

Article

Microbial Diversity and Chemical Dynamics in Karanda Juice Kefir During Fermentation

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Abstract

Water kefir is a non-dairy fermented beverage widely recognised for its health benefits. The supplementation of fruits can further diversify product offerings while enhancing nutritional and functional value. In this study, karanda juice, derived from a fruit native to Thailand and known for its beneficial health properties, was used as a substrate for water kefir production, with the aim of increasing both product diversity and the value of this raw material. The study aimed to investigate changes in microbial diversity and chemical characteristics during fermentation using this specific substrate. The results demonstrated that the fermentation process is driven by a consortium of microorganisms, with *Lactobacillus* spp. and *Saccharomyces* spp. identified as the dominant genera. Formic acid was the predominant organic acid produced, while propionic, isobutyric, and butyric acids were detected in trace amounts. Notably, valeric acid, an organic acid associated with potential health benefits, was identified in karanda juice kefir (KJK). Overall, the findings highlight dynamic changes in both microbial diversity and chemical composition throughout fermentation. These results demonstrate that karanda juice is a promising substrate for water kefir production, with the resulting beverage containing diverse beneficial microorganisms and bioactive organic acids with potential functional properties.



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1. Introduction

Karanda (*Carissa carandas* Linn.) is a berry-like fruit belonging to the Apocynaceae family. In Thailand, it is commonly known as Nham Dang, Manao Ho, or Mamuang Hao Manao Ho. Karanda is widely distributed in Thailand and several other countries, including India, Pakistan, Myanmar, Afghanistan, Nepal, Sri Lanka, and Malaysia [1,2]. Ripe karanda fruits have a distinctive sweet-and-sour flavour and are rich in vitamin C and various phytochemicals, including phenolic compounds, anthocyanins, and organic

acids [2]. Furthermore, karanda fruits have been reported to exhibit several pharmacological properties, including antioxidant, anticancer, anti-inflammatory, antimicrobial, and antidiabetic activities. Due to these beneficial properties, karanda has potential applications in food processing, i.e., food and beverage products, including pickles, jams, jellies, juices, and wines [1].

Water kefir is a non-dairy fermented beverage known for its characteristic sour, sweet, lightly fizzy, and slightly alcoholic flavour, as well as its potential health-promoting properties. Reported benefits include anti-inflammatory, antimicrobial, antioxidant, antidiabetic, and gut health-supporting effects, which are largely attributed to the presence of beneficial microorganisms and fermentation-derived metabolites [3]. In recent years, water kefir has gained increasing popularity due to rising consumer awareness of health and wellness, particularly among individuals with lactose intolerance and those following vegan or plant-based lifestyles [4].

Water kefir is traditionally produced by fermenting water kefir grains in a sugar solution, often with the addition of dried fruits, fresh fruits, or fruit juices to enhance fermentation and flavour development [4,5]. The grains are typically translucent and fragile, with colours ranging from white to yellow depending on the type of sucrose and fruit used during production. Structurally, they consist of a polysaccharide matrix (dextran) that provides a natural habitat for diverse microorganisms, including lactic acid bacteria, yeasts, and acetic acid bacteria [6]. During fermentation, these microorganisms generate a variety of metabolites, such as lactic acid, acetic acid, ethanol, and other bioactive compounds, which contribute to the unique sensory characteristics and functional properties of the beverage [7].

Although studies on water kefir have increased in recent years, research remains relatively limited compared with that on milk kefir, particularly for fruit juice-based water kefir products [7,8]. In addition, variations in the sources of water kefir grains and the raw materials used during production may significantly influence the microbial diversity and characteristics of the final product [7,9]. Therefore, this study aimed to expand current knowledge of the microbiological and chemical characteristics of water kefir by fermenting karanda juice with water kefir grains to develop a novel karanda-based fermented beverage. Changes in the microbial diversity of karanda juice kefir (KJK) were investigated at 0, 1, and 2 days of fermentation using next-generation sequencing (NGS), while changes in the chemical characteristics of KJK were monitored during the fermentation period (days 0–2) and the prolonged fermentation period (days 3–7).

2. Materials and Methods

2.1. Materials

Water kefir grains were purchased from an online shopping platform and stored at 4 °C prior to the experiment. Pasteurised karanda juice was purchased from an online shopping retailer displaying approval from the Thailand Food and Drug Administration.

2.2. KJK Production

KJK production was adapted from Ozcelik et al. [10]. Water kefir grains were activated twice by inoculating into a 15% (*w/v*) brown sugar solution at a ratio of 1:10 (*w/v*). The mixture was incubated at 37 °C for 2 days for each activation cycle.

After activation, the solution medium, including brown sugar solution and karanda juice fermentation medium, was prepared in order to produce the control and KJK samples, respectively. The karanda juice fermentation medium was prepared by mixing the karanda juice with a brown sugar solution at a ratio of 60:40 (*v/v*), resulting in a final brown sugar concentration of 15% (*w/v*). Activated water kefir grains were added to the brown sugar

solution and karanda juice fermentation medium at a ratio of 1:10 (*w/v*), incubated at 37 °C for 2 days (days 0–2) and continuously incubated under the same conditions until day 7 to investigate changes in physicochemical characteristics during prolonged fermentation. Shelf-life determination was beyond the scope of this study.

2.3. Study of Changes in Microbial Diversity

The study of changes in microbial diversity was adapted from Laureys and De Vuyst [7] and Cufaoglu, Eser and Erdinc [9], which found that most significant shifts in microbial community structure are expected to occur within two days [7,9]. Moreover, preliminary knowledge and previous studies indicate that the dominant microbial populations in kefir are lactic acid bacteria (LAB) and yeasts. These microorganisms typically exhibit a short lag phase followed by a rapid exponential (log) growth phase during the first 48 h of fermentation [11]. To capture these critical changes while avoiding unnecessary sampling points, microbial diversity was monitored on days 0, 1, and 2 only. The control and KJK samples were collected on days 0, 1, and 2 of fermentation. Fourteen millilitres of control and KJK were centrifuged at 12,000 rpm for 10 min at 4 °C. Pellets were collected and stored at −80 °C for further analysis. DNA extraction was performed using a DNA extraction kit (PureLink Microbiome DNA Purification Kit, Thermo Fisher Scientific, Waltham, MA, USA). The extraction was performed according to the manufacturer's instructions, with slight modifications. The DNA concentration was measured using a nanodrop spectrophotometer (ND8000, Thermo Fisher Scientific, Waltham, MA, USA).

The DNA was amplified using polymerase chain reaction (PCR) and sequenced using Illumina Miseq (Illumina, San Diego, CA, USA) with forward 5' ACTCCTACGGGAG-GCAGCAG 3' and reverse 5' GGACTACHVGGGTWTCTAAT 3' primers targeting the V3–V4 variable region of the 16S rRNA gene, and forward 5' ANCCATTCAATCGGTANT 3' and reverse 5' CGWTAACGAACGAGACCT 3' primers targeting the V7–V8 variable region of the 18S rRNA gene. 16S rDNA amplicon next-generation sequencing and 18S rDNA amplicon sequencing were used for bacterial and fungal profiling, respectively.

The initial quality assessment of the raw Illumina paired-end sequences was performed using FASTQC [12] and MultiQC [13]. Sequences that had an average Phred score of less than 30 were removed, and reads were trimmed according to barcode and primer sequences utilising FASTP [14]. Paired-end reads were merged with FLASH [15]. The sequences of the microbial communities, which were merged and quality-filtered, were examined using the workflow in QIIME 2 version 2022.2 [16]. For 16S rRNA gene sequences, reads longer than 400 bp were denoised, chimeras were eliminated, and the reads were clustered into amplicon sequence variants (ASVs) utilising DADA2 [17] within QIIME2. The parameters were established with a minimum total frequency of 10 and a condition that each ASV must be present in at least two samples. Taxonomical classification of representative sequences for each ASV was conducted using a BLAST+ consensus taxonomy classifier against the SILVA database [18] (version 138.1), applying an identity cutoff of 80%. Sequences identified as chloroplast or mitochondria were excluded before additional analysis. For the 18S region, sequences longer than 290 bp were chosen and analysed with DADA2 to generate ASVs. The fungal community's taxonomic classification was executed utilising a consensus BLAST method with reference to the SILVA database [18] (version 138.1). Microbial diversity was assessed using the alpha diversity index, which comprised species richness, the inverse Simpson index, and the Shannon diversity index derived from the genus table. Beta diversity assessment utilising the Bray–Curtis dissimilarity matrix was employed to evaluate the similarity between the samples. Moreover, PICRUST2 and FUNGuild were used to predict functional profiles of bacterial and fungal communities, respectively. Bioinformatic and statistical analyses were performed using R version 4.3.2.

2.4. Study of Changes in Chemical Characteristics

The study of changes in chemical characteristics was adapted from Laureys and De Vuyst [7]. Chemical composition was monitored from day 0 to day 7 because metabolite production and biochemical transformations continue throughout the fermentation process. Monitoring over seven days allowed us to evaluate both the early fermentation changes and the evolution of chemical compounds during prolonged fermentation [7]. The control and KJK samples were collected during the fermentation period (0, 1, and 2 days) and the prolonged fermentation period (3, 4, 5, 6, and 7 days) to investigate chemical changes, including pH, total soluble solids, alcohol content, and organic acid content, measured using a pH metre (S20 SevenEasy, Mettler Toledo, Greifensee, Switzerland), hand refractometer (Master-PM, ATAGO, Minato-ku, Tokyo, Japan), ebulliometer (Dujardin Salleron, Noizay, France) and high-performance liquid chromatography (HPLC) (Alliance HPLC, Waters, Milford, MA, USA), respectively.

To analyse the organic acid content (citric acid, lactic acid, formic acid, acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid), the control and KJK samples were centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant of each sample was collected and filtered through a nylon syringe filter. Samples were stored at −80 °C for further analysis. The organic acid content in the samples was measured using HPLC with a 2998 Photodiode Array Detector, equipped with an Aminex HPX-87H column (BIO-RAD®, Hercules, CA, USA) at 65 °C. Analyses were carried out isocratically with a mobile phase of 5 mM H₂SO₄ and a flow rate of 0.6 mL/min [19].

2.5. Statistical Analysis

All experiments were conducted in quadruplicate, except for alcohol content determination, which was performed in duplicate. Duncan's new multiple range test of one-way ANOVA and independent-samples *t*-test were used for statistical analysis of the generated data.

3. Results

3.1. Microbial Composition, Alpha Diversity, and Beta Diversity

The relative abundances of bacteria and fungi in the control and KJK samples at 0, 1, and 2 days of fermentation are shown in Figures 1 and 2. Both the control and KJK present lactic acid bacteria (*Lactobacillus* spp. and *Oenococcus* spp.), acetic acid bacteria (*Acetobacter* spp. and *Komagataeibacter* spp.), and yeast (*Saccharomyces* spp., *Brettanomyces* spp., *Schizosaccharomyces* spp., and *Torulaspora* spp.). The dominant bacteria and yeast, which had relative abundances of more than 99% and 80%, respectively, in both the control and KJK at all fermentation times were *Lactobacillus* spp. and *Saccharomyces* spp., respectively. In addition, other bacteria found in all of the samples were *Oenococcus* spp. and *Acetobacter* spp., followed by *Komagataeibacter* spp. These bacteria had relative abundances of less than 1% in all of the samples. The microbial community identified in KJK was generally consistent with previous studies on water kefir and kefir-like fermented beverages. Similar to earlier reports, lactic acid bacteria and yeasts were the dominant microorganisms throughout fermentation, particularly *Lactobacillus* spp. and *Saccharomyces* spp., which represented the major bacterial and fungal populations, respectively. These findings agree with the studies of Arrieta-Echeverri [20], who reported that *Lactobacillus hilgardii* as is the dominant bacterium in water kefir, and Laureys and De Vuyst [7], who found *Lactobacillus* spp. and *Saccharomyces cerevisiae* to be the predominant microorganisms in fig-based water kefir. Similarly, Azi et al. [21] observed that although dominant species varied among different kefir grains, *Lactobacillus*, *Acetobacter*, and *Saccharomyces cerevisiae* were consistently detected in all of the samples. In the present study, KJK also contained minor populations

of *Oenococcus* spp., *Acetobacter* spp., *Komagataeibacter* spp., *Brettanomyces* spp., *Schizosaccharomyces* spp., and *Torulaspora* spp. The detection of these microorganisms is in agreement with previous reports describing the microbial diversity of water kefir. For example, Verce, De Vuyst, and Weckx [22] reported the presence of *Oenococcus* and *Brettanomyces bruxellensis* in water kefir, while Pidoux [23] described the occurrence of minor yeast populations such as *Torulaspora* spp. in kefir grains. Compared with previous studies, the microbial composition of KJK showed a relatively stable bacterial community during fermentation, with *Lactobacillus* spp. remaining dominant at all fermentation times. However, the yeast community exhibited slight shifts during fermentation. In KJK, the relative abundance of *Saccharomyces* spp. slightly increased, whereas *Schizosaccharomyces* spp., *Brettanomyces* spp., and *Torulaspora* spp. gradually decreased. This trend differed slightly from the control sample, in which *Brettanomyces* spp. and *Torulaspora* spp. tended to increase during fermentation. These results suggest that KJK fermentation conditions may selectively support the growth of *Saccharomyces* spp. while suppressing some non-dominant yeast populations.

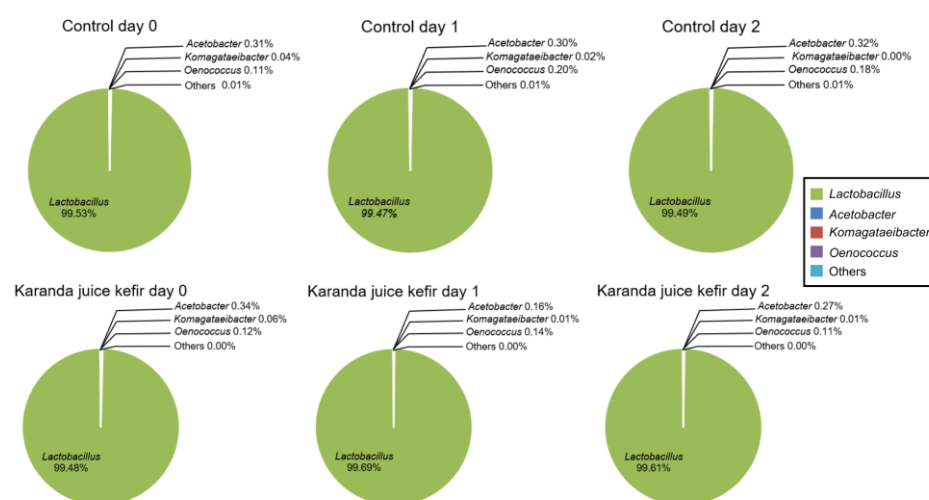


Figure 1. The relative abundances of bacterial genera in the control and KJK.

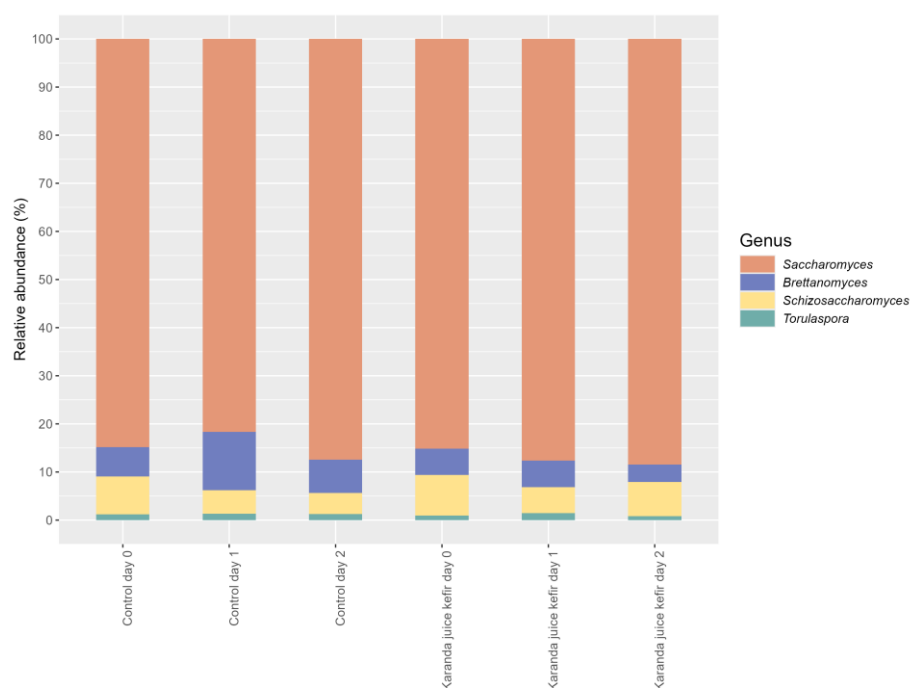


Figure 2. The relative abundances of fungal genera in the control and KJK.

The alpha diversity of bacterial and fungal communities in the control and KJK groups, including evenness, richness, and Shannon diversity indices, is shown in Figures 3 and 4. Alpha diversity reflects the variety and abundance of species present within a local ecological community [24]. Evenness, which represents the equitable distribution of ASV abundances, was evaluated using the inverse Simpson index. Richness was assessed using the Chao1 index, which estimates the abundance of ASVs within the samples. The Shannon diversity index (Shannon–Wiener index) was used to evaluate overall ASV diversity [25,26]. The results showed no significant differences ($p \geq 0.05$) in alpha diversity, including evenness, richness, and Shannon diversity indices, for both bacterial and yeast communities between the control and KJK groups at all fermentation time points.

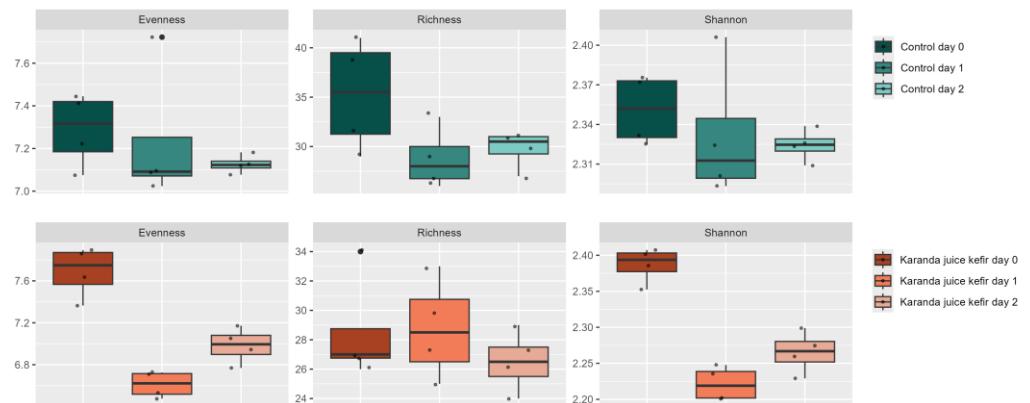


Figure 3. Alpha diversity (evenness, richness, and Shannon index) of bacteria in control and KJK.

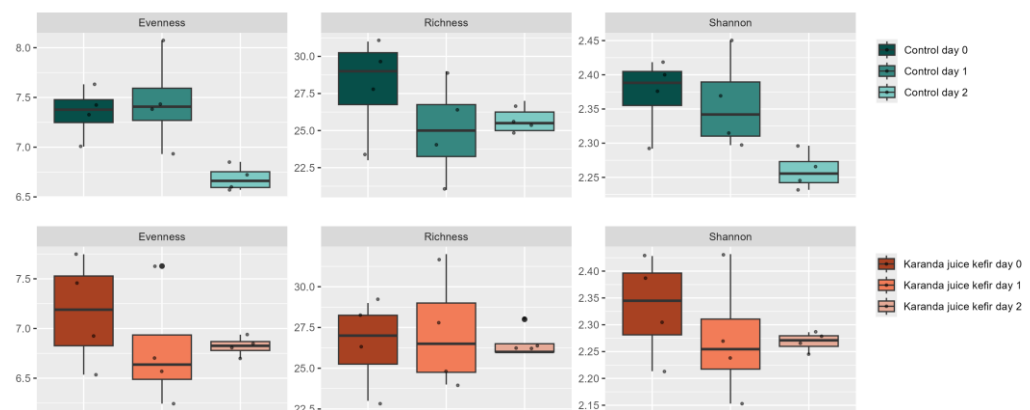


Figure 4. Alpha diversity (evenness, richness, and Shannon index) of fungi in control and KJK.

For principal coordinates analysis (PCoA) plots, a form of metric multidimensional scaling based on Bray–Curtis dissimilarity was used to evaluate the beta diversity of bacterial and fungal communities in the control and KJK samples (Figures 5 and 6). Beta diversity assesses differences between two or more microbial communities [27] by analysing sequence abundance as well as the presence or absence of sequences [28]. PCoA enables the visualisation of variation among samples and facilitates the identification of clustering patterns [29]. The results demonstrate significant differences ($p < 0.05$) in bacterial and yeast community composition among the samples.

In Figure 5, PCoA1 and PCoA2 accounts for 55.92% and 30.56% of the variation, respectively, whereas in Figure 6, PCoA1 and PCoA2 explain 38.23% and 33.05% of the variation, respectively. These percentages represent the contribution of each axis to the overall sample diversity. In addition, the distance between the samples reflects the degree of dissimilarity in microbial composition among the groups [30].

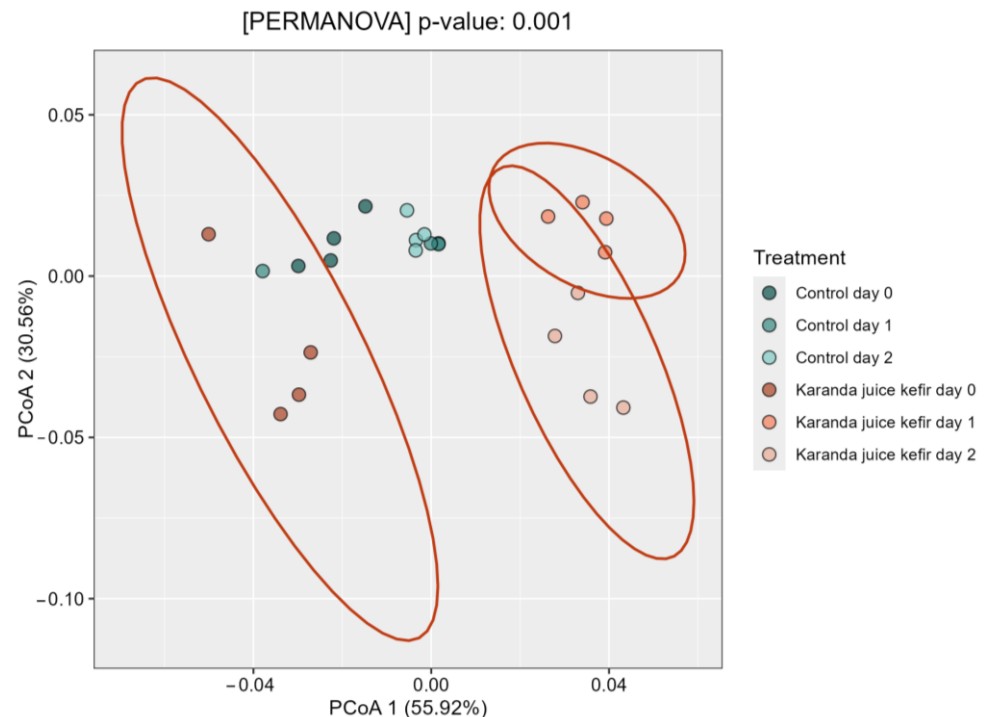


Figure 5. Principal coordinates analysis (PCoA) of bacteria in control and KJK. The ellipses represent the 95% confidence intervals for karanda juice kefir days 0, 1, and 2.

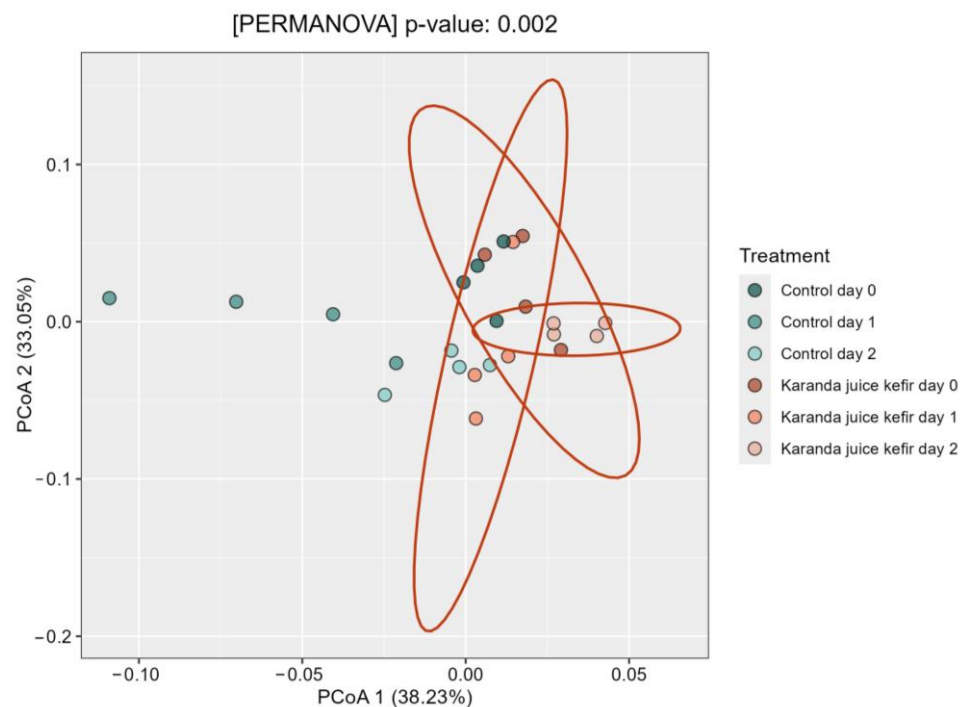


Figure 6. Principal coordinates analysis (PCoA) of fungi in control and KJK. The ellipses represent the 95% confidence intervals for karanda juice kefir days 0, 1, and 2.

Additionally, Figure 5 also shows that the bacterial communities of the control and KJK samples at day 0 were not clearly separated, indicating no significant difference ($p \geq 0.05$) in bacterial composition between these groups at the initial stage of fermentation. However, the bacterial composition of KJK changed significantly ($p < 0.05$) throughout fermentation, particularly at day 0. KJK samples at 1 and 2 days of fermentation differed significantly ($p < 0.05$) from all of the control samples and from KJK at day 0. In contrast, no significant

differences ($p \geq 0.05$) were observed between control samples at 0 and 1 day of fermentation, or between those at 1 and 2 days of fermentation.

Furthermore, Figure 6 demonstrates that there was no significant difference ($p \geq 0.05$) in yeast composition between the control and KJK samples at day 0. However, a significant difference ($p < 0.05$) in yeast composition was observed between the control and KJK samples after 2 days of fermentation.

Regarding the predicted metabolic pathways of bacteria in the control and KJK samples, the results show that the metabolic pathways could be classified into five groups, including carbohydrate fermentation, amino acid metabolism, vitamin and cofactor biosynthesis, cell structure, and membrane metabolism (Figure 7). The dominant metabolic pathways observed in both the control and KJK samples were pyruvate fermentation to acetate and lactate, followed by sucrose degradation, gluconeogenesis, and saturated fatty acid elongation. Moreover, the results reveal that the metabolic pathway profiles of the control samples at 0, 1, and 2 days of fermentation, together with the KJK sample at 0 days of fermentation, clustered together. In contrast, the KJK samples at 1 and 2 days of fermentation formed a separate cluster.

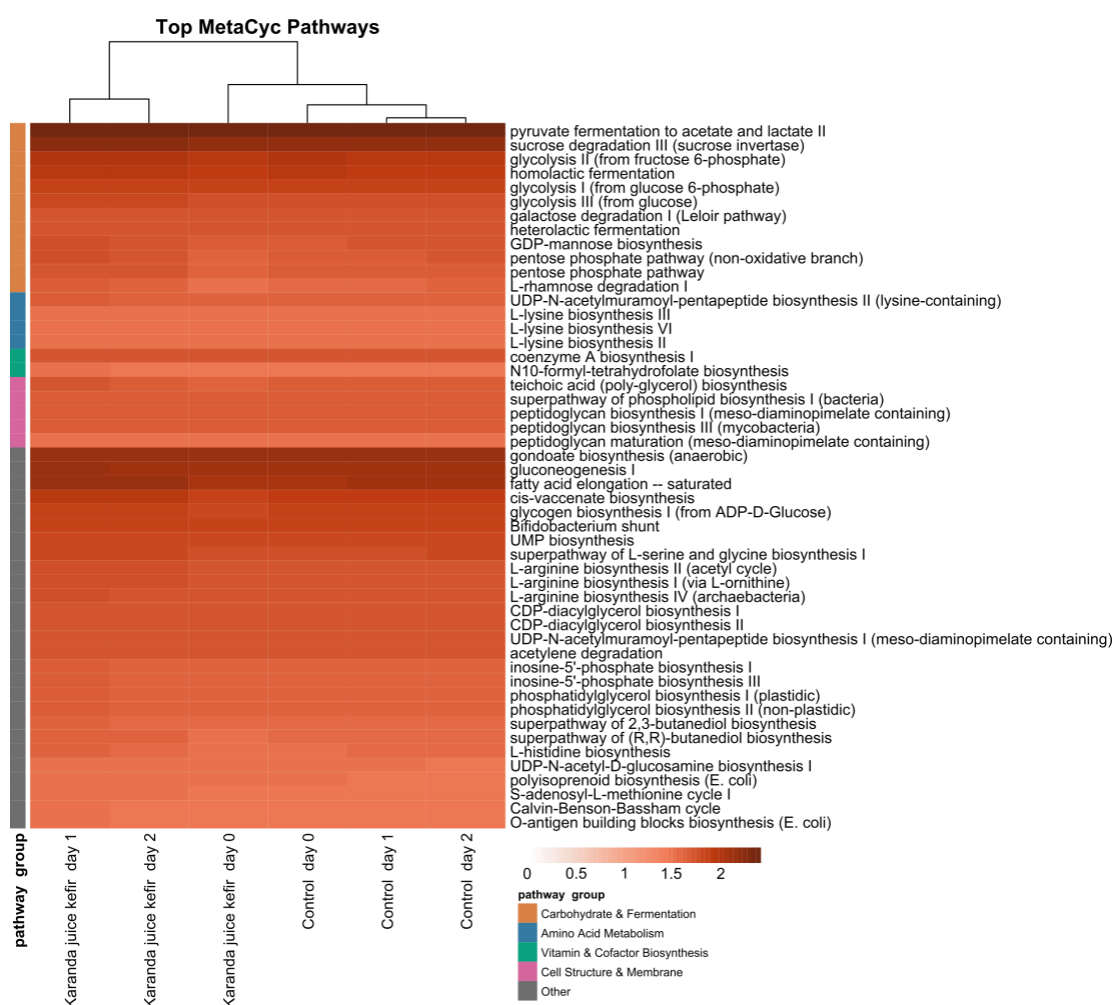


Figure 7. Heatmap of metabolite pathways of bacteria in control and KJK.

The predicted metabolic pathways of yeasts in the control and KJK samples are shown in Table 1. *Saccharomyces* spp., *Brettanomyces* spp., *Schizosaccharomyces* spp., and *Torulaspora* spp. were identified in both the control and KJK samples. These yeast genera were predicted to be associated primarily with sugar degradation pathways. In addition, *Torulaspora* spp. was also associated with the degradation of organic compounds.

Table 1. Metabolite pathways of fungi in control and KJK.

Genus	Metabolite Pathway
<i>Saccharomyces</i>	Sugar degradation
<i>Schizosaccharomyces</i>	Sugar degradation
<i>Brettanomyces</i>	Sugar degradation
<i>Torulaspora</i>	Organic compounds degradation, Sugar degradation

3.2. pH, Total Soluble Solids, Alcohol Content and Organic Acid Contents

The pH, total soluble solids, and alcohol content of the control and KJK samples during the fermentation period (0, 1, and 2 days) and prolonged fermentation period (3, 4, 5, 6, and 7 days) are presented in Table 2, while the organic acid profiles are shown in Table 3. At day 0 of fermentation, the pH value of KJK was lower than that of the control. During fermentation, the pH of KJK increased slightly, whereas the pH of the control decreased. Nevertheless, at the end of prolonged fermentation period (day 7), the pH values of both the control and KJK were below 4.5. Similar findings have been reported in previous studies, where the pH values of fruit-based water kefir products were also lower than 4.5, including plum juice water kefir [31], water kefir produced from *Aronia melanocarpa* pomace and juice [32], as well as apple, fig, and raisin water kefir beverages [33].

Total soluble solids primarily reflect the concentration of soluble sugars, including monosaccharides, disaccharides, and polysaccharides, as well as minerals, organic acids, vitamins, and other soluble compounds [34]. The total soluble solid content of KJK was consistently higher than that of the control throughout fermentation. In both samples, total soluble solids gradually decreased and became relatively stable at day 4. Similar reductions in total soluble solids during fruit-based water kefir fermentation have been widely reported. Dikmetas et al. [35] observed decreases in total soluble solids in water kefir produced from apple, dragon fruit, and kiwi fruit juices. At the end of fermentation, the total soluble solid values ranged from 9.00 to 11.88 °Brix, depending on the type of fruit juice used. Likewise, Randazzo et al. [36] reported that kefir-like beverages produced from apple, grape, kiwifruit, pomegranate, prickly pear, and quince showed decreases in total soluble solids during fermentation, reaching values between 5.87 and 9.97 °Brix.

The alcohol content of KJK was higher than that of the control throughout the fermentation and prolonged fermentation periods. In addition, the alcohol content of both the control and KJK increased during the fermentation and prolonged fermentation periods. The highest alcohol levels were observed on day 7 in the control and on day 4 in KJK. Similar increases in alcohol production during water kefir fermentation have been reported in previous studies, including water kefir produced from orange, apple, dragon fruit, and kiwi fruit juices [35], water kefir made from *Aronia melanocarpa* pomace and juice [32], and lemon juice water kefir [37]. Puntillo et al. [37] further reported that water kefir containing lemon juice exhibited higher alcohol content than water kefir produced without lemon juice.

During both control and KJK fermentation, the concentrations of organic acids changed significantly. Citric acid, lactic acid, formic acid, acetic acid, and isovaleric acid increased during fermentation in both the control and KJK samples ($p < 0.05$). Moreover, the KJK samples contained significantly higher levels of citric acid, formic acid, acetic acid, propionic acid, isovaleric acid, and valeric acid than the control samples ($p < 0.05$).

Table 2. pH value, total soluble solids and alcohol content of control and KJK during fermentation.

Chemical Characteristic	Sample	Time (Days)							
		0	1	2	3	4	5	6	7
pH	Control	4.85 ^{a,X} ± 0.01	3.45 ^{b,X} ± 0.02	3.27 ^{c,X} ± 0.01	3.23 ^{d,X} ± 0.01	3.17 ^{e,X} ± 0.02	3.28 ^{c,X} ± 0.00	3.23 ^{d,X} ± 0.01	3.18 ^{e,X} ± 0.01
	KJK	3.30 ^{d,Y} ± 0.01	3.34 ^{cd,Y} ± 0.01	3.42 ^{bc,Y} ± 0.00	3.49 ^{ab,Y} ± 0.01	3.40 ^{bc,Y} ± 0.00	3.55 ^{a,Y} ± 0.00	3.56 ^{a,Y} ± 0.16	3.44 ^{bc,Y} ± 0.01
Total soluble solid (°Brix)	Control	13.60 ^{a,X} ± 0.10	11.08 ^{b,X} ± 0.08	9.85 ^{c,X} ± 0.17	8.53 ^{d,X} ± 0.08	7.20 ^{e,X} ± 0.12	6.95 ^{e,X} ± 0.09	5.85 ^{f,X} ± 0.26	5.65 ^{f,X} ± 0.22
	KJK	21.70 ^{a,Y} ± 0.30	16.95 ^{b,Y} ± 0.09	14.50 ^{c,Y} ± 0.22	12.90 ^{d,Y} ± 0.10	12.10 ^{e,Y} ± 0.10	11.93 ^{e,Y} ± 0.13	12.05 ^{e,Y} ± 0.09	11.88 ^{e,Y} ± 0.13
Alcohol (%)	Control	0.00 ^{f,X} ± 0.00	1.75 ^{e,X} ± 0.05	3.40 ^{d,X} ± 0.10	4.80 ^{c,X} ± 0.10	5.90 ^{b,X} ± 0.10	6.15 ^{b,X} ± 0.15	6.25 ^{b,X} ± 0.25	6.75 ^{a,X} ± 0.25
	KJK	0.00 ^{e,X} ± 0.00	3.85 ^{d,Y} ± 0.05	6.90 ^{c,Y} ± 0.10	8.50 ^{b,Y} ± 0.10	9.00 ^{a,Y} ± 0.10	8.90 ^{ab,Y} ± 0.20	8.55 ^{ab,Y} ± 0.15	8.90 ^{ab,Y} ± 0.20

^{a–f} Different letters within the same row (different fermentation times for the same type of sample) indicate a statistical difference ($p < 0.05$). ^{X,Y} Different letters within the same column (different sample) indicate a statistical difference ($p < 0.05$).

Table 3. Organic acids (citric acid, lactic acid, formic acid, acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid) in the control and KJK samples during fermentation.

Organic Acid(g/L)	Sample	Time (Days)							
		0	1	2	3	4	5	6	7
Citric acid	Control	0.03 ^{e,X} ± 0.03	0.41 ^{d,X} ± 0.01	0.56 ^{c,X} ± 0.02	0.59 ^{c,X} ± 0.01	0.65 ^{b,X} ± 0.04	0.66 ^{b,X} ± 0.02	0.68 ^{ab,X} ± 0.02	0.70 ^{a,X} ± 0.01
	KJK	1.53 ^{e,Y} ± 0.04	2.08 ^{d,Y} ± 0.01	2.21 ^{cd,Y} ± 0.06	2.40 ^{b,Y} ± 0.15	2.35 ^{bc,Y} ± 0.04	2.48 ^{ab,Y} ± 0.06	2.48 ^{ab,Y} ± 0.19	2.60 ^{a,Y} ± 0.05
Lactic acid	Control	0.17 ^{d,X} ± 0.17	2.28 ^{c,X} ± 0.07	4.34 ^{b,X} ± 0.08	5.30 ^{a,X} ± 0.20	5.35 ^{a,X} ± 0.09	5.38 ^{a,X} ± 0.11	5.30 ^{a,X} ± 0.03	5.22 ^{a,X} ± 0.05
	KJK	0.29 ^{c,X} ± 0.01	1.00 ^{a,Y} ± 0.02	1.03 ^{a,Y} ± 0.03	1.09 ^{a,Y} ± 0.02	1.00 ^{a,Y} ± 0.06	0.96 ^{ab,Y} ± 0.02	0.71 ^{b,Y} ± 0.41	1.01 ^{a,Y} ± 0.05
Formic acid	Control	0.51 ^{d,X} ± 0.51	2.64 ^{c,X} ± 0.02	3.89 ^{b,X} ± 0.03	4.60 ^{a,X} ± 0.14	4.60 ^{a,X} ± 0.09	4.63 ^{a,X} ± 0.05	4.56 ^{a,X} ± 0.02	4.48 ^{a,X} ± 0.03
	KJK	1.09 ^{f,X} ± 0.02	4.34 ^{e,Y} ± 0.06	5.70 ^{ab,Y} ± 0.06	5.81 ^{a,Y} ± 0.08	5.59 ^{bc,Y} ± 0.05	5.45 ^{cd,Y} ± 0.06	5.33 ^{d,Y} ± 0.28	5.50 ^{cd,Y} ± 0.05
Acetic acid	Control	0.08 ^{d,X} ± 0.08	0.43 ^{c,X} ± 0.02	0.62 ^{c,X} ± 0.03	0.58 ^{c,X} ± 0.34	1.03 ^{b,X} ± 0.07	0.99 ^{b,X} ± 0.20	1.30 ^{a,X} ± 0.03	1.44 ^{a,X} ± 0.04
	KJK	0.32 ^{d,Y} ± 0.01	0.91 ^{c,Y} ± 0.03	1.32 ^{bc,Y} ± 0.03	1.61 ^{ab,Y} ± 0.03	1.80 ^{a,Y} ± 0.01	1.95 ^{a,Y} ± 0.02	1.14 ^{c,X} ± 0.66	1.57 ^{ab,Y} ± 0.05
Propionic acid	Control	0.13 ^{a,X} ± 0.13	nd	nd	nd	nd	nd	nd	0.03 ^{b,X} ± 0.06
	KJK	0.51 ^{a,Y} ± 0.13	0.10 ^{b,Y} ± 0.01	0.08 ^{b,Y} ± 0.02	0.09 ^{b,Y} ± 0.01	0.08 ^{b,Y} ± 0.01	0.09 ^{b,Y} ± 0.02	0.08 ^{b,X} ± 0.05	0.10 ^{b,X} ± 0.01
Isobutyric acid	Control	nd	0.08 ^{a,X} ± 0.01	0.09 ^{a,X} ± 0.00	0.05 ^{ab,X} ± 0.06	0.08 ^{a,X} ± 0.05	0.08 ^{a,X} ± 0.05	0.10 ^{a,X} ± 0.01	0.11 ^{a,X} ± 0.02
	KJK	nd	0.08 ^{a,X} ± 0.01	0.05 ^{a,X} ± 0.03	0.06 ^{a,X} ± 0.04	0.02 ^{a,X} ± 0.04	0.04 ^{a,X} ± 0.04	0.02 ^{a,Y} ± 0.03	0.05 ^{a,X} ± 0.08

Table 3. Cont.

Organic Acid(g/L)	Sample	Time (Days)							
		0	1	2	3	4	5	6	7
Butyric acid	Control	0.65 ^{a,X} ± 0.66	0.19 ^{a,X} ± 0.00	0.31 ^{a,X} ± 0.01	0.36 ^{a,X} ± 0.26	0.51 ^{a,X} ± 0.15	0.29 ^{a,X} ± 0.17	0.36 ^{a,X} ± 0.02	0.30 ^{a,X} ± 0.17
	KJK	0.55 ^{a,X} ± 0.26	0.14 ^{b,X} ± 0.04	0.08 ^{b,Y} ± 0.04	0.15 ^{b,X} ± 0.05	0.22 ^{b,Y} ± 0.05	0.18 ^{b,X} ± 0.08	0.12 ^{b,Y} ± 0.08	0.15 ^{b,X} ± 0.06
Isovaleric acid	Control	nd	0.64 ^{b,X} ± 0.01	0.98 ^{ab,X} ± 0.03	0.67 ^{b,X} ± 0.45	0.92 ^{ab,X} ± 0.20	0.96 ^{ab,X} ± 0.12	1.07 ^{a,X} ± 0.03	0.99 ^{ab,X} ± 0.18
	KJK	nd	0.83 ^{c,Y} ± 0.10	1.20 ^{abc,Y} ± 0.14	1.45 ^{a,X} ± 0.09	1.33 ^{ab,Y} ± 0.03	1.39 ^{ab,Y} ± 0.11	1.02 ^{bc,X} ± 0.59	1.38 ^{ab,Y} ± 0.14
Valeric acid	Control	nd	nd	nd	nd	nd	nd	nd	nd
	KJK	2.38 ^{b,Y} ± 0.16	2.65 ^{a,Y} ± 0.05	2.66 ^{a,Y} ± 0.06	2.67 ^{a,Y} ± 0.03	2.65 ^{a,Y} ± 0.02	2.68 ^{a,Y} ± 0.04	2.63 ^{a,Y} ± 0.10	2.76 ^{a,Y} ± 0.05

^{a–f} Different letters within the same row (different fermentation times for the same type of sample) indicate a statistical difference ($p < 0.05$). ^{X,Y} Different letters within the same column (different sample) indicate a statistical difference ($p < 0.05$). nd—not detected.

In the control samples, most organic acids, including lactic acid, formic acid, isobutyric acid, and butyric acid, reached their highest concentrations on day 4 ($p < 0.05$). In contrast, most organic acids in the KJK samples, including lactic acid, formic acid, isobutyric acid, isovaleric acid, and valeric acid, reached their peak levels on day 3 ($p < 0.05$). Lactic acid was the predominant organic acid in the control samples, followed by formic acid and acetic acid. However, in the KJK samples, formic acid was the dominant organic acid, followed by citric acid and acetic acid. In addition, propionic acid, isobutyric acid, and butyric acid were detected only in trace amounts throughout fermentation. Similarly, Esatbeyoglu et al. [32] reported the presence of valeric acid in *Aronia melanocarpa* pomace and juice water kefir. Dikmetas et al. [35] observed increases in lactic acid, acetic acid, and citric acid during the fermentation of water kefir produced from apple, dragon fruit, and kiwi juices. Gökırmaklı, Yüceer, and Guzel-Seydim [38] also reported increased levels of lactic acid and acetic acid in water kefir prepared with dried figs, although citric acid decreased during fermentation. Furthermore, Chomphoosee et al. [39] found that both extracted coffee cherry water kefir and sucrose-supplemented extracted coffee cherry water kefir showed increased levels of lactic acid, acetic acid, citric acid, and butyric acid after fermentation.

4. Discussion

Karanda fruit is widely cultivated in Thailand and is recognised for its numerous health benefits, which have led to its incorporation into a variety of food products [2]. Fruit-based water kefir has also attracted considerable interest, resulting in a range of value-added products derived from raw agricultural materials [5]. In water kefir fermentation, sugar serves as the primary carbon source, while fruits provide essential nitrogen sources. These substrates can influence microbial fermentation performance, microbial diversity, and the dominant microbial populations [6,40]. In this study, karanda juice was fermented to produce KJK, and the resulting changes in microbial diversity and chemical characteristics were investigated.

The microorganisms involved in water kefir fermentation include lactic acid bacteria, yeasts, and acetic acid bacteria [41]. These microorganisms play a crucial role not only in fermentation kinetics but also in the probiotic properties of water kefir products [9]. During fermentation, they produce various metabolites, including organic acids, ethanol, and carbon dioxide, which influence the flavour, taste, and overall characteristics of water kefir [35]. In addition, certain microorganisms and their metabolites have been reported to provide various health benefits, such as anti-inflammatory, antimicrobial, antioxidant, antidiabetic, and gut health-promoting effects [3]. The results of this study demonstrated that the microorganisms present in both the control and KJK samples included lactic acid bacteria, acetic acid bacteria, and yeasts. The dominant microorganisms identified in both groups were *Lactobacillus* spp. and *Saccharomyces* spp. These genera are widely recognised for their probiotic potential and are commonly found in water kefir [42]. Furthermore, other microorganisms detected in both the control and KJK samples, including *Oenococcus* spp., *Acetobacter* spp., *Komagataeibacter* spp., *Brettanomyces* spp., *Schizosaccharomyces* spp., and *Torulaspora* spp., have also been previously reported in water kefir fermentation studies [7,21–23,43]. Compared with milk kefir, which is a fermented milk beverage produced using milk kefir grains, both similarities and differences in microbial composition were observed. Microorganisms commonly reported in milk kefir include *Lactobacillus* spp., *Acetobacter* spp., *Streptococcus* spp., *Leuconostoc* spp., *Bifidobacterium* spp., *Saccharomyces* spp., *Geotrichum* spp., *Kazachstania* spp., and *Dekkera* spp., as well as various other bacteria and yeasts depending on the origin of the kefir grains and fermentation conditions [44–46]. Changes in the relative abundance of microorganisms

during fermentation may be influenced by several factors, including substrate composition and fermentation time [9].

No significant differences ($p \geq 0.05$) in the alpha diversity indices (evenness, richness, and Shannon index) of bacterial and yeast communities were observed between the control and KJK samples. However, beta diversity analysis revealed significant differences ($p < 0.05$) in both bacterial and yeast communities between the control and KJK samples. No significant differences ($p \geq 0.05$) in bacterial and yeast community composition were detected between the control and KJK samples at day 0 of fermentation; however, significant differences emerged at day 2. These findings indicate that the addition of karanda juice influenced microbial diversity during the fermentation process.

Other results obtained in the present study revealed the predicted metabolic pathways of bacteria and yeast during fermentation. The bacterial metabolic pathways were divided into two main clusters: the control and KJK samples at day 0 of fermentation were grouped within the same cluster, whereas the control and KJK samples at days 1 and 2 formed a separate cluster. These findings indicate that metabolic pathways changed throughout fermentation, most likely due to shifts in microbial diversity within the samples. During water kefir fermentation, *Lactobacillus* spp. and *Oenococcus* spp. were identified as lactic acid bacteria capable of hydrolysing sucrose into glucose and fructose, while also producing lactic acid, ethanol, and acetic acid and converting pyruvate into acetoin [22]. Moreover, *Lactobacillus* spp. was associated with gondoate biosynthesis, which is involved in the synthesis and elongation of long-chain fatty acids [47,48], as well as saturated fatty acid elongation pathways [49]. *Acetobacter* spp. was associated with gluconeogenesis, a metabolic process that generates glucose from pyruvate [50,51]. The predicted metabolic pathways of yeasts, including *Saccharomyces* spp., *Brettanomyces* spp., *Schizosaccharomyces* spp., and *Torulaspora* spp., suggested their involvement in sugar degradation. In addition, *Torulaspora* spp. was associated with the degradation of organic compounds. Furthermore, *Saccharomyces cerevisiae* and *Dekkera bruxellensis* (*Brettanomyces bruxellensis*) are capable of hydrolysing sucrose into D-glucose and D-fructose [22]. *Saccharomyces cerevisiae* can also convert carbohydrates into ethanol and carbon dioxide while utilising metabolic by-products such as organic acids to produce flavour compounds [52].

Water kefir can be fermented at 20–37 °C for 1–3 days (24–72 h) [6]. However, in this study, the control and KJK samples were fermented at 37 °C, with the fermentation time extended to 7 days in order to continuously monitor the chemical changes throughout the fermentation process. Specifically, we observed the kinetics of these changes and the effects of prolonged fermentation on the chemical characteristics of the final product. This extended fermentation period enabled a more comprehensive assessment of changes in pH, total soluble solids, alcohol content, and organic acid contents. Moreover, the temperature and duration of fermentation are factors that affect the characteristics of water kefir [7,9].

The pH value of KJK was below 4.5 at the beginning of fermentation. Similarly, previous studies on water kefir and milk kefir have reported pH values below 4.5 after fermentation [31–33,44]. The pH of water kefir is typically below 4.5, which contributes to its microbiological safety by inhibiting the growth of undesirable microorganisms [4]. In addition, the initial pH of KJK was lower and changed only slightly during fermentation compared to the control. The rapid decrease in the pH value of the control suggests a lower buffering capacity of the medium. Water and fruit may provide calcium and buffering capacity, both of which are important factors influencing water kefir grain growth, pH reduction, and overall fermentative activity [33].

The decrease in total soluble solids observed in both the control and KJK during fermentation correlated with increased alcohol and organic acid production. This reduction in total soluble solids results from the conversion of sugars into carbon dioxide, ethanol,

and organic acids during fermentation [32,53]. During water kefir fermentation, yeasts produce invertase enzymes that hydrolyse sucrose into glucose and fructose, which are subsequently metabolised to produce ethanol and carbon dioxide. In addition, lactic acid bacteria and acetic acid bacteria utilise glucose and fructose to produce organic acids. Furthermore, acetic acid bacteria can convert ethanol into acetic acid [41,54,55].

Alcohol content increased during fermentation in both the control and KJK samples. The increase in alcohol content during fermentation is attributed to the activity of yeasts and heterofermentative lactic acid bacteria, which are capable of producing considerable amounts of ethanol in water kefir [37]. The rise in alcohol content was correlated with a decrease in total soluble solids, as well as the dominance of *Saccharomyces* spp. in both the control and KJK samples. However, the KJK sample exhibited a higher alcohol content than the control. The addition of fruit further enhanced alcohol production in the water kefir product, as fruits contain high levels of sugars that provide sufficient nutrients for yeast growth. In addition, fruits create an acidic environment that improves the ability of yeasts to utilise sugars efficiently [56]. Ethanol levels in water kefir can range from trace amounts to more than 3% (*v/v*). The alcohol content in the product depends on several factors, including the dominant yeast strain, initial sugar concentration, fermentation time, and fermentation temperature [5,37,57].

During water kefir fermentation, organic acids are produced by bacteria and yeasts, contributing to flavour development while also controlling and inhibiting spoilage microorganisms and foodborne pathogens [58,59]. In this study, the organic acids identified in both the control and KJK samples were citric acid, lactic acid, formic acid, acetic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid. These organic acids can be classified into two groups: volatile acids (formic acid, acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid) and non-volatile acids (citric acid and lactic acid). Volatile acids are associated with strong aromas and short aftertastes, whereas non-volatile acids help regulate acidity and provide a milder taste in fermented products [60]. Citric acid contributes a fruity tartness, while lactic acid imparts the characteristic sour taste of the product [61,62]. In addition, the fruity flavour and aroma of water kefir are partly attributed to esters derived from acetic acid [63].

Organic acids not only improve the flavour and sensory properties of water kefir but may also provide health benefits to consumers. Acetic acid, formic acid, and lactic acid can reduce pathogen levels in the gastrointestinal tract by lowering the pH, inhibiting harmful bacteria, and promoting the growth of beneficial microorganisms [64]. Water kefir contains both homofermentative and heterofermentative lactic acid bacteria, which produce organic acids such as acetic acid, formic acid, and citric acid that contribute to product flavour and aroma development [35].

In the present study, most organic acids were detected at higher levels in KJK than in the control, and the concentrations of most organic acids increased during fermentation, corresponding with decreases in total soluble solids and pH. However, the pH of KJK slightly increased during fermentation, possibly due to the ability of certain microorganisms to metabolise organic acids [65]. Furthermore, increases in citric acid, lactic acid, formic acid, acetic acid, and isovaleric acid during fermentation were associated with the growth of lactic acid bacteria, particularly *Lactobacillus*, which was dominant in both the control and KJK samples. Acetic acid was not the predominant organic acid in either sample, which is consistent with the low abundance of acetic acid bacteria detected. Nevertheless, some heterofermentative *Lactobacillus* species are capable of producing acetic acid by utilising fructose as an electron acceptor [33].

The composition and predominant organic acids in water kefir products are influenced by the fermentation substrate. For example, butyric acid is the dominant organic acid in

sucrose-based extracted coffee cherry water kefir, and tartaric acid predominates in plum water kefir, while lactic acid is the major organic acid in milk kefir [31,39,66]. In this study, formic acid was the predominant organic acid in KJK, whereas lactic acid was dominant in the control sample. In addition, karanda juice fermentation enhanced the production of citric acid and formic acid. Valeric acid, detected only in KJK, is a short-chain fatty acid naturally found in some fruits. It has been reported to exhibit therapeutic potential for insomnia and seizure disorders and may also serve as a novel therapeutic agent for cancer treatment [67]. Moreover, the results showed that valeric acid slightly increased after one day of fermentation, while propionic acid and butyric acid decreased, potentially due to reverse β -oxidation reactions [68].

The prolonged fermentation resulted in kefir products that have a low pH, but are high in ethanol and organic acid content [7]. Furthermore, temperature influences the products: fermentation at 37 °C results in a biomass increase of the kefir grains and high organic acid content in the product [69,70]. On the other hand, not only the fermentation time and temperature, but also the raw materials are a factor influencing the characteristics of the product [41].

This study found that milk kefir and water kefir exhibit distinct chemical and microbiological characteristics [44,71]. These differences are mainly attributed to the raw materials and starter cultures used during fermentation. Despite these differences, water kefir remains a suitable alternative for consumers who are lactose intolerant or those following vegetarian or plant-based diets.

Functional beverages are non-alcoholic drinks that provide additional health benefits due to the presence of bioactive compounds derived from plants, animals, marine sources, or microorganisms. Currently, functional beverages are attracting increasing consumer interest because of their perceived health benefits, and the global functional beverage market is expected to continue expanding [72]. KJK has strong potential for development as a functional beverage because it contains beneficial microorganisms, including lactic acid bacteria, acetic acid bacteria, and yeast, as well as organic acids associated with health-promoting properties. However, the presence of alcohol in KJK necessitates its removal before the product can be developed as a functional beverage. Several post-fermentation alcohol removal techniques are available and can generally be classified into heat treatment processes and membrane-based technologies. Heat treatment methods, such as evaporation and distillation, may adversely affect the taste and flavour of the product. In contrast, membrane-based technologies, including reverse osmosis, nanofiltration, and pervaporation, are more effective in preserving the nutritional and sensory qualities of food and beverage products [73].

5. Conclusions

To summarise, KJK is a fermented beverage that is produced by adding karanda juice during water kefir fermentation. This study indicated the changes in microbial diversity and chemical characteristics during fermentation. During fermentation, bacteria are relatively stable, while yeast has slightly changed. *Lactobacillus* and *Saccharomyces* were dominant microorganisms in KJK. Moreover, differences in microbial and yeast composition between KJK at 0 and 2 days of fermentation were found. During fermentation, several predicted metabolic pathways in bacteria are observed. Pyruvate fermentation to acetate and lactate, sucrose degradation, gondoate biosynthesis, gluconeogenesis, and saturated fatty acid elongation were the most dominant predicted metabolic pathways. Additionally, predicted metabolic pathways in yeast are associated with sugar degradation and organic compound degradation. The change in chemical characteristics of KJK can be observed during fermentation, including a slight increase in pH, a decrease in total soluble solid,

an increase in alcohol content, and an increase in most organic acids. Moreover, KJK has formic acid as the dominant organic acid, and valeric acid was also found. The changes in the chemical characteristics of the product result from the production of metabolites by microorganisms in the product. The prolonged fermentation led to KJK having a low pH, high organic acid content, and high alcohol content. In addition, the type of raw materials and fermentation time are among the key factors influencing changes in the microbial and chemical characteristics of water kefir products. Adding karanda juice during water kefir fermentation can improve the health advantages of the product and increase product variety of karanda fruit and fermented beverages. Furthermore, KJK can develop into a functional beverage.

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Abbreviations

The following abbreviations are used in this manuscript:

ASVs	Amplicon sequence variants
KJK	Karanda juice kefir
PCR	Polymerase chain reaction

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