

Effect of Varying Concentration of Kefir Grains on the Microbial, Physiochemical, Antioxidant and Sensory Properties of Water Kefir Fermented Drink

Anisha Adya¹, Prashant Sharma², Muskan Chadha¹, Sunil Kumar³, Karuna Singh¹, and Ratnakar Shukla^{4*}

¹Department of Nutrition and Dietetics, Sharda School of Allied Health Sciences, Sharda University, Greater Noida 201310, Uttar Pradesh, India

²Department of Pharmacology, School of Medical Sciences and Research, Sharda University Greater Noida 201310, Uttar Pradesh, India

³Department of Microbiology Graphic Era (Deemed to be) University, Clement Town, Dehradun, Uttarakhand, India.

⁴Department of Clinical Research, Sharda School of Allied Health Sciences, Sharda University, Greater Noida 201310, Uttar Pradesh, India.

*Corresponding author email: ratnakarshukla2021@gmail.com

Abstract. Water kefir is a vegan fermented probiotic-rich beverage that has potential health benefits include improving digestive health and preventing chronic disorders like obesity, gastrointestinal disorders, hyperlipidaemia and hypertension. This study aims to formulate the water kefir fermented drink, using 3 different concentrations of water kefir grains (Sample A- 2%, Sample B-4% and Sample C-6%) and examine the microbial and chemical properties of the all three samples. The physiochemical (pH, titratable acidity, water activity and total soluble solids) and microbial (total bacterial and yeast) counts were estimated using AOAC methods. The DPPH antioxidant potential and total phenolic content were assessed using UV spectroscopy method. The sensory analysis of the water kefir samples was examined using 9-point hedonic scale. The chemical, microbial antioxidant and sensory analysis revealed that the sample C was more desirable in all the aspects as compared to sample A and B with pH: 3.8 ± 0.01 ; titratable acidity (TA): 0.041 ± 0.001 meq/mL and water activity: 0.947 ± 0.003 Aw. The TBC, DPPH percentage and phenolic compound of sample C was found to be 2.91×10^7 CFU/mL, 28.74 ± 0.05 and 14.86 ± 0.45 g/100 GAE, respectively. The overall acceptability score of sample C was 7.00 ± 0.18 . In conclusion, 6% kefir grains concentration were optimum for production of water kefir drink which have higher antioxidant and bacterial count. These findings contribute to a better understanding of the fermentation process that influence the quality and potential health benefits of water kefir drink.

KEY WORDS: Water Kefir, Probiotics, Nutrition, Microbial, Chemical, Antioxidant

1. INTRODUCTION

The market for functional food items has increased significantly worldwide in recent times due to increased consumer knowledge of the connection between diet and illness [1]. Therefore, the use of fermented drinks high in probiotics as a preventative healthcare strategy may enhance gastrointestinal and systemic health and modulate the host immune response [2]. The growing popularity of veganism and conditions such as lactose intolerance or allergic responses to dairy products are other reasons for this trend. As a result, there is a noticeable interest in non-dairy based fermented drinks and functional meals are increasing [3]

Water kefir grain, also known as Tibico grain, has been collected from the Tianshan region in China's Xinjiang province [4]. It represents a non-dairy variant of kefir, produced through the fermentation of a carbohydrate-rich solution using 'kefir grains, which harbor numerous probiotic microorganisms. The predominant microorganisms found in water kefir grains include lactic acid bacteria (LAB), acetic acid bacteria (AAB) and yeast. [5]. The principal microorganisms identified were *Saccharomyces cerevisiae*, *Lentilactobacillus hilgardii*, *Liquorilactobacillus negelii* and *Lactocaseibacillus paracasei* [6]. Aerobic conditions are typically used for water kefir fermentation [5].

High concentrations of acetic acid develop because of aerobic conditions that allow oxygen to promote the development of acetic acid bacteria (AAB) [5]. During the fermentation of water kefir, sucrose is the main substrate that is broken down by microbes to generate mannitol, ethanol, glycerol, lactic acid and acetic acid [8, 9]. Additionally, glucoamylase from LAB enzymatically converts sucrose into water kefir grain dextran exopolysaccharides (EPS), which increase the bulk of water kefir grains during fermentation [9].

Water-kefir fermented drinks are obtained by fermenting water-kefir grains in distilled or mineral water along with different sources of carbohydrates, including sucrose, honey and various fresh or dried fruits. The water kefir grain exhibits a gelatinous composition, consisting of a symbiotic blend of bacteria and yeast encapsulated within a predominantly polysaccharide matrix or a flexible matrix composed of water-soluble glucogalactan, known as kefiran. Kefiran is a vital component that supports the structural integrity, microbial functionality and health benefits of water kefir grains, indirectly ensuring successful fermentation and production of high-quality probiotic beverage.

According to previous reports, water kefir introduces beneficial bacteria into the gut, supporting digestion and maintaining a healthy gut

microbiome. Its probiotic effects promote the growth of beneficial native gut bacteria. It also contains bioactive compounds that have antioxidant and anti-inflammatory properties. Regular consumption may also enhance the immunity. It also potentially improves lipid metabolism and regulates blood sugar levels owing to its low glycemic impact. It also has many other therapeutic functions such as anti-carcinogenic or anti-mutagenic qualities [10].

Previously, 0.4-30% sugar and 6-20% water kefir grains were used to incubate water kefir at 20-37 °C for 24-72 hours with addition of different sugars along with substrates such as fruits, its juice, or pomaces. Thus, the current study aimed to evaluate and analyze the physiochemical, microbial, antioxidant, and sensory properties of water kefir fermented drink having three different concentration such as 2,4 and 6% (w/v) of kefir grains with 10% (w/v) brown sugar in mineral water without inclusion of any fruit or vegetable product as a substrate.

2. MATERIAL AND METHODS

2.1 Procurement of the Water kefir grains:

Water kefir grains were procured from the Feel-Good Kefirs Company, situated in Kannur District, Kerala, India. The kefir grains were allowed to ferment at temperature between 30-32°C in brown sugar solution. The brown sugar solution was changed once a week to keep kefir grains viable.

2.2 Preparation of water kefir fermented drink:

To develop the water kefir fermented drink, three samples (A, B, and C) were prepared, in which 10% of brown sugar was first dissolved in 100 mL of water. Subsequently, a homogenizer was used to ensure that the mixture was well mixed. Following homogenization, the samples were pasteurized for 5 min on a hot plate at 60°C. Following pasteurization, 2%, 4%, and 6% gm of water kefir grain were added to each samples A, B, and C, respectively, and it was then incubated for 24 hours at the experimental temperature of 32°C to initiate the fermentation process, as shown in Figure 1. Kefir grains after fermentation were sieved out and stored in sugar solution for further use, and the fermented drinks were stored at 4°C until further analysis and characterization [11].

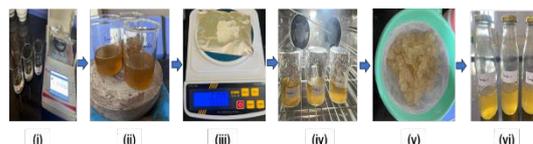


Figure 1: Process flow of production of water kefir fermented drink. (i) Addition of 10gms of brown sugar in each sample, (ii) Heating it at 60°C for 5 minutes, (iii) weighing and adding water kefir grains 2% in sample A, 4% sample B and 6% in sample C (iv) Incubation at 32°C for 24 hours for fermentation, (v) sieving out kefir grain for reutilization, (vi) storing the water kefir fermented drink at 4°C for further analysis.

2.3 Physiochemical analysis

2.3.1 Total Soluble Solid:

The total soluble solid (TSS) has been analyzed manually with the help of digital bench refractometer (Hanna Equipment Pvt. Ltd, Mumbai, India).

2.3.2 pH:

A potentiometric pH meter (Labman Scientific Instruments PVT. LTD., Chennai, India) was used to measure the pH of each sample.

2.3.3 Titratable acidity (TA%):

Utilizing a standardized 0.1N NaOH solution and the standard titration procedure, the titratable acidity (%) was ascertained. In an Erlenmeyer flask, 5mL of each sample was diluted with 25 mL distilled water. Next, 2-3 drops of a 1% phenolphthalein indicator were applied. After that, it was titrated using a standardized 0.1N NaOH solution until a light pink color became stable.

Lactic acid (%) were calculated using the formula:

$$TA (\%) = \frac{\text{Volume of NaOH} \times \text{milliequivalent factor}}{\text{Volume of sample}} \times 100$$

2.3.4 Water activity:

The water activity of each sample has been analyzed using a water activity meter.

2.4 FTIR analysis of water kefir fermented drink:

Cary 630 FTIR (Agilent Technologies, Pune, India) was used to analyze the water kefir fermented drinks. To this end, an adjusted total reflectance (ATR) accessory was used along with a deuterated triglycine sulfate (DTGS) detector with a resolution of 4 cm⁻¹ were employed. Every sample was utilized in a 50µl drop, and the best signal to noise ratio was selected to produce the spectra. Each spectrum was then collected and rotated against the background spectrum of the clear crystal surface to obtain absorbance spectra. The spectrum of each sample was recorded at room temperature. Immediately every measurement, the surface of the crystal was

cleaned with ethanol and dry tissue paper, and the next sample was analyzed.

2.5 Microbial analysis:

The conventional pour plating procedure or total plate count method was used to determine the quantity of viable cells and yeast count present in the products. Inoculants were plated in two replicates on nutrient agar medium (NAM) for total plate count and yeast glucose chloramphenicol agar (YGCA) for yeast counting. The plates were then incubated for 48h at 37°C [12].

The colony-forming units (CFU) were calculated using the following formula:

$$\frac{\text{CFU}}{\text{ml}} = \frac{\text{Average colonies counted} \times \text{dilution factor}}{\text{Volume plated}}$$

2.6 DPPH:

The antioxidant activity of water kefir samples was analyzed in terms of their capacity to donate hydrogen or scavenge radicals. It was analyzed using the Stable DPPH radical, where 5 mL of the methanolic solution of DPPH (0.1 mmol/L) was added to water kefir filtered sample at varying concentration (100, 200 and 300 µl) that had been placed into a cuvette. The mixture was then allowed to sit at room temperature for at least 30 min in the dark. A UV-spectrophotometer was used to measure the drop in the absorbance at 517 nm. Methanol and ascorbic acid served as blank and positive reference standards, respectively.

The formula was used to determine of the percentage of DPPH inhibition by the kefir sample.

$$\text{Scavenging effect (\%)} = \frac{A_c - A_s}{A_c} \times 100\%$$

Where,

A_c= Absorbance of the standard solution

A_s= Absorbance of the sample.

2.7 Total Phenolic content analysis:

The total phenolic content of each of the three samples was determined using the Folin-Ciocalteu technique. After diluting around 2gm of each sample with 10mL of 80% v/v acetone, the samples were centrifuged for 10 min at 20°C and 5000 rpm. A dual-beam UV spectrophotometer was used to measure the absorbance of the samples at 769 nm after 1 mL of the collected supernatant, 2mL of distilled water, 0.5 mL of FC (Folin-Ciocalteu)

reagent, and 0.5 mL of sodium carbonate (10%) were added to a cuvette. The results of the triple TPC analysis are reported as mg gallic acid extract (GAE)/100 mL.

2.8 Sensory evaluation

Fifty semi-trained sensory panelists were selected for sensory evaluation, and a 9-point hedonic scale was employed in the assessment of taste, aroma, flavor, and sweetness of the drink. The drink score for the overall acceptability of the sample served as a response to optimization.

2.9 Statistical analysis:

All experimental analyses were performed in triplicate, and all results are expressed as mean ± standard deviation (SD). All statistical analysis were performed using SPSS version 15.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism version 5.0 (GraphPad Software, Inc.). All categorical and continuous data were analyzed using Chi-square and Mann–Whitney U tests, respectively. Kruskal–Wallis test followed by Bonferroni correction was used to compare the means of different kefir grain concentrations (Sample A, 2%; Sample B, 4%; and Sample C,6%) for physicochemical, microbial, and antioxidant parameters. P value less than 0.05 was considered as significant.

3. RESULT AND DISCUSSION

3.1 Physicochemical analysis:

3.1.1 Total soluble solid (TSS)

TSS is a crucial metric for measuring the quantity of dissolved substances including sugar, organic acids etc. TSS for sample A, B and C was found 3.6° Brix, 8.1° Brix and 9° Brix respectively. At the beginning of fermentation, the TSS in the unfermented drink of all three samples A, B, and C was approximately 9.7 brix. During fermentation, carbon dioxide and alcohol are produced, as bacteria or yeasts consume sugar. As a result of carbohydrate consumption, the TSS of sample C reached to 9° Brix, which was higher that of samples A (3.6 ° brix) and B (8.1° brix), respectively with P value=0.018. It is possible to observe certain differences in TSS between this simple water kefir fermented drink and other kefir drinks that include any fruit as a substrate. In a prior investigation, TSS for jackfruit concentrate water kefir ranged from 20-25° brix. The TSS of jackfruit concentrate water kefir was reduced to 10.5-15° brix after dilution with water. Furthermore, TSS of jackfruit kefir drink was reduced to 3.85-11.90° brix after fermentation [13].

Similarly, another study showed that before to and after the completion of fermentation, the TSS level in a water kefir drink produced from *Aronia melanocarpa* juice and pomace exhibited a very

similar outcome. The fermentation substrates based on pomace and *Aronia melanocarpa* juice had Brix values of 10.4° and 11.8° brix before fermentation. However, after fermentation, brix values significantly decreased to 6.10° and 7.61° Brix, respectively. Similar differences were observed in our study where initial TSS of all three samples was 9.7 ° Brix before fermentation. However, after fermentation, the TSS of samples with different concentration of kefir grains, ranged from 3.6° to 9° Brix. This can be explained by the mechanism by which the bacteria and yeast in the kefir grains ferment the sugar to produce metabolites. Similar to the jackfruit concentrate water kefir drink, it was demonstrated that the TSS value of the drink increased as the amount of jackfruit increased, and the amount of kefir grains decreased. However, our study revealed that higher levels of kefir grain could lower TSS because an increase in the microbial population increases the fermentation of sugar.

3.1.2 pH

The initial pH of samples A, B, and C of the water kefir fermented drink was 7 before fermentation. However, it got decreased significantly after 24h of fermentation, as shown in figure 2. The pH was initially neutral, but during fermentation, the bacteria changed the sugar to lactic acid and acetic acid. The pH of the water kefir grains was reduced by this organic acid thereby gradually increasing its acidity. The pH of sample C (3.833 ± 0.01) was more statistically significant than that of samples A (4.88 ± 0.08) and B (4.467 ± 0.02), (P-value = 0.0273). According to the previous studies, the pH of water kefir generated from *Aronia melanocarpa* juice and pomace after fermentation were 3.39 ± 0.01 and 3.59 ± 0.01 , respectively. Another study showed that each water kefir drink made from the juice of hawthorn, rosehip, cornelian cherry, red plum, and pomegranate had pH from 3.45-3.97 by the end of fermentation [11]. Similarly, water kefir made from the Mediterranean fruit juice provided a pH below 4 [26]. These variations can be explained by the fact that different concentrations of kefir grain and different strains of lactic acid bacteria produce varied amounts of lactic acid, leading to a reduction in pH .

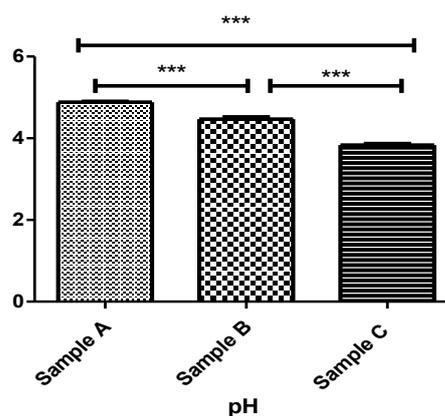


Figure 2: pH analysis of sample A, B and C with 2-6% kefir grain concentration.

*** p<0.001 was considered to be statistically significant. Mann-Whitney U test has been used for subgroup analysis.

A Bonferroni corrected p< 0.017 was considered statistically significant for the analysis of subgroup.

3.1.3 Titratable acidity

The titratable acidity of sample C was higher than of samples A and B (0.025 ± 0.001 meq/mL vs. 0.029 ± 0.08 meq/mL and 0.041 ± 0.001 meq/mL with p=0.027), as shown in Figure 3. The microbiota of water kefir consists of both homofermentative and heterofermentative lactic acid bacteria that produces the lactic acid, a primary organic acid. So titratable acidity is expressed as lactic acid. As a result, organic acids such as citric, formic, and acetic acids are generated, which help to give the aroma and flavors to the product.

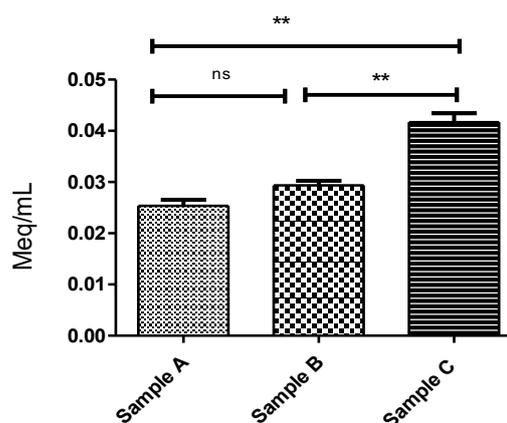


Figure 3: TA (meq/mL) of sample A, B and C with 2-6% kefir grain concentration.

** P<0.01 was considered to be statistically significant. Mann-Whitney U test has been used for subgroup analysis.

A Bonferroni corrected $P < 0.017$ considered to be statistically significant for the analysis of subgroup.
ns: Not significant

3.1.4 Water activity:

Every food product must possess water activity (aw). The water activity of samples A and B of the simple water kefir fermented drink was 0.9931 ± 0.0005 Aw and 0.9710 ± 0.0005 Aw, respectively, whereas it was 0.9473 ± 0.0003 Aw in sample C (P-Value=0.026), as shown in Figure 4. This indicates the presence of water for enzymatic reactions, microbial reactions and chemical stability. The relatively high Aw level suggests that there is sufficient free water in the water kefir fermented drink for the growth of yeast and bacteria. However, it is still slightly below that of pure water, which has an Aw of 1.0. This is sufficient to prevent the formation of pathogenic microorganisms, yet it is still favorable for the growth of yeast and lactic acid bacteria, two helpful fermentation-related germs. A low aW value indicates limited microbial growth. The kefir drink made from 100% cow milk had the greatest water activity, which was 0.89 ± 0.02 Aw. Whereas the combined cow's milk and colostrum produced an average of 0.85 to 0.89 aW. Water is also necessary for enzymatic reactions and chemical stability. The sample C of our study had water activity of 0.947 ± 0.003 Aw, which is lower than that of pure water, which has an Aw of 1.0. This might be because we did not use any fruit substrate, which could have further reduced aw. This is sufficient to prevent the growth of harmful microorganisms.

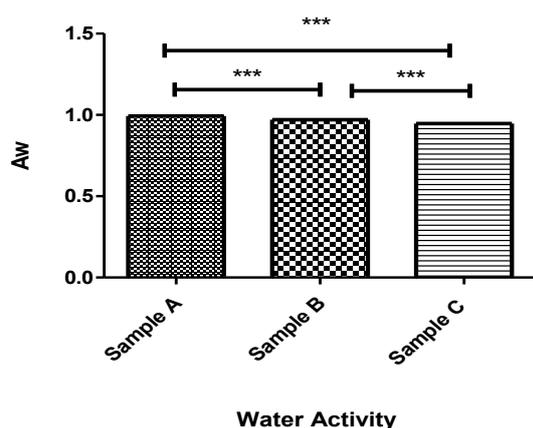


Figure 4: Water activity of sample A, B and C with 2-6% kefir grain concentration.

*** $P < 0.001$ was considered to be statistically significant. Mann-Whitney U test has been used for subgroup analysis.

Bonferroni corrected $P < 0.017$ considered to be statistically significant for the analysis of subgroup.

3.1.5 FTIR Analysis:

The FTIR spectra of Samples A, B, and C revealed peaks at 998 cm^{-1} and 1054.8 cm^{-1} , indicating the presence of carbohydrates and alcohols, which supports the fermentation process [14]. Sample A exhibited an additional low-intensity peak at 1636.3 cm^{-1} which corresponded to water molecules. The reduced intensity of this peak may be due to the presence of proteinaceous compounds in the water kefir. A broad absorption band at 3265.1 cm^{-1} was also observed for sample A which is the characteristic of O-H stretching vibrations and this is mostly attributed to the presence of water and alcohol.

Sample B showed an additional peak at 1420.1 cm^{-1} , suggesting C-H bending vibrations, which may originate from carbohydrates or organic acids formed during fermentation. Both samples B and C showed a peak at 1632.6 cm^{-1} , which corresponded to O-H bending of water and the amide I band, indicating the presence of proteinaceous material. Additionally, both samples (B and C) showed broad peaks at 3257.7 cm^{-1} , representing O-H stretching vibrations, primarily due to water, alcohols, and carboxylic acids produced during fermentation.

So, the FTIR analysis of samples A, B, and C shown in Figure 5, indicates a diverse mixture of organic compounds with potential hydrogen bonding, unsaturated bonds, oxygenated functional groups and proteinaceous compounds. These findings align with the expected biochemical profile of water kefir, where microbial activity results in the breakdown of sugar and production of various organic compounds.

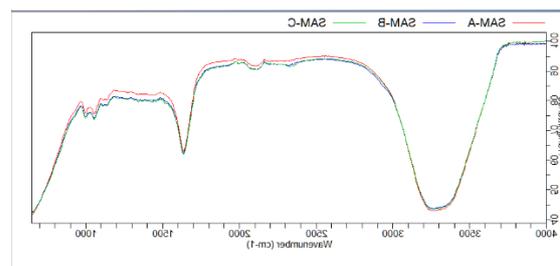


Figure 5: FTIR analysis of sample A, B and C

3.2 Microbial analysis of water kefir fermented drink

The microbial counts in sample A, B and C were $1.95 \times 10^5 \pm 2 \times 10^3$ CFU/mL, $4.55 \times 10^5 \pm 1.5 \times 10^3$ and $2.91 \times 10^7 \pm 1.5 \times 10^4$ respectively shown in Table 1. The CFU count indicates the number of living and functioning bacteria that can proliferate and form colonies. Despite not using any fruit or vegetable as a substrate, the range of sample C fell within the predicted range of the bacterial count to be claimed as probiotics (10^7 - 10^9 CFU/mL) [34].

The yeast count in sample C was determined to be 1.7×10^4 CFU/mL, whereas samples A and B exhibited counts of 1.2×10^3 CFU/mL⁻¹ and 2.4×10^3 CFU/mL⁻¹, respectively (table-1). No mold growth was observed in any of the samples, suggesting that the fermented water kefir drink maintained a high level of hygienic quality.

According to *Muganga et al.*, for fermented foods to have a beneficial effect on health, the recommended viable cell count is 10^6 to 10^8 CFU mL⁻¹. Depending on the types of grain, the microbiota of water kefir is composed of yeast, LAB, and AAB. However, the inclusion of fruits like figs, apples and grapes can alter the acidic environment and carbohydrate content, which can lead to development of dominating groups of microbiotas. It was also established in a prior study that water kefir bacteria are more viable in water kefir drinks made with 2% lactose than in dwhey (demineralized whey) with 5% lactose. The lactose in the dWhey water kefir was used by the microorganism and fermented to glucose and galactose and the mesophilic bacterial count ranged from more than 6 log CFU/mL in the samples [10]. However, in sample C of our study, it was found to have 2.91×10^7 CFU/mL without adding any fruit substrate. This microbial analysis revealed a symbiotic relationship between beneficial bacteria and yeast in a fermented water-kefir drink. However, this interaction could potentially be enhanced by increasing the concentration of kefir grains or incorporating any fruit as an additional substrate. This result is quite fulfilling with the guideline of Food and Agricultural organization of the United Nations (FAO), which states that the minimum level of viable probiotic bacteria in a food product should be at least 10^6 CFU/g.

Table: 1 Microbial analysis of all three samples (A, B, and C)

	Sample A (Mean ± SD)	Sample B (Mean ± SD)	Sample C (Mean ± SD)	P-value
TBC (CFU mL ⁻¹)	$1.95 \times 10^5 \pm 2 \times 10^3$	$4.55 \times 10^5 \pm 1.5 \times 10^3$	$2.91 \times 10^7 \pm 1.5 \times 10^4$	0.032
Yeast count (CFU mL ⁻¹)	$1.2 \times 10^3 \pm 60.83$	$2.4 \times 10^3 \pm 152.8$	$1.7 \times 10^4 \pm 57.74$	0.026

TBC: Total bacterial count; CFU: Colony forming unit.

P < 0.05 was considered to be statistically significant.

Kruskal-Wallis test has been used to analyze the P-value.

3.3 DPPH radical scavenging antioxidant analysis:

Table 2 and figure 6 present the DPPH radical scavenging activities of water kefir samples with varying concentrations of kefir grains. Sample C exhibited markedly elevated scavenging activity (28.74%) compared with samples A (2.715%) and B (4.8%), indicating a substantial difference among the samples (p < 0.05). Furthermore, an increase in the concentration of kefir grain inoculum was directly associated with an increase in antioxidant potential. This substantial increase in the antioxidant activity of the water kefir samples can be attributed to the fermentation process, which results in elevated levels of bioactive compounds. Further analysis using unpaired t-tests revealed that all subgroup analysis samples A, B, and C yielded p-values less than 0.0001, confirming that the differences in scavenging activity were statistically significant.

In previous studies, DPPH scavenging activity was reported to be 11%, when water kefir was cultured by inoculating it with sugar solution (6.5%) in mineral water containing 5% water kefir grains and 5 g/L fresh apple pieces. In contrast, sample C in our study had a DPPH scavenging activity of 28.83%, without any fruit being added as a substrate. The investigation of water kefir fermented with aronia pomace and aronia juice revealed that the DPPH radical-scavenging activity of aronia pomace kefir was higher than that of aronia juice [11]. According to different studies, the DPPH activity of vegetable juice water kefir fermented drinks made from carrot, fennel, onion, melons, tomatoes, and strawberries was 14.53%, 20.12%, 78.67%, 20.24%, 78.31%, and 95.38%, respectively [10]. Similarly, soy whey, pomegranate juice, whey, peanuts, Russian olive fruit, and flaxseed oil cake were found to have high antioxidant activities in fermented plant beverages [40]. The high level of DPPH activity was primarily due to the presence of fruits and vegetables which are already high in antioxidants, as substrates for kefir fermentation.

Bioactive chemicals in the exopolysaccharide structure generated during fermentation and lactic acid bacteria present in the kefir grain are also two major factors contributing to the high antioxidant activity of water kefir fermented drinks [11, 22]. This high level of antioxidant activity in sample C shows that it may have positive effects on health, since antioxidants shield cells from free radical damage, which may lower the risk of chronic illness and improve general health.

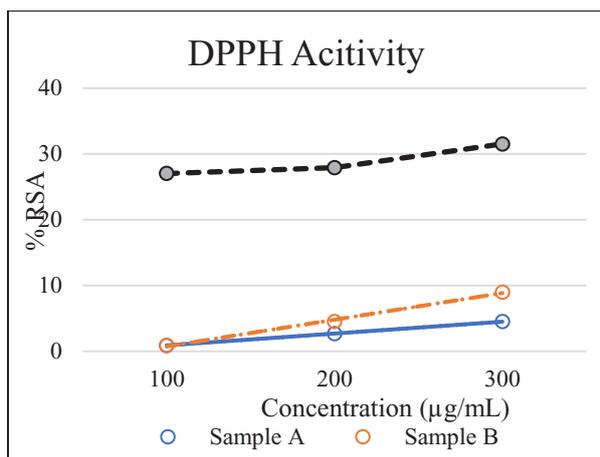


Figure 6: DPPH radical scavenging activity (RSA) % of sample A, B and C with 2%,4% and 6% kefir grain concentration.

Table 2: DPPH RSA (µg/mL) of Sample A, B and C

Samples	RSA (µg/mL)	P-Value (Anova)	Unpaired t-test	P-Value
A	2.715 ± 0.007		A vs B	<0.0001
B	4.806 ± 0.002	<0.05	B vs C	<0.0001
C	28.74 ± 0.05		C vs A	<0.0001

*P value < 0.05 was considered to be statistically significant. Kruskal-Wallis analysis has been used to compare DPPH radical scavenging activity among samples A, B, and C. Bonferroni corrected P < 0.017 considered to be statistically significant for the subgroup analysis.

3.4 Phenolic compounds

Figure 7 illustrates a significantly higher number of phenolic compound content in Sample C (14.86 ± 0.45 g/100 g GAE) of the water kefir fermented drink compared to Sample A (2.64 ± 0.03 g/100 g GAE) and Sample B (8.56 ± 0.45 g/100 g GAE) (P < 0.05) without the addition of any additional plant-based substrate. In previous study it was approximately 191 mg GA/I phenolic compound when 5% kefir grains were used for the fermentation of pomegranate juice with the addition of whey [14]. This demonstrates that a simple water kefir fermented drink without the addition of any other fruit substrate and made with kefir grain, brown sugar, and mineral water can also offer preventative health care against many health issues because of its adequate phenolic content.

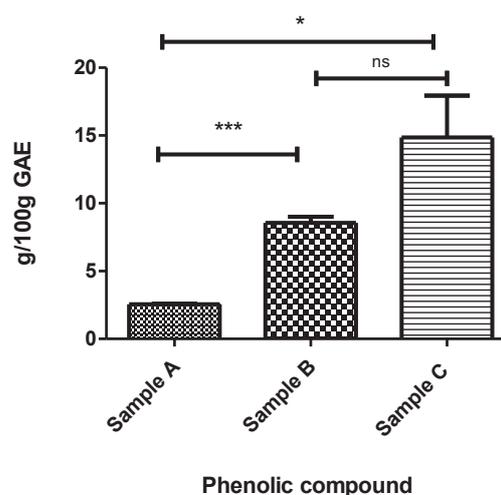


Figure 7: Phenolic compound of sample A, B and C with 2-6% kefir grain concentration.

* Indicates P < 0.05 and *** indicates P < 0.001 was considered to be statistically significant. Mann-Whitney U test was used for subgroup analysis. A Bonferroni corrected P value < 0.017 considered to be statistically significant for the subgroup analysis. ns: Not significant

3.5 Sensory analysis of the water kefir fermented drink:

Samples A, B, and C were evaluated based on their appearance, sourness, odour, texture, and overall acceptability using a nine-point hedonic scale from liked extremely to dislike extremely. Figure 8 shows the radar graphs of the sensory attributes of samples A, B, and C. The values plotted on each axis of the radar chart correspond to one of the sensory attributes. Each attribute value is placed on the corresponding axis and linked by lines to create a polygon. Sample C formed a larger, more expansive polygon than samples A and B, reflecting higher scores across most attributes. However, samples A and B formed comparatively smaller polygons, reflecting a lower score for a few attributes. Hence, sample C was more acceptable for all parameters than samples A and B [15].

Table 3: Sensory analysis of sample A, B, and C

Parameter	Sample A	Sample B	Sample C	P-value
Appearance	5.3 ± 0.31	5.8 ± 0.42	6.9 ± 0.25	0.0048
Texture	5.8 ± 0.21	6.2 ± 0.25	6.8 ± 0.25	0.0126
Odour	5.67 ± 0.15	5.85 ± 0.12	6.90 ± 0.35	0.0145
Sourness	5.12 ± 0.17	5.62 ± 0.18	6.97 ± 0.22	0.0072
Overall acceptability	4.32 ± 0.17	6.25 ± 0.20	7.0 ± 0.18	0.0073

P < 0.05 considered to be statistically significant. Kruskal-Wallis test has been used to analyze the P-value.

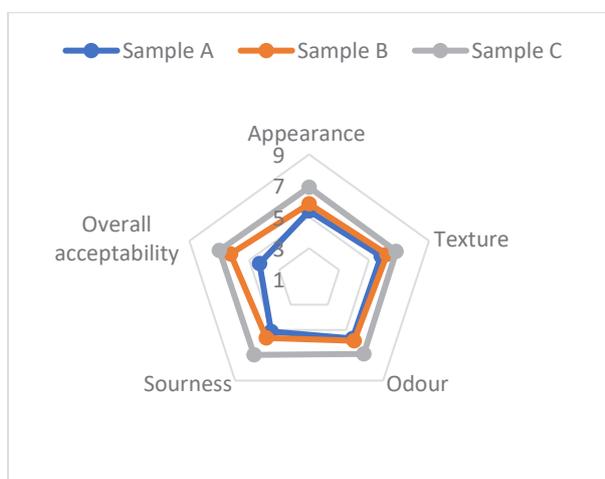


Figure 8: Average result of T analysis of all the sample of water kefir after 24 hours of fermentation.

4. CONCLUSION

In conclusion, this study demonstrated that water kefir fermented with different concentrations of kefir grains without the inclusion of any fruit or fruit product as a substrate exhibits significant variations in microbial, physicochemical, and antioxidant properties. Among the 3 samples, the 6% kefir grain concentration (sample C) showed optimal results, with the highest microbial viability (2.91×10^7 CFU/mL), antioxidant activity (28.83% DPPH), and improved physicochemical characteristics, including a desirable pH, TA and TSS. The acceptability evaluation also indicated that the fermented water kefir drink, particularly higher kefir grain concentration can serve as an important functional beverage with potential health benefits, especially for individuals seeking non-dairy probiotic alternatives.

REFERENCES

- I. Siró, E. Kápolna, B. Kápolna, & A. Lugasi. Functional food. Product development, marketing and consumer acceptance—A review. *Appetite*. **51**, 456–467 (2008).
- M. D. Pendón, A. A. Bengoa, C. Iraporda, M. Medrano, G. L. Garrote, & A. G. Abraham. Water kefir: Factors affecting grain growth and health-promoting properties of the fermented beverage. *Journal of Applied Microbiology*. **133**, 162–180 (2022).
- M. Min, C. R. Bunt, S. L. Mason, & M. A. Hussain. Non-dairy probiotic food products: An emerging group of functional foods. *Critical Reviews in Food Science and Nutrition*. (2024)
- C. Cao, Q. Hou, W. Hui, L. Kwok, H. Zhang, & W. Zhang. Assessment of the microbial diversity of Chinese Tianshan tibicos by single molecule, real-time sequencing technology. *Food Sci Biotechnol*. **28**, 139–145 (2019).
- D. Laureys *et al.* The Type and Concentration of Inoculum and Substrate as Well as the Presence of Oxygen Impact the Water Kefir Fermentation Process. *Front. Microbiol*. **12**, (2021).
- D. Laureys & L. De Vuyst. The water kefir grain inoculum determines the characteristics of the resulting water kefir fermentation process. *Journal of Applied Microbiology*. **122**, 719–732, Mar. (2017).
- D. Laureys & L. De Vuyst. Microbial Species Diversity, Community Dynamics, and Metabolite Kinetics of Water Kefir Fermentation. *Applied and Environmental Microbiology*. **80**, 2564–2572 (2014).
- D. Laureys, A. Van Jean, J. Dumont, & L. De Vuyst. Investigation of the instability and low water kefir grain growth during an industrial water kefir fermentation process. *Appl Microbiol Biotechnol*. **101**, 2811–2819 (2017).
- D. Laureys, M. Aerts, P. Vandamme, & L. De Vuyst. The Buffer Capacity and Calcium Concentration of Water Influence the Microbial Species Diversity, Grain Growth, and Metabolite Production During Water Kefir Fermentation. *Front. Microbiol*. **10**, (2019).
- K. Saleem *et al.* Nutritional and functional properties of kefir: review. *International Journal of Food Properties*. **26**, 3261–3274 (2023).
- Cufaoglu & A. N. Erdinc. An alternative source of probiotics: Water kefir.” *Food Frontiers*. **4**, 21–31 (2023).
- M. Chadha, R. Shukla, R. K. Tiwari, S. Choudhary, A. Adya, & K. Singh. Optimizing

- the Formulation of Homemade Milk Kefir Drink from India: Comprehensive Microbial, Physicochemical, Nutritional, and Bioactivity Profiling. *Engineering Proceedings*. **67** (2024).
- 13 A. G. Pablo & L. Cimafranca. Development of a Water Kefir Beverage Made from Jackfruit Processing By-product and Its Potential Probiotic Property. *Philipp J Sci*. **151** (2022).
- 14 S. Toschkova. Structural characterization of an exopolysaccharide produced by *Lactobacillus plantarum* Ts. *Acta Scientifica Naturalis*. **9**, 71–83 (2022).
- 15 S. Choudhary, K. Singh, M. Chadha, & R. Shukla. Assessment of techno-functional properties of optimized popped sorghum grains. *Indian Journal of Agricultural Biochemistry*. **37**, 142–154 (2024).