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Original Article

Zooming into the microbial and metabolic effects of citicoline use in kefir

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Abstract

This study focused on the metabolic and microbiological changes induced by citicoline in kefir. To demonstrate the microbial effects of citicoline in kefir, metabolite analysis was conducted using 16S rRNA and 28S rRNA metabarcoding. Additionally, kefir metabolite analysis was performed using gas chromatography–mass spectrometry (GC–MS). As a result of 16S rRNA analysis, the total bacterial abundances in the milk, kefir-citicoline (kefir-CT), and kefir samples were detected at rates of 82.83%, 79.23%, and 88.72%, respectively. The most dominant species in milk and kefir-CT samples were *Halorubrum sp.*, accounting for 9.03% and 10.98%, respectively, whereas the most predominant species in kefir samples was *Halostella sp.* (9.5%). As a result of 28S rRNA analysis, the most dominant species in milk, kefir-CT, and kefir were *Candida albicans* (76.8%), (65.3%), and (67.68%), respectively. Furthermore, the GC–MS method was used to characterise the qualitative and quantitative changes of metabolites in milk, kefir, and kefir-CT samples and to determine the differences between the samples. As a result, a total of 25 different compounds were detected in these samples. Metabarcoding and GC–MS analyses have shown that citicoline use alters bacteria, yeast, and total volatile components. However, the addition of novel ingredients to foods with a wide range of health benefits, given their high diversity, creates opportunities for scientific research and diverse applications in the food market. Nevertheless, further research is needed in the future to understand the mechanisms within the body and in foods before these supplements can be added to foods or replace their natural variants.

Keywords kefir, citicoline, 16S rRNA, 28S rRNA, volatile compounds

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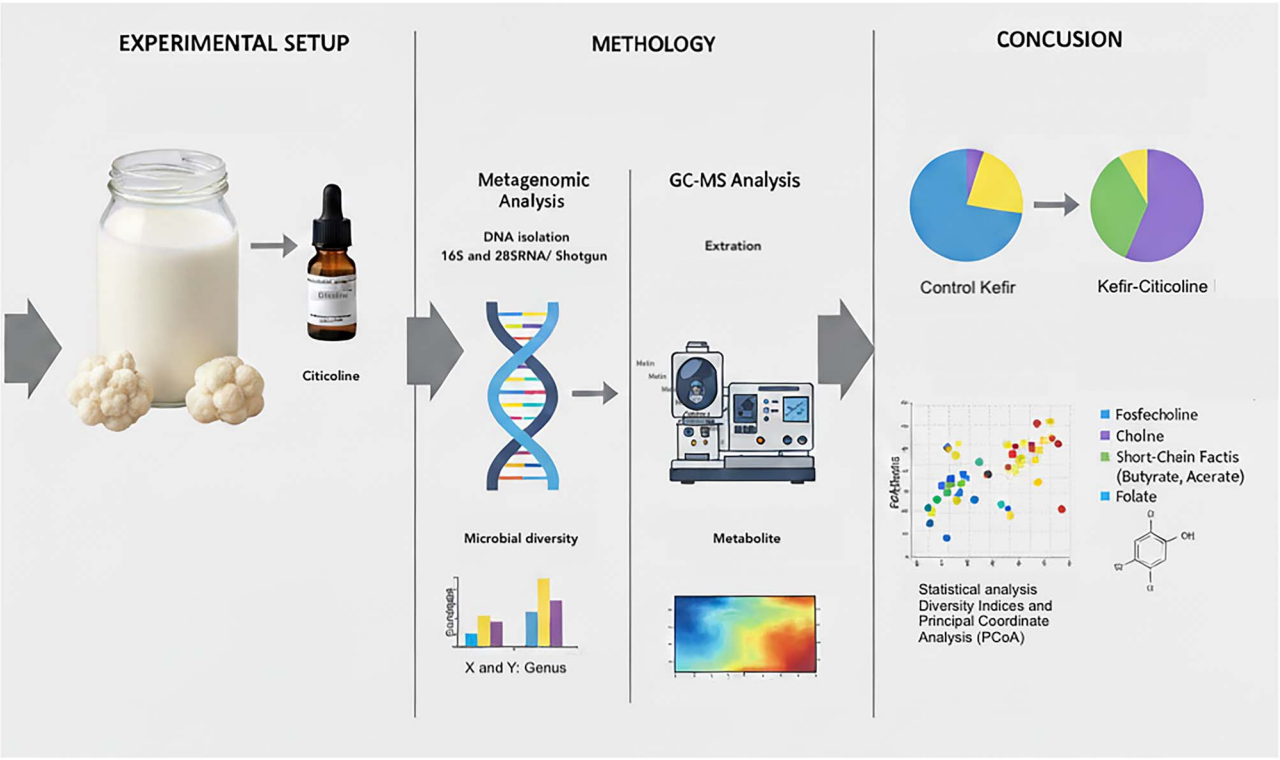
Graphical abstract



Introduction

Kefir is a slightly alcoholic fermented food originating from the Caucasus Mountains, characterised by its low acidity and creamy texture (Grønnevik et al., 2011). Traditional kefir is produced by adding kefir grains to milk, leading to the formation of an exopolysaccharide matrix during fermentation (Dallas et al., 2016; Prado et al., 2015). Among probiotic foods, milk kefir has gained popularity in recent years and attracted consumer interest due to its potential health benefits (Cufaoglu & Erdinc, 2023). Fermented foods such as kefir provide numerous public health benefits, including hypocholesterolemic, anticancer, wound-healing, anti-inflammatory, and antimicrobial effects (Azizi et al., 2021; Prado et al., 2015). These beneficial properties are largely attributed to the bioactive compounds formed during fermentation and the product's microbial composition; however, both factors vary depending on the source of the milk, fermentation parameters, and the origin of the kefir grains (Farag et al., 2020; González-Orozco et al., 2022; Satir et al., 2016). Kefir grains generally contain microorganisms belonging to the genera *Streptococcus*, *Lactococcus*, *Acetobacter*, *Lactobacillus*, and *Leuconostoc* (Bengoa et al., 2019; González-Orozco et al., 2022). Additionally, several probiotic yeast species *Kluyveromyces marxianus*, *Pichia pastoris*, *Kluyveromyces lactis*, *Pichia kluyveri*, and *Saccharomyces boulardii* have recently been shown to contribute to the sensory characteristics of probiotic and functional foods (de Souza et al., 2024). The metabolites produced during kefir fermentation including organic acids, ethanol, carbon dioxide, and bioactive peptides play a crucial role in defining its sensory and functional qualities (Ströher et al., 2025). Advances in metabolomics, transcriptomics, proteomics, and genomics have enhanced the characterisation of microbial communities in fermented and functional foods (Alraddadi et al., 2023). While some studies have focused on metagenomic profiling of kefir grains, recent research increasingly relies on rDNA amplicon sequencing using next generation sequencing (NGS) technologies to identify microbial populations in kefir (Ilıkkan et al., 2021; Zeng et al., 2022). Mass spectrometry (MS)-based approaches have also been widely employed to characterise bioactive molecules present in kefir (Al-Mohammadi et al., 2021; Amorim et al., 2019; Izquierdo-González et al., 2019; Piovesana et al., 2018; Santini et al., 2020; Savastano et al., 2020).

The incorporation of novel components into health-promoting foods has created new opportunities for scientific research and broadened potential applications in the food industry (Hossain et al., 2025). For example, synthetic novel foods enriched with vitamins and minerals are often developed to complement or replace natural sources, given that they can provide comparable health benefits. Citicoline ($C_{14}H_4N_4O_{11}P_2$) is an essential compound involved in cellular phospholipid biosynthesis and is naturally present in all living cells, where it plays a key role in maintaining cell membrane integrity and function (Świątkiewicz & Grieb, 2023). It has been widely used for its neuroprotective effects and in the management of chronic ischemic stroke and vascular cognitive impairment (Sufianova et al., 2023). With low toxicity and rapid metabolism in the body, citicoline exhibits antioxidant, anti-apoptotic, and anti-inflammatory properties and has shown potential in mitigating diabetic neuropathy, liver damage, and brain injury-induced retinopathy (Anandan et al., 2024). However, studies evaluating the use of citicoline as a food supplement remain

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scarce. Therefore, the present study focuses on characterising the effects of citicoline supplementation in kefir produced from traditional kefir grains, with particular emphasis on changes in bacterial and fungal populations as well as metabolic profiles.

Materials and methods

Sample preparation

Kefir grains (Doğadan, Türkiye) were continuously cultured in pasteurised fatty milk (3.7%) at 25 °C for 1 week before the experiments began. After 48 hr, the grains were separated using gauze (sterile), washed with water (sterile), and transferred to pasteurised milk. Citicoline (500 mg/ml) added during kefir fermentation was determined according to Matamoros et al. 2025 and Rossi et al. (2025).

DNA extraction process

Bacterial and fungal genomic DNA extractions of kefir and milk samples were performed using DNeasy PowerFood Mikrobiyal Kiti (QIAGEN, Germany). The amount of isolated DNA was determined fluorometrically using Qubit 3.0.

Minimal inhibitory concentration

The antibacterial activity of citicoline was determined using the broth microdilution method in a 96-well plate with nutrient broth (Merc, Germany). *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Klebsiella pneumonia* ATCC 1705 strains were used. For this purpose, the turbidity of all bacterial strains was adjusted to 10^6 CFU/ml MacFarland. Citicoline (500 mg/ml) was added to the first well, and dilutions of 250, 125, 62.5, 31.25, 15.625, and 7.8125 mg/ml were applied to the other wells. Bacteria-free broth was added to the last wells as a negative control and then incubated at 37 °C for 18 hr. After the incubation period, optical density values were measured at 620 nm, following the procedure described in previous studies. The analysis was performed in duplicate (Liu et al., 2020).

Metabarcoding analysis

Amplification of 16S rRNA

The V5–V7 regions of the 16S rRNA gene to be used in species determination were amplified using Simpli Amp Thermal Cycler with primer pairs (799F,5′-AACMGGATTAGATACCCKG-3′ and 1191R:5′-ACGTCATCCCCACCTTTCC-3′). Polymerase chain reaction (PCR) amplification conditions: 95 °C for 10 min, 95 °C for 30 s, 53–50 °C for 30 s, 72 °C for 90 s, and 35 cycles were performed. The temperature was reduced to 4 °C.

Amplification of 28S rRNA

Polymerase chain reaction was performed for 28S rRNA (D1 and D2 region) using ITS1 and ITS2 primer pairs (ITS1-F: 5′-CTTGGTCATTTAGAGGAAGTAA-3′ and ITS2-R: 5′-GCTGCGTTCTTCATCGATGC-3′). The Hot Star Master Mix Kit (BioRad) was used for the PCR mix. Polymerase chain reaction amplification conditions: 95 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min, 72 °C for 2 min, and 35 cycles were carried out. Sequencing of the prepared libraries was performed using the MiSeq system and paired-end sequencing (2 × 300 bp) with the MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA).

NGS and sample library preparation

To library preparation (16S rRNA) V5–V7 amplicon products were purified with the "Qiaseq beads Clean-Up Kit, Cat. No.: 180795" from Qiagen before sequencing. Library preparation for 16S rRNA V5–V7 amplicon products was done using Illumina's "Nextera XT DNA Library Prep Kit, Cat. No.: FC-131-1096" and indexing was done using "TG Nextera XT Index Kit v2 Set A (96 Indices, 384 Samples), Cat. No.: TG-131-2001". The concentrations of the libraries were determined using iQuant dsDNA HS Assay Kit (ABP Bioscience, USA). Sequencing was performed using the Miseq (Illumina) platform as paired end (PE) 2×150.

Polymerase chain reaction amplicons of the D1/D2 regions of the fungal 28S rRNA gene amplified with ITS1F/ITS2R primer sequences were purified with Qiagen's "Qiaseq beads Clean-Up Kit, Cat. No.: 180795" before sequencing. Library preparation studies were performed with Illumina's "Nextera XT DNA Library Prep Kit, Cat. No.: FC-131-1096" and indexing was performed with "TG Nextera XT Index Kit v2 Set A (96 Indices, 384 Samples), Cat. No.: TG-131-2001". The concentrations of the libraries were determined using the iQuant dsDNA HS Assay Kit (ABP Bioscience, USA) kit. Sequencing was performed with the iSeq100 (Illumina) platform as PE 2×150.

Gas chromatography–mass spectrometry analyses

Milk and kefir samples were dried with a liquid nitrogen process (Mudoor Sooresh et al., 2025). Samples were resuspended in freshly prepared methoxylamine hydrochloride (Sigma) solution (50 μl, 15 g L^{-1}). After the suspension period, the mixtures were homogenised at room temperature for 1 hr. 100 μl of N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was added to the homogenised samples. Then, they were incubated at 180 W for 2 min (Félix et al., 2018). During the derivatisation process, 0.100 g L^{-1} ribitol (sigma) was added to the sample as a standard. Gas chromatography–mass spectrometry (GC–MS) data were collected by repeatedly subjecting the same analytical procedures applied to the control and experimental groups. Metabolites were analysed using Shimadzu brand GCMS/GP2010 instrument (Japan). Rtx-5MS GC capillary column (length 30.0 m, diameter 0.25 mm) was used for separation. For the samples, the oven was set at 70 °C (1 min); the column temperature was set at 170 °C, and the column temperature was set at 280 °C (8 min). Helium (2 ml per minute) was used as the carrier gas. The GC injector was set at 280 °C in non-split mode. Measurements were carried out under electron impact (EI) ionisation (70 eV) at a frequency of 3.9 Hz in full scan mode (m/z 45–650). The temperatures of the EI ion source and the quadrupole mass filter were kept at 200 and 280 °C, respectively. Each compound was identified by comparing their experimental mass spectra with those in the NIST library.

Physico-chemical analysis of milk, kefir, and kefir-CT

pH analysis

After fermentation (48 hr), the pH values of kefir and milk samples were measured using a pH metre.

Lactose analysis

Lactose in kefir and milk samples was analysed using a Lactoscan Sp60 milk analyser.

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Table 1 Distribution of relative abundance of samples for bacterial and fungal.

Distribution ratios of bacterial							
Samples	**Superkingdom**	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**
Kefir-CT	100.00%	100.00%	100.00%	92.73%	85.78%	80.56%	64.11%
Kefir	100.00%	100.00%	100.00%	95.26%	88.32%	78.15%	61.76%
Milk	100.00%	100.00%	100.00%	86.29%	86.13%	85.99%	67.83%
Distribution rates of fungal							
Kefir-CT	100.00%	100.00%	99.07%	98.43%	98.43%	98.36%	98.26%
Kefir	100.00%	100.00%	96.94%	96.94%	97.01%	96.81%	96.56%
Milk	100.00%	100.00%	99.11%	98.79%	98.83%	98.83%	98.79%

Note. CT = citicoline.

Titratable acidity

Determination of titratable acidity was done according to the protocol reported by Palamutoğlu et al. (2023). Briefly, 10 ml of kefir samples were transferred into an Erlenmeyer (250 ml), and then 10 ml of distilled water at 20 °C and four drops of phenolphthalein were added. The mixture was titrated with 0.1 N NaOH until a permanent pink colour appeared. The lactic acid content was calculated based on the volume of NaOH used.

Ash analysis

For analysis, the crucibles were first heated in a muffle furnace at 550 °C for 2 hr and cooled in a desiccator for 30 min. Approximately, 100 ml kefir samples obtained after 48 hr of fermentation were weighed and heated at 250 °C preheat for 1 hr. Then, it was heated to 550 °C to obtain white ash. After cooling, the mass was weighed again, and the ash percentage was calculated.

Results

Antimicrobial effect

Antimicrobial effect was determined with the broth microdilution method in a 96-well plate. The results show that the citicoline at 500 mg/ml had antibacterial properties against *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *K. pneumonia* ATCC 1705.

Distribution of relative abundance (kefir, kefir-CT, and milk)

Family-level abundance rates were 85.78% *Streptomycetaceae* in kefir-CT, 88.32% *Pseudomonadaceae* in kefir, and 86.13% *Flavobacteriaceae* in the milk sample. At the genus level, *Pseudomonas* (80.56%), *Streptomyces* (78.15%), and *Acinetobacter* (85.99%) were dominant (Table 1, Figures 1 and 2). Furthermore, the species-level distributions were 64.11% *Halostella sp.* in kefir-CT, 61.76% *Halorubrum sp.* in kefir, and 67.83% *Halorubrum sp.* in milk (Figure 1). Based on the 16S rRNA analysis, the total bacterial abundances in the milk, kefir-CT, and kefir samples were determined to be 82.83%, 79.23%, and 88.72%, respectively. The most dominant species in the milk and kefir-CT samples was *Halorubrum sp.*, whereas *Halostella sp.* was the predominant species in kefir. Conversely, the least abundant species were identified as *Catenovulum sediminis* in milk, *Micromonospora krabiensis* in kefir, and *Myroides odoratimimus* in kefir-CT. Interestingly, *Klebsiella michiganensis*, *K. pneumoniae*, and *E. coli* were detected in both the milk and kefir samples but were absent from kefir-CT. Additionally, an increase in *Cyanobacteriota* (6.28%) was observed specifically in the kefir-CT sample, in contrast to the others (Table 2).

Distribution of relative abundance (kefir, kefir-CT, and milk)

The levels of *Debaryomycetaceae* in the kefir-CT, kefir, and milk samples were 98.43%, 97.01%, and 98.83%, respectively. In these samples, the proportions of *Candida spp.* were determined as 98.36%, 96.81%, and 98.83%. Notably, the distribution of *Candida albicans* was found to be 98.26%, 96.56%, and 98.79% in the respective samples (Table 1, Figure 2).

As a result of 28S rRNA analysis, the most dominant species in milk, kefir, and kefir-CT was *C. albicans* (76.8%), (65.3%), and (67.68%). In contrast, the least species were identified as *Trichophyton rubrum* in milk, *Pyrenophora tritici-repentis* in kefir, and *Neurospora tetrasperma* in kefir-CT. Interestingly, while the *Sordaria macrospora* species was detected in kefir with added citicoline, this species was not found in kefir, milk and kefir-CT (Figure 2 and Table 2).

Metabolomic analysis of milk kefir and kefir-CT

GC-QqQ-MS was used to characterise the qualitative and quantitative changes of metabolites in milk, kefir, and kefir-CT samples during fermentation, to identify differences between the samples, and to determine volatile compounds. In total, 25 unique compounds were detected across the samples. In the milk sample, the identified compounds included cyclopentanone, O-methyloxime, trans-1-butene, 1-butoxy, 2-furaldehyde O-methyloxime, 1-butanol, tert-butyldimethylsilyl ether, orthoformic acid, tri-sec-butyl ester, and 1-butoxy-1-isobutoxy-butane. Additional compounds, such as butane, 1,1-dibutoxy, 2,5-cyclohexadiene-1,4-dione dioxime, and various fatty acids were also detected. In contrast, the kefir-CT sample contained 2-isopropyl(dimethyl) silyloxypropane, tert-butyldimethylsilyl formate, 2-heptanol, 2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one, isomaltol, propionic acid (chloro-2-hydroxy), methyl 1-methylpyrrole-2-carboxylate, and 2,3-methylenedioxyphenol, among others. For the kefir sample, the detected metabolites included 3-hydroxy-8,9-didehydroadamantan-2-one, hexane-1,6-dimethoxy, 4-cyclopentene-1,3-dione, 1-propylmethyl ether, 2-butanol-1-methoxy, alpha and beta-crotonolactone, acetic acid 1-dimethyl(isopropyl) silyloxybutane, L-phenylalanine N-formyl, isomaltol, and 2,3-methylenedioxyphenol.

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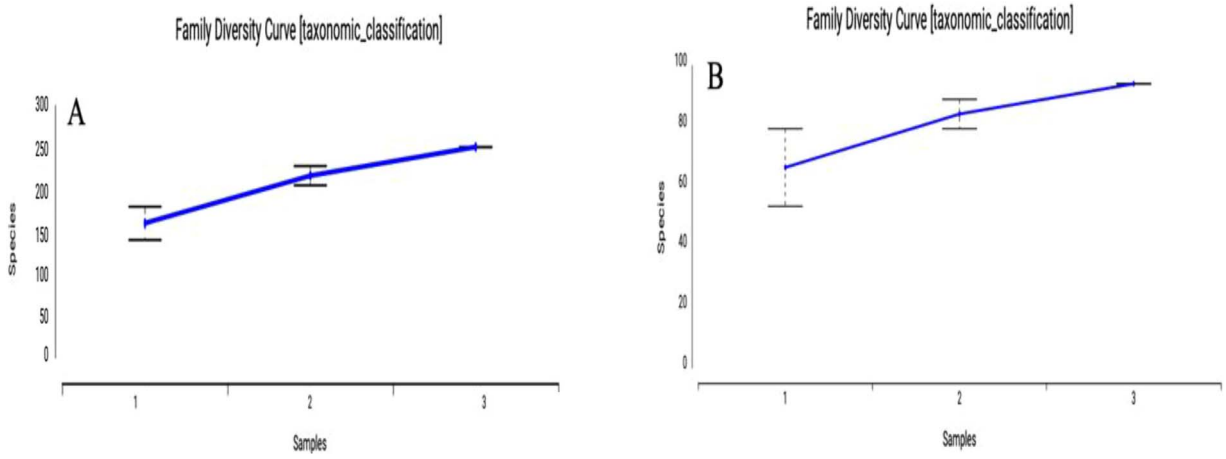

Figure 1 (A) Cumulative bacterial species richness showing the change in total species number detected across three different samples (Samples 1: milk, 2: kefir, and 3: kefir-CT). (B) Cumulative fungal species richness showing the change in total species number detected across three different samples (Samples 1: milk, 2: kefir, and 3: kefir-CT).

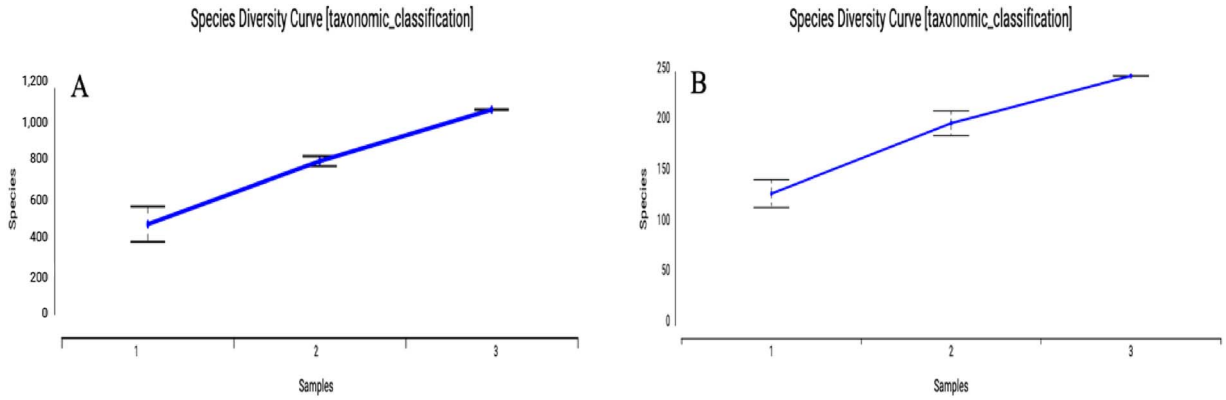

Figure 2 (A) Bacterial species richness and cumulative bacterial species curve obtained within the scope of the samples (Samples 1: milk, 2: kefir, and 3: kefir-CT). (B) Fungal species richness and cumulative fungal species curve obtained within the scope of the samples (Samples 1: milk, 2: kefir, and 3: kefir-CT).

Notably, only isomaltol and 2,3-methylenedioxyphenol were common to both kefir samples (Table 3 and Figure 3).

Results of physico-chemical

The pH of kefir was 4.35 after fermentation, while the pH of kefir-CT was 4.24. After 48 hr of fermentation, the titratable acidity was observed to be between 0.62 and 0.74 lactic acid in kefir-CT, while it was between 0.78 and 0.80 in kefir. While the lactose content in the milk sample was recorded as 3.1 g, the lactose concentration in mature kefir-CT was recorded as 3.9 g/100. However, it was determined as 4.06 g/100 g in mature kefir without citicoline. While the ash content in the matured kefir-CT was 0.843%, the ash content in the kefir was found to be 0.829%.

Statistical analyses

Statistical analysis was performed using the Shannon–Wiener diversity index. The Shannon index usually takes a value between 1.5 and 3.5, and as this index increases, diversity also increases. Simpsons index takes a value between 0 and 1.1 means diversity, 0 means no diversity (Table 4). According to the 16S rRNA and 28S rRNA analysis results, bacterial and fungal statistics were found to be significant in all samples ($p < .05$). Based on diversity indices and principal coordinate analysis (PCoA), the highest family diversity for bacteria was seen in milk, followed by kefir (with CT) and kefir (without CT). The artificial variation axis separating the kefir (with CT) and kefir (without CT) samples was found to be 62%. The main difference separating the milk group vertically between the two kefirs was found to be 37%. The highest family diversity for fungal was observed in kefir (with CT), kefir (without CT), and milk, followed by kefir (with CT). The artificial variation axis separating the kefir (with CT) and kefir (without CT) samples was found to be 62%. The main difference separating the milk group vertically between the two kefirs was found to be 37% (Figure 4). According to PCoA, the highest species diversity for bacteria was seen in milk, followed by kefir (with CT) and kefir (without CT). The artificial variation axis separating the kefir (with CT) and kefir (without CT) samples was found to be 60%. The main difference separating the milk group vertically between the two kefirs was found to be 39%.

The highest species diversity for fungal was observed in kefir (with CT), kefir (without CT), and milk, followed by kefir (with CT). The artificial variation axis separating the kefir (with CT) and kefir (without CT) samples was found to be 60%. The main difference separating the milk group vertically between the two kefirs was found to be 39% (Figure 5).

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Table 2 Species distribution (bacterial and fungal) relative abundance of samples.

Bacteria					
Kefir-CT	**%**	**Kefir**	**%**	**Milk**	**%**
Halorubrum sp. SS7	10.98	*Halostella sp. PRR32*	9.65	*Halorubrum sp. SS7*	9.03
Syntrophobotulus glycolicus	9.17	*Acidovorax sp. BLS4*	8.82	*Tenacibaculum singaporense*	6.41
Qipengyuania psychrotolerans	5.47	*Acidiplasma cupricumulans*	6.03	*Escherichia coli*	5.05
Defluviimonas sp. SAOS-178_SWC	4.97	*Klebsiella pneumoniae*	5.85	*Thermococcus sp. Bubb.Bath*	5.01
Phycisphaera mikurensis	4.26	*Bacillus amyloliquefaciens*	5.27	*Mucilaginibacter gotjawali*	3.74
Pontibacillus sp. ALD_SL1	3.56	*Methanobrevibacter smithii*	5.25	*Flavobacterium sp. KACC 22763*	3.42
Tenacibaculum singaporense	2.75	*Halorubrum sp. SS7*	4.8	*Methanobrevibacter olleyae*	3.18
Acidovorax sp. BLS4	2.73	*E. coli*	3.9	*Micromonospora krabiensis*	2.94
M. krabiensis	2.73	*Myroides odoratimimus*	2.94	*Klebsiella michiganensis*	2.63
M. odoratimimus	2.35	*M. krabiensis*	2.13	*Catenovulum sediminis*	2.63
Fungal					
Candida albicans	65.3	*C. albicans*	67.68	*C. albicans*	76.8
Capronia epimyces	13.36	*Lasiodiplodia theobromae*	9.96	*Cadophora gregata*	3.64
Cadophora gregata	2.76	*Cadophora gregata*	3.2	*Rhizomucor pusillus*	2.9
Aspergillus versicolor	2.61	*Leptographium clavigerum*	3.0	*Trametes versicolor*	2.82
Rhizophagus irregularis	2.04	*Alternaria metachromatica*	3.0	*Aspergillus glaucus*	2.12
Rhizomucor pusillus	1.82	*Alternaria hordeiaustralica*	1.1	*Rhizophagus irregularis*	1.27
Trametes versicolor	1.75	*Alternaria infectoria*	1.1	*Neurospora tetrasperma*	0.78
Leptographium clavigerum	1.42	*Rhizophagus irregularis*	0.9	*Alternaria metachromatica*	0.53
Sordaria macrospora	1.29	*Trametes versicolor*	0.87	*Leptographium clavigerum*	0.45
Neurospora tetrasperma	0.7	*Pyrenophora tritici-repentis*	0.47	*Trichophyton rubrum*	0.41

Microorganism (bacterial and fungal)–metabolite relationship analysis

Following false discovery rate method to identify strong positive and negative correlations observed in the obtained *p*-values. Correlation heatmap visualisation was used to present the correlation pattern between all microorganisms (bacterial and fungal) metabolite pairs. As a complement to the correlation heatmap, only the strongest relationships were highlighted using a network graph. The potential relationships between the bacterial compositions and metabolite profiles obtained in the study were evaluated using heatmaps (Figure 6) and network graphs based on Spearman correlation analysis (Figure 7). A wide range of correlations, both positive and negative, was identified between bacteria and metabolites. Metabolites, such as 1-butanol-tert-butyldimethylsilyl-ether, 1-butoxy-1-isobutoxy-butane, and cyclopentanone_o-methyloxime, exhibited strong positive associations with many bacteria, while correlations with some metabolites, such as isomathol and acetic acid, were found to be weaker or negative. Potential relationships between microorganism data (bacterial and fungal) and metabolite profiles were further analysed. For this purpose, the Spearman correlation coefficient (ρ) was calculated for all microorganism (bacterial and fungal)–metabolite pairs. Multiple testing correction was performed using the Benjamini–Hochberg Potential relationships between fungal compositions and metabolite profiles obtained in the study were assessed using heatmaps and network graphs based on Spearman correlation analysis. Some metabolites exhibited positive correlations with multiple fungal, while others showed negative associations. The correlation between bacteria and metabolite profiles was visualised using a network graph complementing the heatmap, highlighting only the strongest relationships. Metabolites, such as 1-butanol-tert-butyldimethylsilyl-ether and 1-dimethyl-(isopropyl)-silyoxybutane, were found to be hubs and associated with numerous bacterial and fungal (*M. odoratimimus*, *Halorubrum sp.*, *Acidovorax sp.*, and *M. krabiensis*) and (*Cadophora gregata*, *Rhizophagus irregularis*, *Trametes versicolor*, and *Neurospora tetrasperma*).

Discussion

In recent years, scientific literature has increasingly supported the health benefits of kefir, a beverage historically known as a probiotic. Additionally, consumers' beliefs and perceptions of functional and beneficial foods have recently gained importance (Valletta et al., 2023). In foods obtained through fermentation and containing certain microbial taxa, it is necessary to make new additions to meet the minimum requirements to improve the food. Recent studies have shown discrepancies between the declared bacterial numbers and

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Table 3 Volatile compounds identified in all samples.

Volatile compounds														
Milk					**Kefir-CT**					**Kefir**				
	Peak IDs	**Aera%**	**Height %**	**A/H**		**Peak IDs**	**Aera%**	**Height %**	**A/H**		**Peak IDs**	**Aera%**	**Height %**	**A/H**
Cyclopentanone, O-methyloxime	2	3.89	5.68	2.83	2-Isopropyl (dimethyl) silyloxypropane	1	1.28	5.87	1.12	3-Hydroxy-8,9-didehydroadamantan-2-one	1	1.75	4.06	1.24
Trans-1-Butene, 1-butoxy	3	5.65	8.20	2.84	Tert-butyldmethylsily formate	2	7.70	14.92	2.62	Hexane, 1,6-dimethoxy	4	4.75	10.81	1.26
2-Furaldehyde O-methyloxime	9	1.97	4.35	1.86	2-Heptanol	7	1.58	1.80	4.48	4-Cyclopentene-1,3-dione	5	2.94	4.53	1.86
1-Butanol, tert-butyldimethylsily ether	11	13.74	10.12	5.61	2,4 Dihydroxy-2, 5-dimethyl-3 (2H)-furan-3-one	8	3.09	3.34	4.74	1-propylmethyl ether	7	3.15	6.99	1.29
Orthoformic acid, tri-sec-butyl ester	15	1.35	1.92	2.90	Isomathol	9	5.46	5.70	4.92	2-Butanol, 1-methoxy	9	2.07	3.48	1.71
1-Butoxy-1-isobutoxy-butane	16	4.25	2.38	7.36	Proponic acid, chloro-2-hydroxy	10	5.08	4.27	6.11	Alpha, Betta, Crotonolactone	10	0.66	5.58	2.88
Butane,1,1- dibutoxy	17	2.51	1.98	5.25	Methyl 1-methylpyrrole-2-carboxylate	11	1.41	1.94	3.71	Acetic acid	11	12.42	11.88	3.00
2–5 Cyclohexadiene-1,4-dione, dioxime	18	2.44	2.37	4.25	2,3-Methylenedioxyphenol	15	13.66	5.55	12.59	1-dimethyl (Isopropyl) silyoxybutane	15	1.62	1.72	2.71
Butane, 1,1-dibutoxy	19	1.54	1.05	6.06						L-Phenylalanine, N-formyl	18	1.81	1.66	3.12
										Isomalthol	13	4.38	2.29	5.48
										2,3-Methylenedioxyphenol	19	18.38	8.30	6.35

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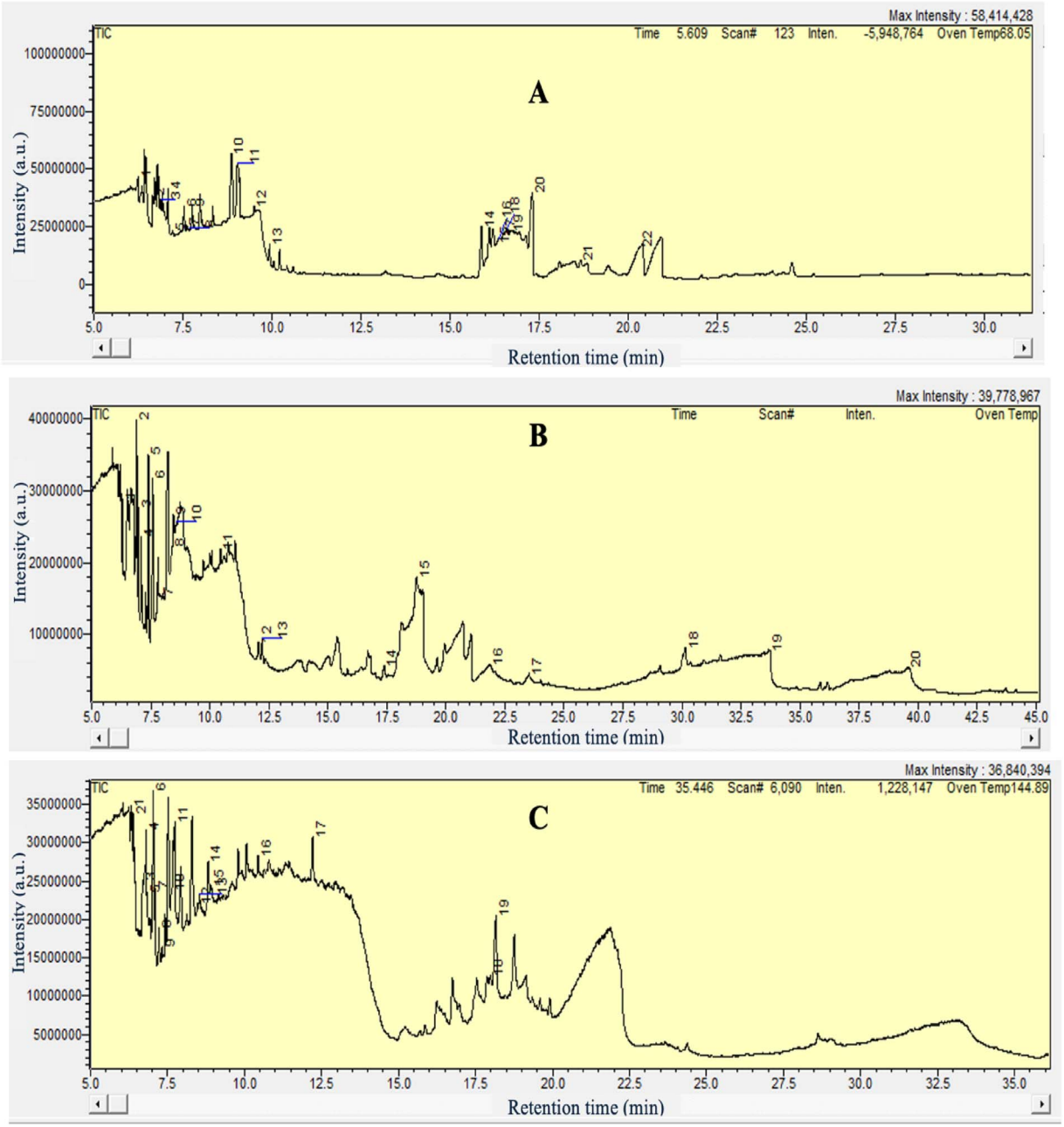

Figure 3 (A) Typical chromatogram profile representing the extraction of volatile aromatic compounds in the milk sample. (B) Typical chromatogram profile representing the extraction of volatile aromatic compounds in the kefir-CT sample. (C) Typical chromatogram profile representing the extraction of volatile aromatic compounds in the kefir.

taxa in probiotic products and those measured experimentally (Chen et al., 2017; Metras et al., 2021; Morovic et al., 2016).

Antimicrobial effect was determined using the broth microdilution method. The results show that citicoline at 500 mg/ml had antibacterial properties against *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *K. pneumonia* ATCC 1705 in this study.

Similar to this study, a study showed a significant increase in *Bacteroides spp.*, *Fusobacterium spp.*, *E. coli*, *Clostridium spp.*, *Providencia spp.*, *Prevotella intermedia*, and *Porphyromonas gingivalis* in rats given 500 mg compared to a normal healthy control group. In this study, it was reported that co-administration of citicoline and *Lactobacillus* significantly increased the intestinal microbiota compared to the positive control group (El-Baz et al., 2023). Recent studies have shown discrepancies between the declared bacterial counts and taxa listed on probiotic product labels and those identified experimentally (Barukčić et al., 2017; Leite et al., 2013). In the current study, the pH of kefir without citicoline was recorded as 4.35 upon fermentation, while the pH of kefir containing citicoline was recorded as 4.24. In a study conducted by Ströher et al. (2025), the pH of kefir was recorded as 4.40 after a 24-hr fermentation period.

After 48 hr of fermentation, the titratable acidity was observed to be between 0.62 and 0.74 lactic acid in kefir with citicoline, while it was observed to be between 0.78 and 0.80 in kefir without citicoline. In another study (Gul et al., 2018), a titratable acidity of 0.64–0.76 g of was recorded in kefir after 24 hr of fermentation, which is lower than the result of this study. In contrast, in another study by

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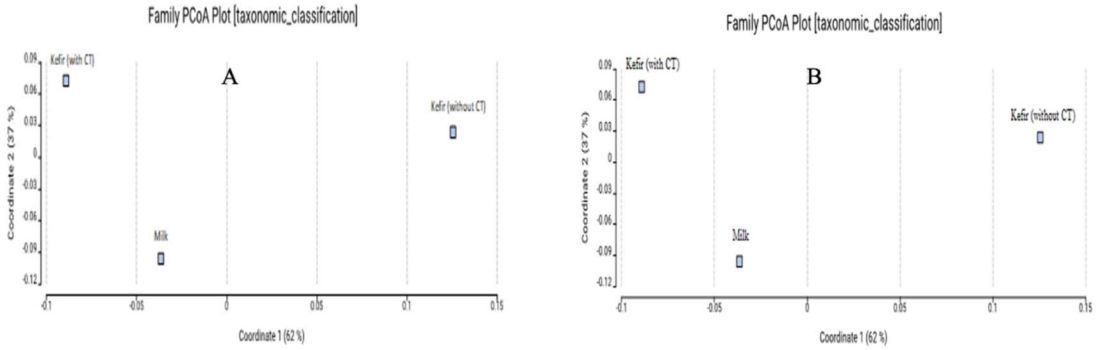

Figure 4 (A) Bacterial family-level PCoA plot, each point represents a sample, and the proximity between points indicates the degree of similarity in their microbial composition. (B) Fungal family-level PCoA plot each point represents a sample and the proximity between points indicates the degree of similarity in their microbial composition.

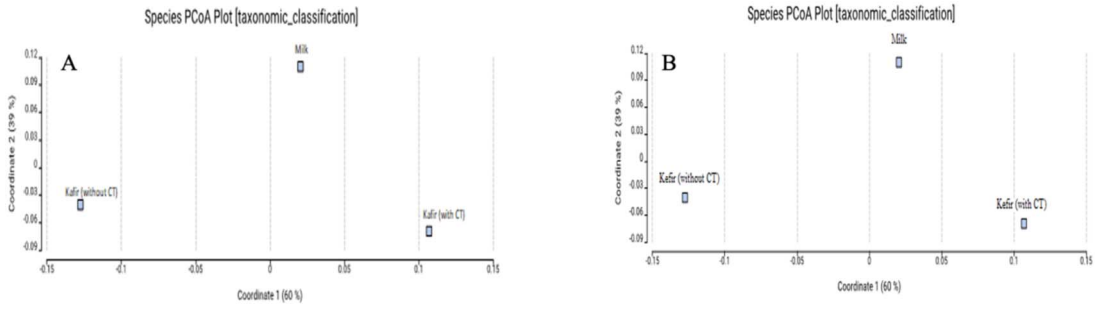

Figure 5 (A) Principal coordinate analysis (PCoA) of bacterial communities at the species level. Points are coloured by (sample) and axes represent the percentage of variation explained. (B) Principal coordinate analysis (PCoA) of fungal communities at the species level. Points are coloured by (sample) and axes represent the percentage of variation explained.

Table 4 Taxonomy statistics for bacterial and fungal.

Diversity of bacterial at the species level		
Samples	**Shannon index (H)/(H/LN(N))**	**Simpsons index (D−1)**
Kefir-CT	4.297/0.6875	0.9641
Kefir	4.261/0.6876	0.9627
Milk	4.451/0.7408	0.9724
Diversity of fungal at the species level		
Kefir-CT	1.598/0.3243	0.5527
Kefir	1.618/0.3319	0.5287
Milk	1.349/0.2813	0.4064

Note. The Shannon index ((H)/(H/LN(N)) generally takes a value between 1.5 and 3.5, and as this index increases, diversity increases. The Simpsons index (D−1) takes a value between 0 and 1. 1 means diversity, and 0 means no diversity.

Windayani et al. (2019), it was reported that titratable acidity was 1.96–2.64 g.

During kefir fermentation, the β-galactosidase enzyme possessed by Lactic Acid Bacteria (LAB) hydrolyses lactose into glucose and galactose, converting it into lactic acid and at the same time transforming into other metabolites. In this way, the product has sensory and nutritional properties (Bengoa et al., 2019). This makes kefir more tolerable for people with lactose intolerance (Rosa et al., 2017). In this study, lactose ratios in kefir with and without citicoline were determined as 3.9 g/100 and 4.06 g/100 g, respectively. In another study, the lactose content of kefir was recorded as 4.01 g/100 g (Ströher et al., 2025).

The coagulation of milk proteins (casein) under the influence of lactic acid bacteria contributes to the high protein content of kefir and therefore to the increase in the ash content. In this study, the ash content in the kefir with added citicoline was found to be 0.843% and the ash content in the kefir without added citicoline was found to be 0.829%. In another study, the ash content of kefir was reported to be 0.831% (Ströher et al., 2025).

While 16S ribosomal RNA (rRNA) is a common choice for bacteria, more variability in the target gene to be used has been highlighted for fungal. The most used method is the internal transcribed spacer (ITS), thanks to the existence of a curated database. However, the unequal ITS length among species plays an important role in obtaining shorter fragments at the PCR stage and obtaining Operational Taxonomic Unit (OTUs) with shorter ITS fragments. Since OTU abundance is proportional to the number of reads obtained, this may lead to incorrect estimation of OTU abundance (Bokulich & Mills, 2013; De Filippis et al., 2014; Ercolini et al., 2013). Therefore, it may also be advisable to use different targets, such as 26S (Garofalo et al., 2015; Stellato et al., 2015; Wang et al., 2015) or 18S rRNA (Liu et al., 2015; Minervini et al., 2015) genes, which requires focusing on different regions.

The most dominant species in milk and kefir-CT was *Halorubrum sp.* while the most dominant species in kefir CT samples was *Halostella sp.* On the other hand, the least species were identified as *C. sediminis* in milk, *M. krabiensis* in kefir CT and *M. odoratimimus* in kefir-CT. The reason for this result can be attributed to the fact that cyanobacteria

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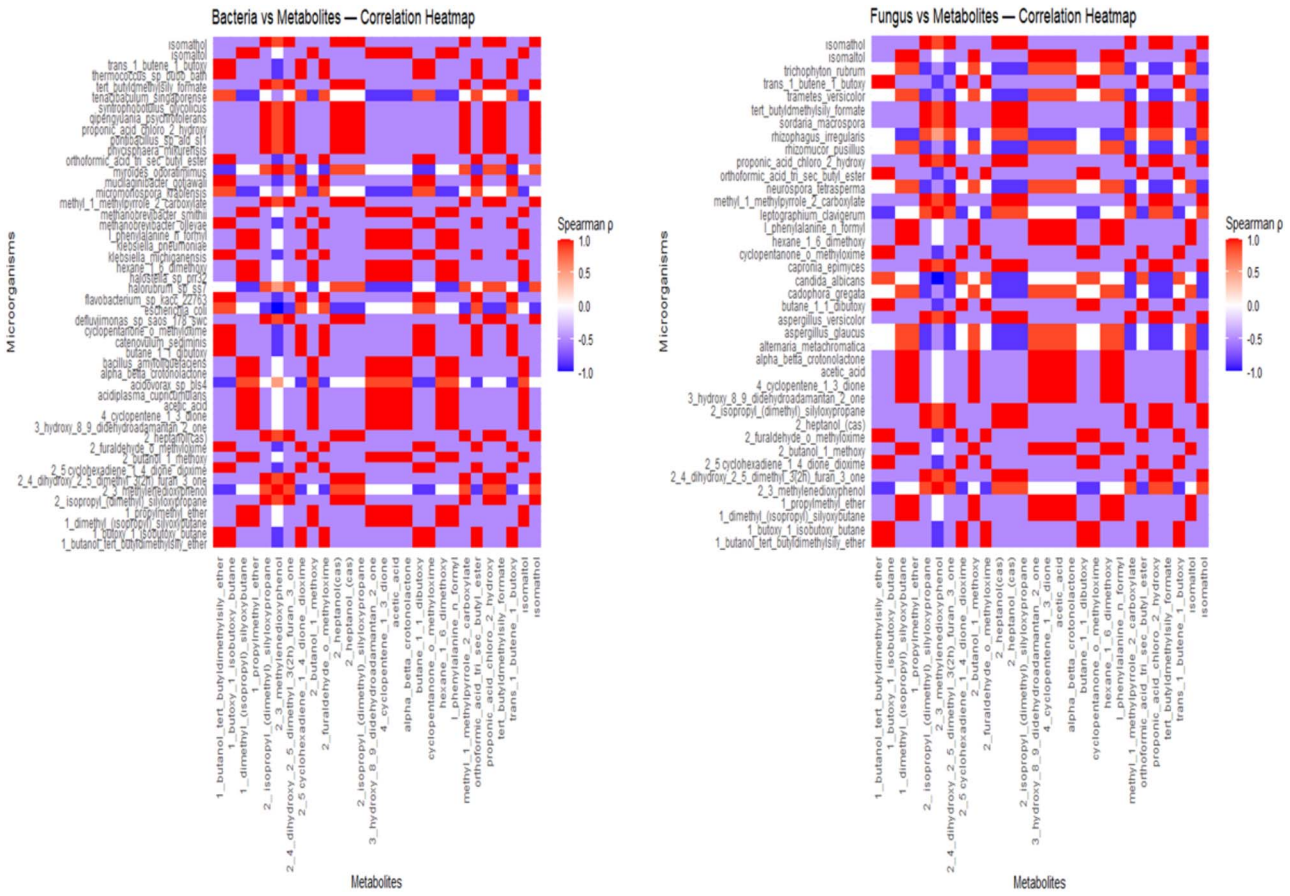

Figure 6 Heatmap visualising relationships between bacterial and fungal metabolite profiles based on spearman correlation coefficients. (Red tones indicate positive correlation and blue tones indicate negative correlation).

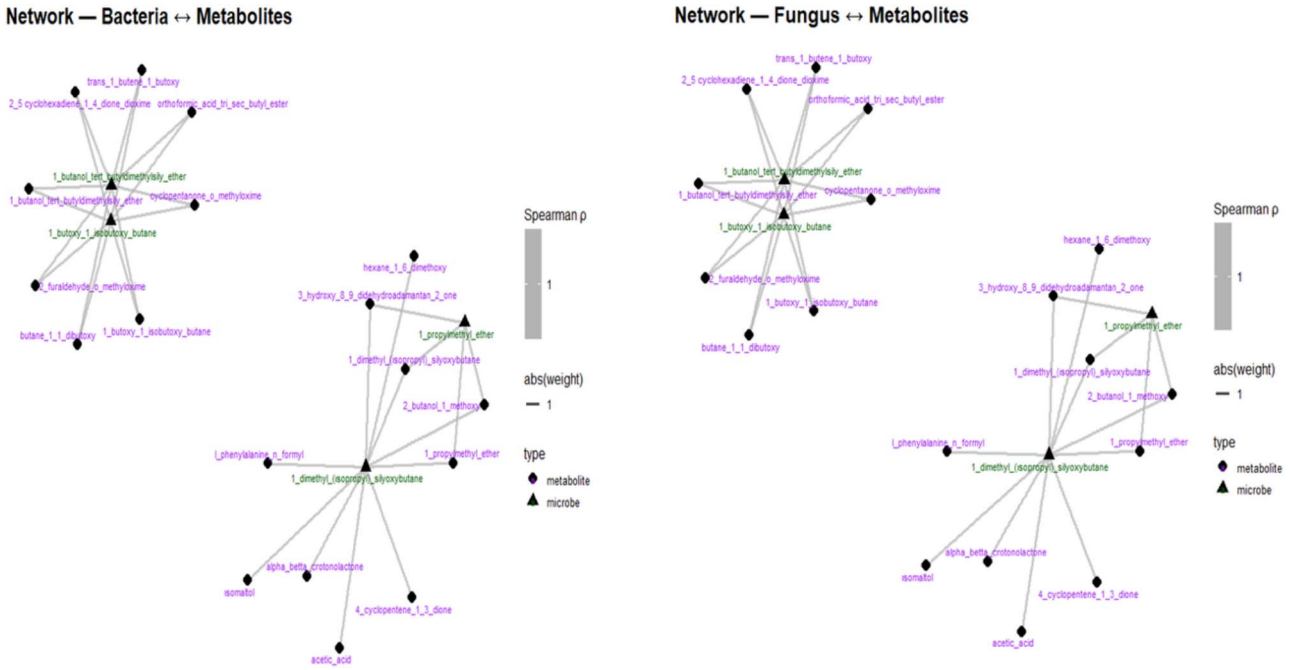

Figure 7 Network map visualising the relationships between bacterial and fungal communities and metabolite profiles using network analysis.

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contribute to fermentation by affecting taste, texture, and nutritional content with their ability to photosynthesise and fix atmospheric nitrogen, and contribute to the diversity of bacterial and fungal by producing exopolysaccharides and antimicrobial substances that contribute to the safety and quality of fermented foods (Wang et al., 2020a; Yang et al., 2022).

In a study conducted by Liu et al. (2025), contrary to the results of this study, kefir 1 sample contained high levels of *Listeria*, but there were many other genera belonging to the *Proteobacteria* phylum. However, the kefir 2 sample was reported to be rich in *Enterococcus*, *Bacillus*, and *Streptococcus*, parallel to the results of this study. In addition, in parallel with the results obtained in the kefir sample in this study, Liu et al. (2025) stated that kefir 3 sample was dominated by the *Proteobacteria* phylum, including *Escherichia*, *Enterobacter*, and *Pseudomonas*, and many other genera. Interestingly, while food pathogens *K. michiganensis*, *K. pneumonia*, and *E. coli* were found in milk and kefir, these bacteria were not found in kefir-CT. The unexpected detection of several common pathogenic bacteria (*Escherichia, Klebsiella*, and *Listeria*) in the kefir sample in this study suggests that the individual pathogen may not have the capacity to cause harmful health effects for consumers (Liu et al., 2025). This result in the study could be explained by the possibility of *Klebsiella* and *Listeria* originating from cattle faeces or contamination from environmental sources. Furthermore, the presence of coliform bacteria in milk can be an indicator of unsanitary conditions in milk production (Martin et al., 2016). However, the absence of these pathogens in the kefir-CT sample is the presence of antimicrobial activity of citicoline.

The most important features of cyanobacteria are that they contain high-quality biomolecules, such as proteins, carbohydrates, and essential fatty acids, in their structures. Passos et al. (2023) conducted a comprehensive systematic analysis of these components in cyanobacteria in a study and reported that they are rich in polyunsaturated fatty acids, such as alpha-linolenic acid and other omega-3 precursors, some of which are known for their cardiovascular and immunological health benefits in humans and animals. The presence of these essential compound's positions cyanobacteria as promising nutritional supplements. In addition to fatty acids, proteins in their structures, when combined with carbohydrates, act as prebiotic agents that can affect the host's intestinal microbiota and offer benefits for animal production systems aiming to optimise intestinal health (Reyes et al., 2025). Interestingly, an increase in *Cyanobacteriota* was observed in kefir-CT unlike the other sample in this study.

In recent years, high-resolution MS approach has been used to identify milk proteins and derived bioactive peptides in cow's milk kefir before and after fermentation (Amorim et al., 2019). Some studies have characterised bioactive peptides produced from milk proteins in organic cow's milk kefir samples (Santini et al., 2020) and peptide profile of goat's milk kefir at different fermentation times using LC-ESI MS/MS-IT. We used GC-QqQ-MS to characterise the qualitative and quantitative changes of metabolites and the differences between samples in milk, kefir samples without and with citicoline during the fermentation period and to identify volatile compounds. In total, we found 25 unique compounds in all different samples. In this study, we characterised the qualitative and quantitative changes of metabolites in milk, kefir-CT samples with and kefir during fermentation and to characterise the differences between the samples. In total, we found 25 unique compounds in all different samples. Cyclopentanone, O-methyloxime, trans-1-butene, 1-butoxy, 2-furaldehyde O-methyloxime, 1-butanol, tert-butyldimethylsilyl ether, Orthoformic acid, tri-sec-butyl ester, and 1-butoxy-1-isobutoxy-butane in milk sample. Propanoic acid present in kefir containing citicoline is released because of lipid and carbohydrate metabolism and has been reported to play a role in the formation of milky, fruity and floral taste in the sensory properties of kefir Dertli & Çon (2017). In kefir containing citicoline, 2-heptanol has been reported to add earthy, oily, and mushroom aromatic notes to kefir (Carunchiawhetstine et al., 2003; Sarhir et al., 2021). Similarly, 2-heptanol has been reported to provide a green and floral aroma to the product (Wang, et al., 2020a). It has been reported to have antimicrobial potential against fungal and *Enterobacteriaceae* (Fraud et al., 2003). Additionally, the isometholes present in both kefirs are released because of amino acid metabolism and give kefir a distinctive cheese and fruity taste (Walsh et al., 2016). *Cyanobacteriota* significantly alter the flavour and aroma characteristics of fermented foods. During the fermentation process, flavour and aroma are primarily determined by the production of esters, alcohols, organic acids, and other volatile compounds. *Cyanobacteria* also synthesise numerous secondary products, including phenolic acids, flavonoids, chlorophylls, and carotenoids, altering the overall flavour and aroma of the product (Nawaz et al., 2024). However, in the present study, metabolites, such as 1-butanol-tert-butyldimethylsilyl-ether and 1-dimethyl-(Isopropyl)-silyoxybutane, were found to be central and associated with many bacterial and fungal (*M. odoratimimus*, *Acidovorax sp.*, and *M. krabiensis*) and (*C. gregata, R. irregularis*, *T. versicolor*, and *N. tetrasperma*). However, unlike milk and kefir samples, 2-heptanol, 2,4-dihydroxy-2, 5-dimethyl-3(2H)-furan-3-one, propionic acid, chloro-2-hydroxy, and methyl 1-methylpyrrole-2-carboxylate metabolites were found in the kefir-CT sample. The reason for this different result may be attributed to the synthesis of secondary metabolites such as alcohols and organic acids by *Cyanobacteriota* in fermented foods (Nawaz et al., 2024; Ströher et al., 2025a).

Conclusion

This study provides a comprehensive evaluation of the microbiological change, as well as the metabolic and physicochemical characteristics, of the kefir microbiota in response to citicoline supplementation. The findings indicate that the inclusion of citicoline results in slight modifications to the microbial composition and metabolic activity of kefir. *Enterococcus* species were identified as the dominant bacterial group in both citicoline-supplemented and control samples, while *Candida* species represented the predominant yeasts in both formulations. Physicochemical analyses revealed that the kefir without citicoline exhibited a lower pH and higher titratable acidity compared to the citicoline-supplemented counterpart. The incorporation of novel bioactive compounds into traditionally fermented foods expands their potential applications in both scientific research and the food industry, as they confer a diversity of functional properties. Nevertheless, further studies are required to elucidate the underlying mechanisms by which such supplements interact with both the human body and food matrices, to ensure safe and effective integration into food products or substitution for natural variants.

Author contributions

Mukaddes Barel (Methodology, Formal analysis, Writing—original draft, Writing—review & editing)

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Conflicts of interest

The authors declare that they have no competing interests.

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