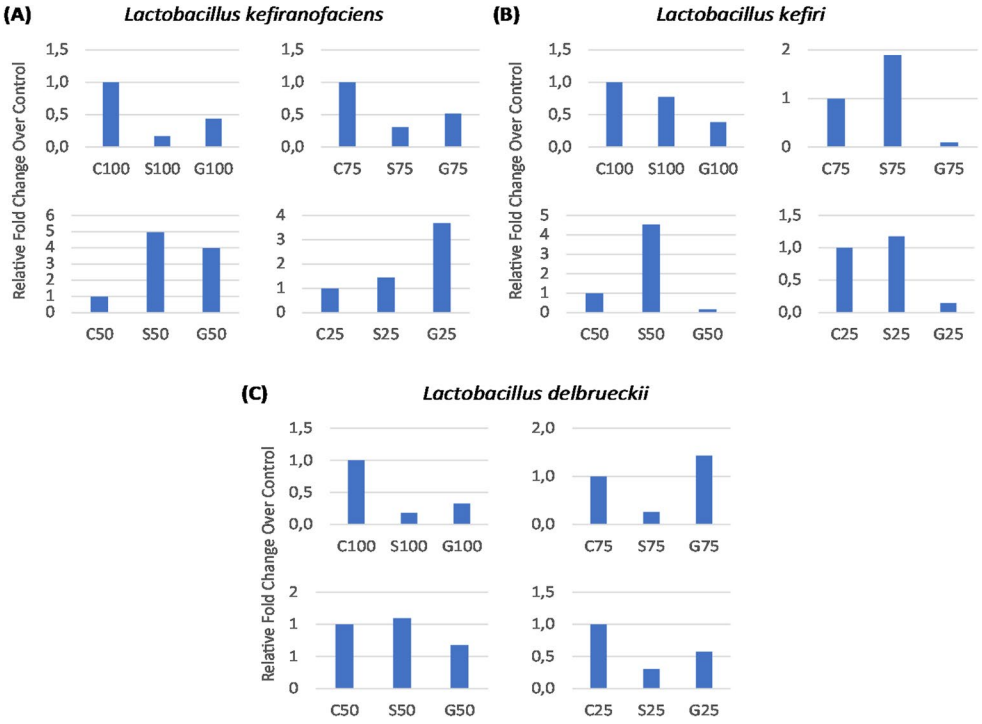


Fig. 3 Relative fold changes of *Lb. kefiranofaciens*(**A**), *Lb. kefiri*(**B**), and *Lb. delbrueckii*(**C**) among the samples

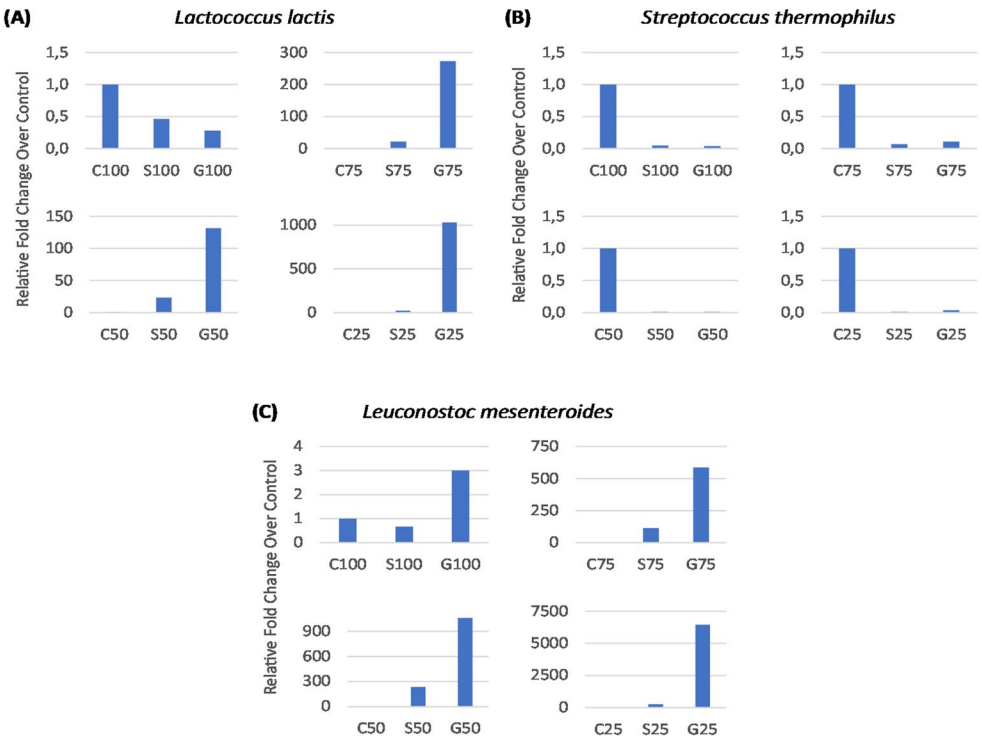


Fig. 4 Relative fold changes of *Lc. lactis*(**A**), *Str. thermophilus*(**B**), and *Leu. mesenteroides*(**C**) among the samples

dominant in S75, and *Enterococcus* was dominant in S100. *Lactobacillus* was dominant in all goat samples, similar to cow samples. *Acetobacter* was detected at levels above 2% in G25, G50, and S25. Notably, the detection of *Acetobacter* above 2% in some cow, sheep, and goat samples points to the occurrence of mild acetic fermentation or exposure to oxygen during the process. Figure 7 presents the relative abundances of the most common (>%2) bacterial genera in all samples. In addition to the dominant genera, several other bacterial groups were detected at varying levels across the samples. Low-abundance genera appeared particularly in C25, S25, S100, and G50 samples, where the relative abundance of the "Others" category exceeded 8%, indicating a more diverse microbial community. The presence of

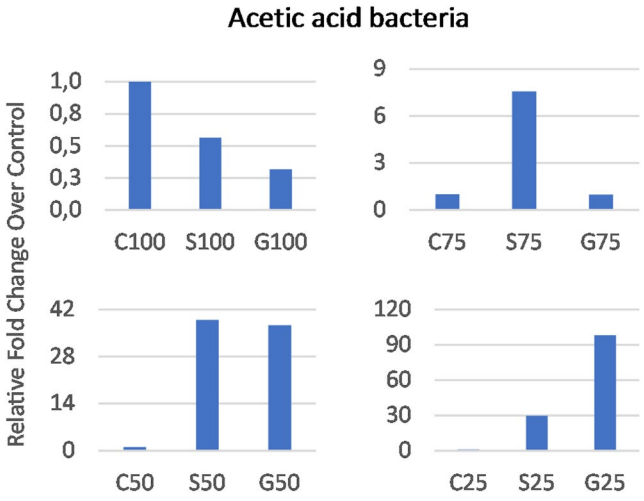


Fig. 5 Relative fold changes of acetic acid bacteria among the samples

these minor genera suggests a complex microbial ecosystem where interactions among dominant and subdominant bacteria may influence the overall fermentation process.

Discussion

Whey, a major byproduct of cheese production, retains approximately 50% of milk's dry matter, including lactose, whey proteins, a significant amount of milk salts, and some fat. Due to its nutrient-rich composition, whey serves as a valuable resource in the food industry, contributing to the production of various food ingredients (Fox et al., 2017). Additionally, whey can support bacterial growth for polysaccharide production from lactose while also reducing biological oxygen demand (Gassem et al., 1997; Fialho et al., 1999). Although whey is not classified as hazardous waste, its high organic content poses environmental concerns (Prazeres et al., 2012). Kefir grains offer a sustainable approach to minimizing the organic content of milk byproducts, thereby reducing their ecological impact (Settanni et al., 2020). Since lactic acid bacteria metabolize lactose as an energy source, milk—being rich in lactose—is commonly used for kefir production. However, whey also contains lactose alongside other essential nutrients, making it a viable substrate for fermentation. Further investigation into the manufacturing process and chemical composition of whey-based kefir is still necessary (Vashisht et al., 2023). Studies indicate that the heat treatment applied to milk before cheese production, along with the amount of rennet used, can influence whey's chemical composition, particularly its protein structure (Miloradovic et al., 2016). To ensure consistency, all milk samples in this study underwent the same heat treatment and received equal amounts of rennet. Traditional kefir grains or starter cultures can be used in whey-based kefir production (Magalhães et al., 2011). However, as noted by Kurnick et al. (2024), certain microbiological, technological, and sensory challenges arise when using sheep and goat whey in beverage production. Various whey types have been used to enhance the quality of whey-based kefir (Magalhães et al., 2010, 2011; Pasotti et al., 2017; Aidarbekova & Aider, 2022). Bilac et al. (2023) examined the physicochemical properties of Brazilian homemade kefir, incorporating 5% homemade kefir grains into whole-fat cow's milk and incubating it at 28 °C for either 24–48 h.

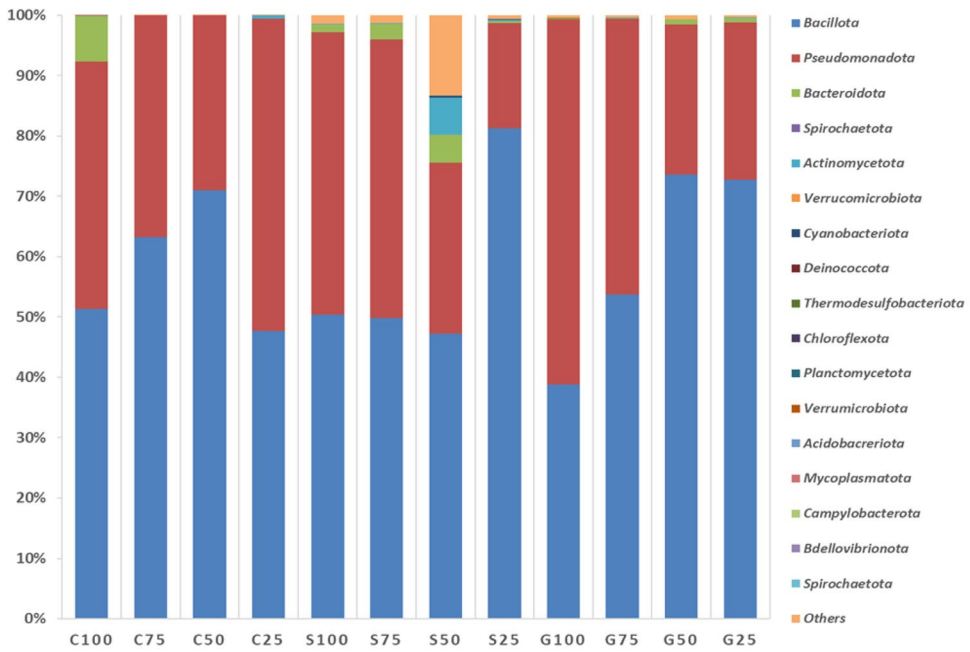


Fig. 6 Relative abundance of operational taxonomic units at the phylum level

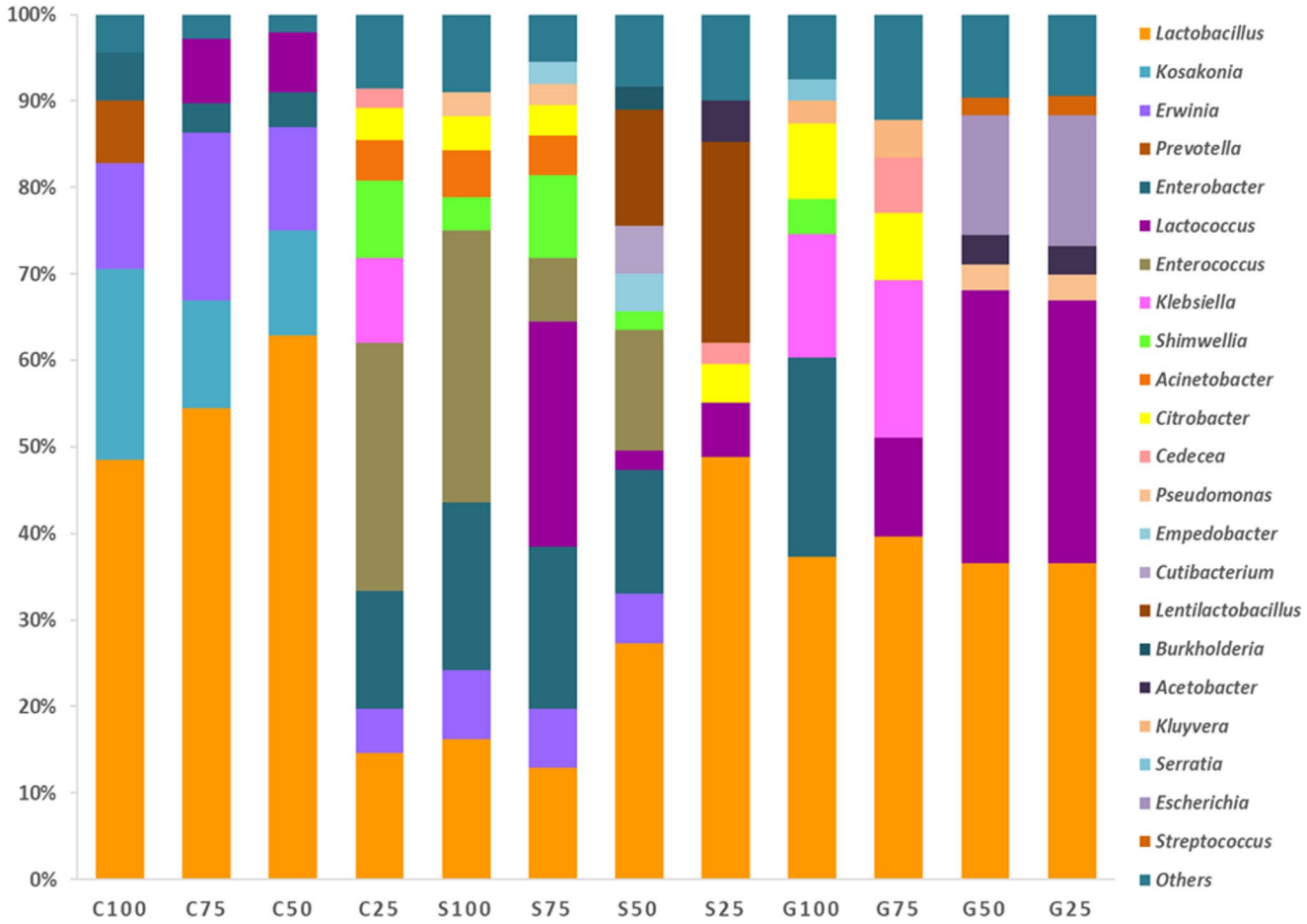


Fig. 7 Relative abundance of operational taxonomic units at the genus level

Their findings showed that the pH declined from 6.60 to a range of 4.58–4.38 after 24 h, and further to 3.74–3.60 after 48 h. The titratable acidity measured between 1.03% and 1.10% at 24 h and increased to 1.70%–1.71% after 48 h. Similarly, Dinkçi et al. (2023) developed probiotic whey-based beverages using cow, sheep, and goat milk, reporting lactic acid concentrations of 0.80%, 0.82%, and 0.76%, respectively. Among these, the highest acidity was found in sheep milk samples, followed by goat and cow samples. A trend was noted where increasing whey levels in sheep and cow milk resulted in lower acidity, whereas in goat milk, acidity increased as whey content rose. The fermentation capacity of starter cultures in dairy products is often assessed by monitoring pH reduction and acidity development. The activity and growth rate of starter culture microorganisms depend on various factors, including the milk's lactose and protein content, fermentation conditions, and milk type. The acidity in fermented dairy products primarily results from the conversion of lactose into lactic acid by lactic acid bacteria (Lee & Lucey, 2010). Yilmaz-Ersan et al. (2018) found that commercial starter cultures were more effective than kefir grains in achieving the desired pH, regardless of the milk type used. The fermentation times in this study aligned with those reported by Yilmaz-Ersan et al. (2018) and Wszolek et al. (2001). Variations in pH and acidity values were observed among the whey-based fermented beverages produced. The alkalinity and buffering capacity of sheep and cow whey are likely linked to their calcium content. It is well-established that calcium levels in milk differ among species and even between breeds within the same species (Park et al., 2007; Raynal-Ljutovac et al., 2008; Mayer & Fiechter, 2012; Singh et al., 2019). High pH and lower acidity may be influenced by calcium's role in calcium phospho-caseinate formation (Galina et al., 2007). As fermentation progresses, whey is released due to the increased solubility of calcium phosphate micelles, which corresponds to declining pH and rising acidity (Gomes et al., 2013). Thus, the observed variations in the pH and acidity of the kefir beverages may be attributed to the specific milk-to-whey ratios used during production. At the end of 24 h of fermentation, all kefir samples reached an acidity level of 0.6% lactic acid, meeting the regulatory standards set by the Turkish Food Codex Fermented Dairy Products Communiqué.

The microbial compositions of dairy products fermented with kefir grains, incorporating whey at varying concentrations, were analyzed through high-throughput sequencing of the V3-V4 region of the 16 S rRNA gene. The total sequence reads from cow, sheep, and goat milk and whey samples were grouped into 4, 13, and 6 distinct phyla, respectively, with Bacillota, Pseudomonadota, and Bacteroidota emerging as the three most dominant phyla. In samples C100 and G100, *Lactobacillus* was the predominant genus, whereas *Enterococcus* was most prevalent in S100. Lactic acid bacteria hold a crucial role in the food industry and probiotic applications, although their abundance can be influenced by various factors, such as environmental stress, available carbon sources, and strain-specific conditions (Angelin & Kavitha, 2020; Abarquero et al., 2022). Among lactic acid bacteria, the *Enterococcus* genus remains a subject of debate. While certain enterococcal strains are utilized in food production for their contributions to ripening, shelf-life extension, and flavor development (Moreno et al., 2006; Morandi et al., 2013), not all strains are considered entirely safe. Reports indicate that *E. faecium* is the most frequently detected *Enterococcus* species in dairy products (Morandi et al., 2006). This species is widely applied in the fermentation industry and has demonstrated various in vitro health benefits (Angelin & Kavitha, 2020; Abarquero et al., 2022). The microbial composition of kefir beverages can vary significantly due to differences in the geographical origin of kefir grains, fermentation techniques, detection methodologies, and maintenance practices (Prado et al., 2015; Guzel-Seydim et al., 2021). Consequently, direct comparisons across studies can be challenging. Identifying the microbial communities within kefir products and quantifying their abundance are key factors in understanding microbial diversity in these complex ecosystems. Utilizing sensitive and reliable detection methods can help address questions related to how microbial populations respond to environmental and internal factors, ultimately influencing the quality of the final product. Based on previous research highlighting the dominant microbiota in kefir (Garofalo et al., 2015; Gao & Zhang, 2019; Nejati et al., 2020), seven microorganisms were selected for comparison in the whey kefir samples produced in this study. The results indicated that microbial species commonly found in raw milk were also present in whey-based kefirs. Even after pasteurization, microbial DNA can persist in the final products. Certain genera, including *Erwinia*, *Kosakonia*, *Klebsiella*, *Shimwellia*, *Citrobacter*, and *Pseudomonas*, remained at high levels in some samples, yet they did not surpass the dominance of *Lactobacillus*, *Lactococcus*, and *Enterococcus*, which were found in higher concentrations in kefir beverages. The findings suggested that a 24-hour incubation period may not be sufficient for kefir-associated bacteria to achieve dominance, particularly in samples containing whey. While extending the incubation period or increasing the starter culture concentration could enhance the presence of kefir microorganisms, these adjustments might also lead to undesirable sensory and textural alterations in the final product (Ding et al., 2020). The observed differences in microbial composition among the kefir samples may be attributed to several factors. The physicochemical properties of whey, such as its lactose, protein, and mineral content, can influence the growth dynamics of kefir-associated bacteria. Additionally, the presence of whey proteins and peptides may provide selective advantages for certain microorganisms by acting as growth promoters or inhibiting competitors. Moreover, variations in milk-to-whey ratios likely modulate the fermentation environment, altering pH, redox potential, and nutrient availability, which in turn shape the microbiota profile. Finally, species-specific tolerance to environmental stresses, such as osmotic pressure and acidification, could have contributed to the observed differences. The microbial shifts observed in this study are not only of academic interest but also have practical relevance for product safety, shelf life, and probiotic functionality. For example, the dominance of lactic acid bacteria such as *Lactococcus* and *Leuconostoc* is essential for developing desirable sensory characteristics and preserving product stability. On the other hand, the presence of *Enterococcus* in some samples, while common in dairy fermentations, warrants careful monitoring due to its potential implications for food safety. Understanding these shifts allows for targeted process optimization to enhance both product quality and consumer health benefits. These findings highlight that microbial diversity is shaped not only by milk type but also by milk concentration and fermentation conditions. Such diversity may contribute to differences in acidification, flavor development, and texture among the final products, emphasizing the importance of controlling fermentation parameters to achieve consistent product quality.

The qPCR analysis conducted in this study showed that *Lactococcus lactis* and *Leuconostoc mesenteroides* were present in significantly higher amounts in goat milk samples compared to those from sheep and cows. Mesophilic starter cultures primarily consist of bacteria from the *Lactococcus* and *Leuconostoc* genera (Parente & Cogan, 2004; Üstünol, 2015). Among these, *L. lactis* is the most widely used mesophilic starter culture due to its strong acid-producing ability (Mayra-Mäkinen & Bigret, 2004; Üstünol, 2015). When these bacteria are employed as starter cultures in the dairy industry, an essential factor is the ability of *Lactococcus* strains to ferment lactose. *L. lactis* efficiently metabolizes lactose, making it well-suited for adaptation to milk and dairy product environments (Gutiérrez-Méndez et al., 2010). This species can be found in various dairy products,

including raw milk, kefir grains, and fermented beverages (Yerlikaya, 2019). Additionally, *L. lactis* plays a crucial role in starter cultures for cheese production, contributing to lactic acid formation and aiding in the breakdown of milk proteins during fermentation, which influences both the sensory and microbiological properties of the final product (Cavanagh et al., 2015). Other commonly used mesophilic starter cultures include *Leu. mesenteroides* (Mayra-Mäkinen & Bigret, 2004) and other *Leuconostoc* species (Bintsis, 2018). *Leuconostoc* is found in a variety of food sources, including dairy products, plant-based foods, meats, and various fermented products. However, this genus has complex nutritional requirements (Huys et al., 2012). Its growth is relatively slow, requiring specific nutrients such as amino acids and various growth factors. Furthermore, *Leuconostoc* species have limited acidification capabilities (Dellaglio et al., 1995; Liu et al., 1997; Huys et al., 2012; Bintsis, 2018). Instead of primarily producing lactic acid, they metabolize glucose to generate *D*-lactate, ethanol, carbon dioxide (CO_2), and small amounts of acetate (Villani et al., 1997; Hemme & Foucaud-Scheunemann, 2004; Bintsis, 2018). In dairy products, the two most frequently encountered *Leuconostoc* species are *Leu. mesenteroides subsp. mesenteroides* and *Leu. lactis*. The sensory characteristics and textures of foods such as meat, milk, cheese, and butter are influenced by *Leuconostoc* species (Hemme & Foucaud-Scheunemann, 2004; Huys et al., 2012). Certain *Leuconostoc* strains, including *Leu. mesenteroides subsp. dextranicum* and *Leu. mesenteroides subsp. cremoris*, are integral to flavor development in fermented dairy products (Carr et al., 2002). Additionally, some strains metabolize citrate to produce diacetyl and acetoin, compounds that enhance the characteristic aroma and taste of dairy products (Dellaglio et al., 1995; Liu et al., 1997; Villani et al., 1997). *Leu. mesenteroides*, *Leu. pseudomesenteroides*, and *Leu. citreum* have been identified in raw milk cheeses (Terzić-Vidojević et al., 2014; Biolcati et al., 2020; Sánchez-Juanes et al., 2020). In fermented milk products, *Leuconostoc* species are known to coexist with acid-producing *Lactococcus* strains and may even promote the growth of citrate-positive *Lactococcus* cultures (Vedamuthu, 1994; Bandell et al., 1998; Hache et al., 1999). The elevated presence of *L. lactis* and *Leu. mesenteroides* in goat milk samples observed in this study supports these findings, suggesting that their co-growth may be more favorable in this specific dairy matrix. Understanding the shifts in microbial composition is essential, as these changes can affect the sensory properties, shelf life, and probiotic potential of the final product. For instance, the dominance of *Lactococcus* and *Leuconostoc* in goat milk samples may enhance flavor and texture, while the presence of *Enterococcus* in some formulations could raise safety or functional considerations. Moreover, tailoring the microbial profile through whey supplementation offers opportunities to develop novel kefir products with specific health benefits and improved industrial performance. Further research optimizing these microbial dynamics may thus contribute to more sustainable and value-added dairy production systems.

Conclusion

Sheep and goat breeding is predominantly carried out on small-scale farms, with the resulting milk largely utilized in cheese production. Whey, a significant by-product of this process, is often discarded. However, this study has shown that whey can be used in kefir production. All samples reached sufficient acidity at the end of 24 h of fermentation. It was revealed that whey-based kefir beverages contain high levels of bacteria that can grow on MRS and M17 agar medium. Through metagenomic analysis of the V3-V4 region of the 16 S rRNA gene, the bacterial microbiota of these kefir beverages was comprehensively characterized. Moreover, the relative abundance of key microorganisms typically found in kefir was effectively quantified using qPCR analysis. Therefore, it was concluded that the whey obtained from sheep and goat milk cheeses produced in family-type small enterprises could be used in kefir production without needing extra equipment. Accordingly, it is recommended that whey be used in kefir production as an environment-friendly solution that would enable whey recycling and waste reduction in the dairy industry. However, further research is needed to optimize fermentation conditions and ensure product quality in whey-based kefir beverages.

Declarations

Conflict of interest The authors declare no potential conflict of interest.

Ethics approval This article does not require IRB/IACUC approval because there are no human and animal participants.

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