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Optimizing the Formulation of Homemade Milk Kefir Drink from India: Comprehensive Microbial, Physicochemical, Nutritional, and Bioactivity Profiling †

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Abstract: Kefir is a naturally fermented milk drink with rich probiotic content. This study aimed to develop and optimize homemade cow milk kefir (HCMK) and evaluate its microbial, chemical, nutritional, and antioxidant properties. HCMK was optimized using response surface methodology, where the independent variables were kefir grain inoculum (2–4%) and fermentation time (20–28 h), and the dependent variables were total bacterial count, pH, and overall acceptability. HCMK was prepared using 3% *w*/*v* Indian kefir grains inoculated into cow milk and fermented at 25 °C for 24 h. Optimized dependent variables were $2.08 \times 10^8 \pm 0.34$ CFU/mL, pH 4.52 ± 0.05, and overall acceptability of 6.55 ± 0.21. Nutritional analysis revealed protein 3.6 g/100 mL, carbohydrates 2.66 g/100mL, fat 3.4 g/100 mL, iron 2.99 mg/100 mL, and calcium 29.3 mg/100 mL. Antioxidant profiling elucidated 54% radical scavenging activity and 18 mgGAE/100 mL total phenolic content. GC-MS revealed the presence of bioactive compounds with documented antioxidant, anti-inflammatory, and antimicrobial activities. This study highlights HCMK as a healthy probiotic functional food with significant antioxidant potential.

Keywords: kefir; nutrition; microbiology; GC-MS; antioxidant

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1. Introduction

Probiotics and bioactive organic compounds have garnered significant attention in recent years [1]. Natural probiotics in fermented foods enhance several metabolic processes, as well as nutritional and functional properties such as antioxidant and anti-inflammatory effects. This is a result of substrate modifications and the production of bioactive and bioavailable end products, making them promising adjuncts to human health [2,3]. Probiotic and bioactive compounds, including biomolecules with alcoholic functional groups, organic acids, acetaldehydes, and bacteriocins, inhibit pathogenic microbes such as *Bacillus cereus*, *Streptococcus pyogens*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonela typhimurium* [2]. Probiotic and bioactive compounds include proteolytic enzymes (trypsin, pepsin, and α-chemotrypsin), which enhance several facets of human nutrition [2]. Among the emerging functional foods, probiotic-based foods and beverages are becoming one of the most acceptable to consumers [4]. Therefore, there is an urgent need for further research on various probiotic-rich fermented foods.

Traditionally, fermented foods have been shown to contain probiotic microorganisms [5]. Kefir is a naturally fermented food that is rich in probiotics. It originated from

the Caucasus mountains regions of Asia and is an acid-alcoholic fermented milk drink that has been consumed since ancient times [6]. Kefir is creamy in texture and tastes slightly sour and acidic. It is now a widely consumed beverage worldwide, mostly in Eastern and Northern Europe and Japan. The term kefir is derived from the Turkish word "keyif" meaning "good feeling", in reference to the emotions one has after consuming the drink [6]. Kefir is differentiated into two main types, namely traditional and industrial kefir, based on the process of its production method. The key difference between these methods lies in the process of inoculation of kefir grains or starter culture in the milk. In the traditional method, kefir varies in microbial content and sensory properties due to its origin, storage, and handling conditions [7]. In contrast, industrial methods rely on standard starter cultures and precise environmental conditions to ensure that every batch has the same microbial content and sensory properties [7]. However, there are variations within each type, and the differences between them can be attributed to factors such as the type of fermentation used, the duration of fermentation, and the specific microorganisms involved in the fermentation process. Traditional kefir preparation involves fermenting milk using kefir grains, a starter culture of yellowish, tiny, amorphous, and gelatinous form [8]. Kefir grains are hard granules resembling cauliflower florets in appearance that vary from 0.3 to 3.5 cm in size. These grains are usually composed of diverse combinations of bacterial species, including lactic acid bacteria (10^8 CFU/g) such as *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Carnobacterium*, and acetic acid bacteria (10^5 CFU/g) such as *Acetobacter cerevisiae*, *Acetobacter pasteurianus*, and yeast (10^4–10^6 CFU/g) [9,10]. Kefir grains are kept viable by maintaining a high bacteria–yeast ratio, which is accomplished by ongoing fermentation cycles that increase the biomass of grains depending on different factors, such as pH, temperature, washing, milk renewal, and availability of nutrients [11]. After a series of successful fermentation processes, kefir grains divide into new grains with the same microbial traits as the originals [12]. To produce kefir in household conditions, kefir grain preservation can be accomplished either by continuous fermentation cycles or 10 weeks of multiplication of biomass growth [13].

However, the production of traditional kefir faces various challenges, such as variability in microbial diversity and physicochemical and nutritional properties. This hinders the production process and quality consistency. To address these challenges, optimizing cow milk kefir can be used to standardize its microbial and nutritional qualities. Some studies have shown that kefir grains and fermentation time directly impact the properties of the drink. Kefir grain concentrations of 3–5% were found to be optimal for yielding the lactic acid bacteria and protein levels [1,14,15]. Additionally, 18 h, 21 h, and 24 h fermentation times have been known to change the intensity of microbial activity and chemical composition of milk, which can influence the quality of the end product [14,16,17]. Apart from preserving the shelf life of cow milk, fermentation through kefir culture encompasses augmentation of its bioavailability and therapeutic benefits, such as antimicrobial and anticancer properties due to the presence of strain-specific microorganisms in kefir [17,18]. Thus, optimizing these variables is critical for enhancing kefir quality and its suitability for meeting customer needs and food safety requirements.

According to reports, probiotics present in milk kefir generate different chemical metabolites that have hypocholesterolemic, antimicrobial, antihypertensive, anti-inflammatory, antioxidant, and anticarcinogenic actions and are generally beneficial to human health [18,19]. Kefir has been shown to possess antibacterial properties against a variety of microbes [19]. Kefir has been acknowledged as a beneficial functional food with alternative therapeutic potential, and further studies on this topic are particularly important [19]. This study aimed to develop and optimize the homemade cow milk kefir drink (HCMK) preparation process under household conditions using response surface methodology (RSM) and to determine the bioactive compounds and the physicochemical, nutritional, and antioxidant properties of HCMK.

2. Materials and Methodology

2.1. Procurement of Kefir Grains

Kefir grains were procured from the culture market, Sri Dhanwantari Probiotics Pvt. Ltd., Vandipalayam, Ukkaram, Tamil Nadu, India. The grains were spherical, resembling cauliflower florets, and ranged in color from white to creamy yellow. They were initially produced by auto-aggregating various yeast, lactic acid, and acetic acid bacteria, generating adherent grain surfaces after consecutive fermentations with pasteurized cow milk.

2.2. Optimization and Preparation of Homemade Cow Milk Kefir Drink (HCMK)

The homemade cow milk kefir drink (HCMK) was optimized using response surface methodology (RSM) coupled with central composite design (CCD). The independent variables used during the study included kefir grain inoculum (2–4%) and fermentation time (20 h–28 h). Thirteen trial runs were conducted with five replicates at the center point, using a CCD setup with two independent variables. The dependent response variables evaluated were the total bacterial count (TBC), pH, and overall acceptability.

For the preparation of the HCMK, 2–4% of kefir grain inoculum was inoculated in commercially available full cream cow milk, which was further allowed to ferment at 25 °C for 20 h–28 h, as per the different trial runs (Figure 1). After completion of the fermentation process, the cow milk kefir drink was filtered, and the kefir grains were separated using a sterile plastic sieve. The drink was formulated using the same procedure in triplicates and was stored at 4 °C for analysis (Figure 1). Thereafter, kefir grains were washed gently with double distilled water and kept at 4 °C with 50 mL of cow milk for future use.

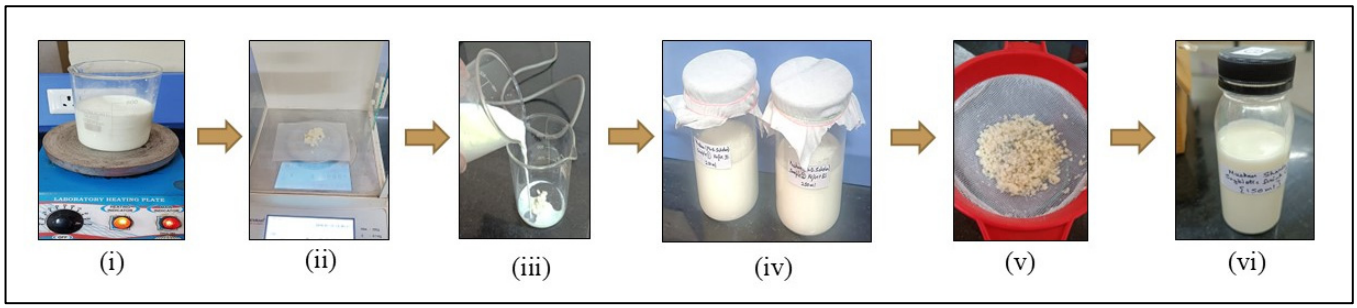

Figure 1. Formulation of milk kefir drink using household conditions. (**i**) Boiling milk at 100 °C, (**ii**) kefir grain inoculum, (**iii**) addition of milk, (**iv**) fermentation at room temperature, (**v**) sieving kefir grains for reuse, and (**vi**) HCMK drink stored at 4 °C.

2.3. Physicochemical Analysis

Physicochemical analyses were performed for both HCMK and cow's milk, which served as the control. The lactic acid content was measured using an acid-base titration method that was conducted using 0.1N NaOH and 1% phenolphthalein indicator [20]. The color parameters lightness (L), redness (a), and yellowness (b) of the samples were measured using a spectrophotometer (NS810 Portable Spectrophotometer, 3nh, Chennai, India).

The viscosity of the samples was measured using a Brookfield digital viscometer (AMETEK Instruments Pvt. Ltd., Mumbai, India) with a spindle at 30 rpm and 25 °C. The pH of all samples was measured using a digital pH meter (Labman Scientific Instruments Pvt. Ltd., Chennai, India).

Total soluble solids were measured using a digital bench refractometer (Hanna Equipments Pvt. Ltd., Mumbai, India). The water activity of the samples was measured using a water activity meter (Rotronic HC2A-AW, PST Pvt. Ltd., Chennai, India).

2.4. Sensory Evaluation Test

A total of fifty sensory semi-trained panelists were recruited for the overall acceptance evaluation of the HCMK and control (cow milk) samples using a 9-point hedonic scale [21].

The panel members were asked to evaluate the appearance, sourness, odor, texture (consistency), and overall acceptability of the formulated HCMK. Different coded fermented milk samples of 50 mL at 8–10 °C were provided in transparent cups with a maximum of four samples to test in one session, and mineral water was given in between samples to rinse the palate.

2.5. Nutritional Analysis

2.5.1. Proximate Analysis

The macronutrient compositions of the HCMK and control (cow milk) samples were determined according to the AOAC methods [22]. Moisture content was determined using the hot air oven method by drying the sample at 100 ± 1 °C for 10 h, and calculated using the following formula [23]:

$$Moisture\% = \frac{Intial\ weight - Final\ dried\ weight}{Intial\ weight} \times 100$$

The moisture-free sample was then kept at 600 °C in a muffle furnace (Bionics Scientific Pvt. Ltd., Delhi, India) for 3 h to estimate the crude ash content using the following equation [24]:

$$Ash\ \%\ by\ mass = \frac{Intial\ weight - Final\ dried\ weight}{Intial\ weight} \times 100$$

The protein content was analyzed using the Kjeldahl method [23]. This method comprises three steps. In the first step, protein digestion was performed using conc. sulfuric acid in the presence of a potassium sulfate, which acts as a catalyst. In the second step, the digested sample was neutralized with alkali to liberate ammonia and collected in boric acid. In the third step, it was further titrated with hydrochloric acid in the presence of a methyl red–bromocresol green indicator until the green distillate changed from colorless to pink. Crude protein content mass was expressed as a percentage using the multiplication of nitrogen content and using a 6.38 specific conversion factor for milk.

Crude fat was estimated using the Rose–Gottlieb method (FSSAI 01.123:2022) [24], which is a gravimetric method in which fat globule membranes of milk fat globules are ruptured by the addition of ammonia to liberate fat. HCMK and control samples were treated with 25% ammonia and 95% ethyl alcohol to precipitate the proteins. Fat was extracted with diethyl ether (peroxide free) and petroleum ether (boiling range 40–60 °C), which was then evaporated, and the residue content was measured.

Carbohydrates were obtained by the difference method using the following equation [22]:

$$\text{Total Carbohydrate(g)} = 100 - \sum(\text{Protein, carbohydrate, fat, moisture})\ \text{g}$$

Total energy was calculated by the summation method using the equation [22]:

$$\text{Energy (kcal)} = 4 \times (\text{protein} + \text{carbohydrate})\ \text{g} + 9 \times (\text{fat})\ \text{g}$$

2.5.2. Micronutrient Analysis

Briefly, 1 g moisture-free HCMK and control were taken for wet digestion of samples kept at 550 °C in a muffle furnace for 3 h to obtain crude ash content. In glass tubes, an ash sample was added with 3 mL nitric acid and 1 mL hydrochloric acid and heated on a hot plate at 60–80 °C for 6–7 h till a colorless solution was obtained. The mixture was left to cool, and the contents of the tubes were adjusted to 50 mL using distilled water [25].

The digested samples were analyzed for calcium, iron, zinc, magnesium, manganese, and phosphorous contents using atomic absorption spectroscopy (AAS-Perkin Elmer (MA 5840), Monmouth County, NJ, USA). Different electrode lamps were employed for each of the minerals. For calibration, the standard solutions of each mineral were run using

the equipment. The dilution factors for magnesium, phosphorus, and other minerals were 10,000, 2500, and 100, respectively. The concentrations were expressed as 'ppm' and then converted to mg [25].

The wet-digested sample solution was used for the estimation of sodium and potassium, which were analyzed using flame photometry (D. Haridas and Company, Pune, India). Standard solutions of 25, 50, and 100 ppm were used for calibration. The concentration of elements was calculated by degree of emission, expressed as 'ppm', and then converted to mg.

2.6. Microbial Analysis

For the determination of the TBC and yeast count, 1 mL HCMK and control samples were added to ten-fold dilutions in 0.1% sterile peptone water. Serial dilutions were performed for 10^{-7} and plated in each medium. The TBCs were quantified on nutrient agar media (NAM) plates (Sisco research laboratory, Pvt. Ltd., Maharashtra, India), which were incubated at 37 ± 1 °C for 24 h. Yeast counts were quantified on yeast-extract glucose chloramphenicol (YGC) (HiMedia®, Mumbai, India) agar plates, which were incubated at 27 ± 1 °C for 48 h under aerobic conditions [26].

The presumptive test for *Escherichia coli* was performed by mixing the samples with Butterfield's phosphate-buffered water (HiMedia Mumbai, India) at pH 7.2. Serial dilutions were made from each sample in sterile Butterfield's phosphate-buffered diluents (Butterfield's buffer). Aliquots from each serial dilution were inoculated in lactose broth (LB) tubes (HiMedia, Mumbai, India) in triplicate to determine the most probable number. The LB tubes were cultured at 37 °C for 48 h to determine gas production. A loopful of each suspension was transferred to E. coli broth (ECB) (HiMedia Mumbai, India) tubes and incubated at 44 °C for 48 h. The E. coli count was quantified by streaking a loopful of suspension from ECB tubes onto eosin methylene blue agar (HiMedia Mumbai, India) plates and incubating at 35 °C for 24 h.

Total counts for HCMK and control samples were measured using a digital colony counter (Tanco, New Delhi, India) and expressed as colony-forming units per milliliter (CFU/mL) determined using the following formula:

$$\frac{CFU}{mL} = \frac{Number\ of\ colonies \times Dilution\ Factor}{Volume\ of\ culture\ plate\ in\ ml}$$

2.7. GC-MS Analysis

Briefly, 0.1 g of HCMK sample was weighed and dissolved in 99% methanol at 25 °C followed by 2 h sonication. The extract was filtered using a syringe filter of 0.22 µm. The identification of bioactive compounds was performed using a GC system (Agilent-8860, Agilent Technologies, Santa Clara, CA, USA) interfaced with a single quadrupole (stainless steel source) mass spectrometer coupled with a DB-5 capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness, J and W Scientific, Folsom, CA, USA). Helium at 1 mL/min was used as the carrier gas, and the retention indices were determined after studying the effect of these chromatographic conditions on the C8–C20 n-alkane series [27]. This analysis took 22 min on the GC-MS instrument. The relative percentage of sample constituents was calculated using the peak area normalization method. The identification of the bioactive compounds of HCMK was performed through the application of retention indices and the mass spectra patterns of the compound by comparison with the database of the National Institute Standard and Technology (NIST). The spectrum of the unknown component was analyzed and matched with the spectrum of the known components in the NIST library.

2.8. Bioactive Compounds

2.8.1. Total Phenolic Content

The total phenolic content of the samples was determined using the Folin–Ciocalteu method, as previously described [28]. Briefly, 2 g of HCMK and control samples was

diluted with 10 mL Acetone (80% v/v) and then centrifuged (5000 rpm, 10 min at 20 °C). In a cuvette, 1 mL of the collected supernatant, 2 mL distilled water, 0.5 mL Folin–Ciocalteu reagent, and 0.5 mL of sodium carbonate (10%) were loaded. The absorbance of the samples was measured at 760 nm by using a dual-beam UV spectrophotometer after incubation in the dark at 30 °C for 1 h. Total phenolic content analysis was performed in triplicates and was expressed as 'mg gallic acid extract (GAE) per 100 mL' [28].

2.8.2. Antioxidant Activity

For the 2,2-Diphenyl-1-picrylhydrazyl (DDPH) radical scavenging activity, 0.2 g samples were added to 10 mL methanol and centrifuged (5000 rpm, 10 min at 25 °C), and the supernatant was filtered. Then, 0.1 mL of the filtered sample was added to 5 mL of 0.1 mmol/L DPPH solution made with methanol (99%) and incubated in the dark for 30 min at 30 °C [28]. The absorbance was recorded using a dual-beam UV spectrophotometer at 517 nm, and percentage radical scavenging activity (RSA) was calculated using the following equation:

$$\% \text{ RSA} = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

The inhibition concentration (IC) value expressed the concentration of the compounds that could inhibit 50% of the total DPPH radical. The IC50 was determined from the scavenging effect percentage graph against the extract concentration through curve fitting of a non-linear regression model. The values are expressed in μg/mL of the sample extracts. The lower the IC50, the higher the antioxidant activity, and vice versa.

2.9. Statistical Analysis

Data were expressed as the mean ± standard deviation. Physicochemical, microbial, and nutritional data of samples were analyzed using Student's *t*-test. Sensory evaluation data were analyzed using the chi-square test. All statistical analyses were performed using GraphPad Prism version 8.0 (GraphPad Software Inc. La Jolla, CA, USA). Data were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. Optimization of HCMK

Thirteen formulations of HCMK drinks were prepared as per the trial runs. The central point was repeated five times in trial runs 1, 4, 6, 7, and 13 (Table 1). The effects of these factors on the response variables included TBC (Y1), pH (Y2), and overall acceptability (Y3). The TBC varied from 7.95×10^6 to 8.73×10^8 CFU/mL, with a mean value 2.67×10^8 CFU/mL (Table 1). An increase in the inoculum level of kefir grains increased the TBC. Similar results were observed in a previously reported study, which noted that the counts of lactic acid bacteria (LAB) increased with an increase in kefir grain inoculum from 5% to 8% [29]. The pH values observed in the 13 trial runs ranged from 3.85 to 6.09, with a mean value of 4.82 ± 0.68. Furthermore, an increase in the quantity of kefir grain inoculum was directly related to a decrease in the pH level. It has been reported that pH is reduced during fermentation, which influences the level of CO_2 generated during cow milk kefir production [30]. The overall acceptability scores ranged from 4.61 to 6.55 across 13 trial runs with a mean value of 6.01 ± 0.61, providing insights into the sensory preferences and satisfaction levels of the participants. These variations in responses demonstrate the sensitivity of fermentation parameters to the overall properties of the drink, providing a foundation for further optimization.

The ANOVA and regression analysis revealed that both independent factors (kefir grains and fermentation time) were critical in determining the response-dependent variables. The ANOVA quadratic model explained a substantial portion of the variability and showed a good fit for the datasets (R^2 = 0.966 for TBC, R^2 = 0.926 for pH, and R^2 = 0.78 for overall acceptability) (Table 2). According to Yaakob et al. [31], the predicted R^2 should

be greater than or equal to 0.8, and higher adjusted R^2 values indicate a good fit for the model dataset. The effects of A-Kefir grains and B-Basil seeds were highly significant across all model dataset response variables ($p < 0.001$), indicating a substantial positive influence on TBC, pH, and overall acceptability (Figure 2). Additionally, the lack of fit data suggests model accuracy in the representation of the datasets, reinforcing the reliability of the optimization process.

Table 1. Matrix of the central composite design (CCD) and observed responses.

Run	Factor A: Kefir Grains (%)	Factor B: Fermentation Time (h)	Response Y1: Total Bacterial Load (CFU/mL)	Response Y2: pH	Response Y3: Overall Acceptability (Score)
1	3	24	2.08×10^8	4.52	6.55
2	4.41421	24	7.03×10^8	4.47	6.02
3	3	29.6569	6.83×10^8	3.97	5.14
4	3	24	2.08×10^8	4.52	6.55
5	2	20	9.87×10^6	5.69	6.21
6	3	24	2.08×10^8	4.52	6.55
7	3	24	2.08×10^8	4.52	6.55
8	4	20	8.99×10^7	5.03	5.46
9	4	28	8.73×10^8	3.85	4.61
10	3	18.3431	8.01×10^6	6.09	5.94
11	2	28	5.74×10^7	5.11	5.85
12	1.58579	24	7.95×10^6	5.8	6.12
13	3	24	2.08×10^8	4.52	6.55

Table 2. Effects of independent variables (kefir grains and fermentation time) on dependent variables (TBC, pH, and overall acceptability).

Source	Sum of Squares	Df	Mean Square	F-Value	*p*-Value
TBC					
Model	1.00×10^{18}	5	2.01×10^{17}	69.7	<0.001
Lack of Fit	2.02×10^{16}	3	6.72×10^{15}		
Pure Error	0	4	0.00×10^0		
Total	1.02×10^{18}	12			
Predicted $R^2 = 0.966$; adjusted $R^2 = 0.860$					
pH					
Model	5.45	5	1.090	31.19	<0.001
Lack of Fit	0.2446	3	0.082		
Pure Error	0	4	0.000		
Total	5.69	12			
Predicted $R^2 = 0.926$; adjusted $R^2 = 0.694$					
Overall acceptability					
Model	3.98	5	0.797	9.67	0.005
Lack of Fit	0.5764	3	0.192		
Pure Error	0	4	0.000		
Total	4.56	12			
Predicted $R^2 = 0.783$; adjusted $R^2 = 0.101$					

Degree of freedom (Df).

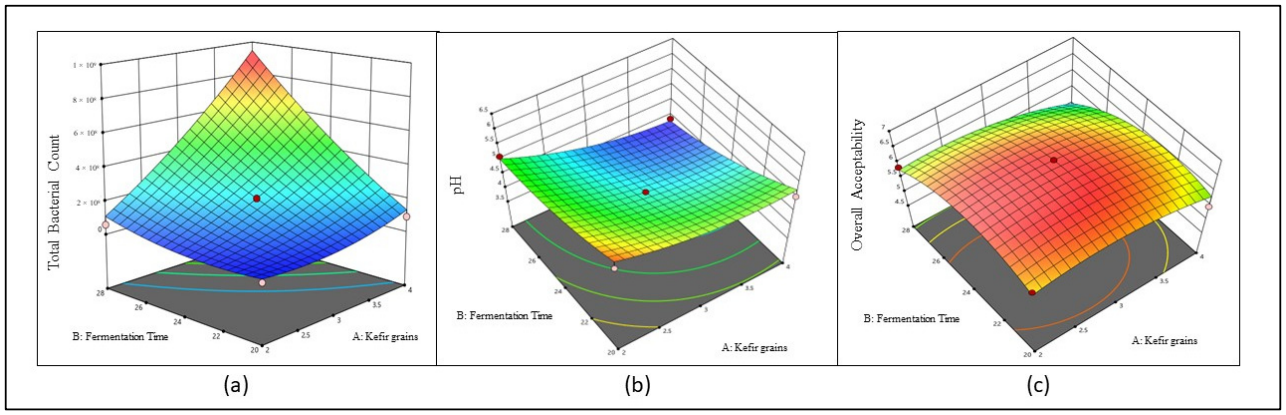

Figure 2. Three-dimensional response graphs showing the effects of kefir grains and fermentation time on (**a**) total bacterial count, (**b**) pH, and (**c**) overall acceptability.

The optimum formulation was the sample prepared with 3% *w*/*v* kefir grain inoculum and 24 h fermentation time. As per previously reported studies [7,32,33], the levels of kefir grains (2–5% *w*/*v)* and fermentation time (18 h–24 h) are consistent with our optimized formulation. In contrast, few studies observed higher nutritional and antioxidant efficacy with 10% *w*/*v* kefir grains [2]; this difference could be explained by the use of different varieties of kefir grain inoculum, which changes the microbial and nutritional parameters of the milk kefir drink. The experimental values were closely aligned with the predicted values of response variables, supporting the optimization hypothesis. The optimized HCMK was prepared in triplicates to confirm the accuracy of the developed model. As shown in Table 3, the optimized results were $2.08 \times 10^8 \pm 0.34$ CFU/mL, pH 4.52 ± 0.05, and overall acceptability 6.55 ± 0.21.

Table 3. Experimental and predicted values of responses under optimized conditions.

Response Variables	Experimental Values	Predicted Values
Total bacterial load (CFU/mL)	$2.08 \times 10^8 \pm 0.34$	2.65×10^8
pH	4.52 ± 0.05	4.32
Overall acceptability	6.55 ± 0.21	6.85

3.2. Physicochemical Analysis of HCMK

Physicochemical analysis of HCMK and control (cow milk) revealed significant changes induced by the fermentation process. The pH of HCMK was lower than that of the control (4.52 ± 0.19 vs. 6.54 ± 0.11, $p < 0.001$), indicating increased acidity, which may have resulted from lactic acid production during fermentation. This acidity level was further supported by the increased titratable acidity ($0.92 \pm 0.14\%$) of HCMK as compared to the control (Table 4). These values were also consistent with the 4–6 pH value and 0.7–1.2% titratable acidity reported for the cow milk kefir [7,11]. The rate of acidification observed during fermentation in our study fits the literature data and could be the result of bacterial ability to acidify the milk [34,35]. Variation in pH and lactic acid percentage during kefir fermentation is an indirect indication of the biological activity of kefir grains [36]. With the increase in the TBC due to the addition of kefir grains, the pH of the sample decreased. However, it does not become increasingly acidic over time, as kefir grains are strained after 24 h of the fermentation cycle [35,37].

Table 4. Physicochemical analysis of HCMK compared to control.

Variables	HCMK	Control	*p*-Value
Viscosity (mPa.s)	41.3 ± 3.08	2.48 ± 0.02	<0.001
pH	4.52 ± 0.19	6.54 ± 0.11	<0.001
Titratable acidity (% lactic acid)	0.92 ± 0.14	0.16 ± 0.03	<0.001
Color *			
L	29.7 ± 3.93	76.41 ± 2.36	<0.001
A	−0.4 ± 0.11	−1.2 ± 0.61	0.084
B	3.5 ± 1.00	6.94 ± 0.73	0.008
Water activity	0.55 ± 0.02	0.99 ± 0.04	<0.001
Total soluble solids	6.54 ± 0.44	13.17 ± 0.92	<0.001

* Lightness (L), redness (A), and yellowness (B) values.

Color measurements showed significant differences, with HCMK having lower lightness (L), redness (A), and yellowness (B) values, likely influenced by the breakdown of pigments and the formation of new compounds during fermentation. HCMK exhibited a notably higher viscosity compared to the control (41.3 ± 3.08 mPa.s vs. 2.48 ± 0.02 mPa.s, $p < 0.001$), indicating a thicker consistency resulting from the interaction of proteins and polysaccharides during fermentation. This is specifically due to the production of kefir's unique polysaccharide known as kefiran, which forms the grain matrix and is also dissolved in the fluid to influence the consistency of the fermented drink [38,39].

Additionally, HCMK exhibited lower water activity and total soluble solids than the control ($p < 0.001$), indicating changes in water content and solute concentration as a result of fermentation (Table 1). These physicochemical alterations establish the characteristics of the kefir fermentation process, which affects texture, acidity, and overall composition.

3.3. Sensory Analysis of HCMK

The radar graph visually depicts the sensory attributes of HCMK and the control (Figure 3). The values plotted on each axis of the radar chart correspond to one of the sensory attributes. Each attribute value was placed on the corresponding axis and linked by lines to create a polygon. The HCMK formed a larger, more expansive polygon compared to the control, indicating higher scores across most attributes. The control drink formed a smaller polygon, reflecting lower scores for some attributes (Figure 3). The sensory evaluation data suggest that HCMK is generally preferred over the control in terms of sourness (7.3 ± 0.53 vs. 4.4 ± 0.71, $p < 0.01$) and texture (7.1 ± 0.82 vs. 5.7 ± 0.34, $p < 0.01$). The overall acceptability of HCMK was moderately lower (6.55 ± 0.33) than that of the control (7.1 ± 0.48), indicating that HCMK closely approached the acceptability level of standard cow milk, which is consumed in most households (Figure 3). The evaluation showed that fermentation enhanced the flavor profile, texture, and aroma. These factors likely led to the higher sensory scores in the HCMK.

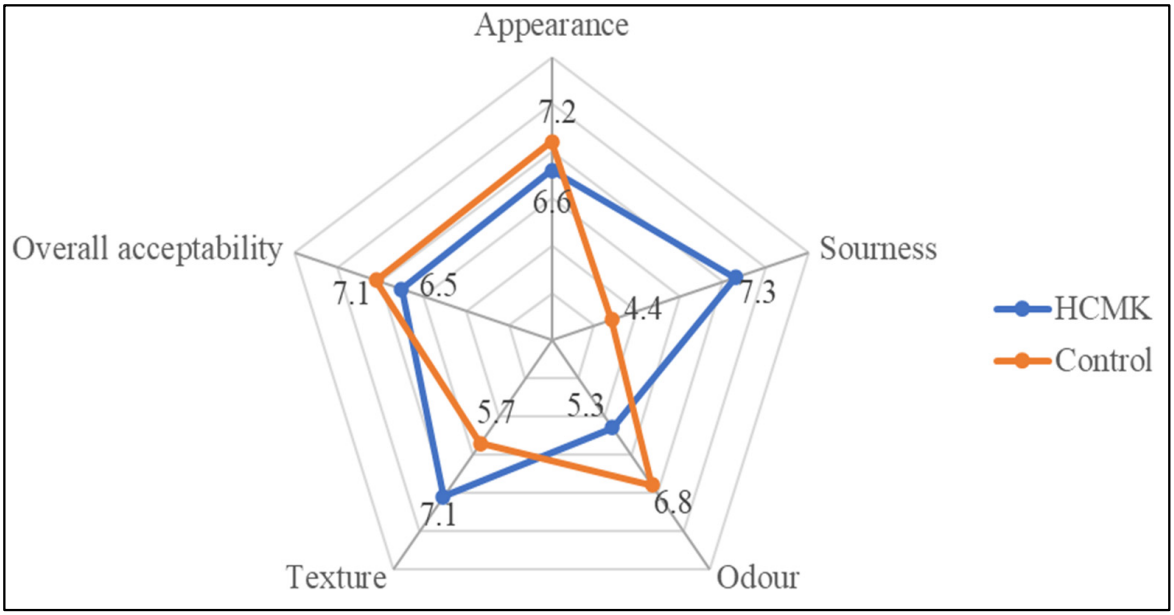

Figure 3. Sensory evaluation of HCMK compared to control.

3.4. Nutritional Analysis of HCMK

The nutritional profile of HCMK compared with that of the control (cow milk) is shown in Table 5. The moisture percentage in HCMK was higher compared to the control (89.7% vs. 86.2%, $p < 0.001$). Our study data comply with the previously reported studies (88–90%), which have depicted a slight decrease in dry matter content in kefir drinks after fermentation due to the lactose change [35,40,41]. The ash percentage of HCMK was higher than that of the control, suggesting an increase in mineral content. This is a result of microbial activity during fermentation, which leads to the breakdown of certain components, such as calcium, magnesium, and phosphorus in milk [41,42].

Table 5. Macronutrient analysis of HCMK compared to control.

Variables	HCMK	Control	*p*-Value
Moisture (%)	89.7 ± 0.06	86.2 ± 0.03	<0.001
Ash (%)	0.643 ± 0.04	0.54 ± 0.08	0.357
Carbohydrate (g/100 mL)	2.66 ± 0.40	4.28 ± 0.87	0.042
Calories (kcal/100 mL)	56.06 ± 3.18	82.24 ± 4.29	<0.001
Protein (g/100 mL)	3.6 ± 0.20	3.14 ± 0.36	0.125
Fat (g/100 mL)	3.4 ± 0.19	5.84 ± 0.71	0.999

Carbohydrate content was significantly lower in HCMK compared to the control (2.66 ± 0.40 g/100 mL vs. 4.28 ± 0.87 g/100 mL), likely due to the consumption of lactose by lactic acid bacteria during fermentation and the presence of polysaccharide kefirans [43].

The protein content is slightly higher in HCMK (3.6 ± 0.20 g/100 mL) compared to the control (3.14 ± 0.36 g/100 mL), indicating potential protein enrichment through fermentation processes. Kefir grains influence milk proteins through proteolysis, resulting in different peptides and non-protein nitrogen compounds that have an impact on the protein profile of kefir [44]. However, the data from this study showed that there was no significant difference in the total protein content of kefir and unfermented milk. During fermentation, casein content remains almost stable, thus reflecting a relatively low level of casein proteolysis [45].

The fat profile of HCMK was found to be reduced in comparison to that of the control, indicating that the process of kefir fermentation can affect the distribution and content of fats; thus, the overall fat content in HCMK was found to be reduced. Our results for fat, protein, and ash contents are in agreement with the results reported in previous studies

(fat 3.3–3.5%, protein 3.09–3.91%, and ash 0.61–1.06%) [9,46–49]. Also, the composition of protein, which is more than 2.7% and fat less than 10% in HCMK, complies with the Codex Alimentarius standards for fermented milk, FAO [50]. The lower caloric content in HCMK than that of control (56.06 ± 3.18 kcal/100 mL vs. 82.24 ± 4.29 kcal/100 mL, $p < 0.001$) reflects changes in carbohydrate and fat content during fermentation.

The values of HCMK exhibited significantly higher levels of certain minerals such as calcium, iron, zinc, magnesium, manganese, and sodium, and lower levels of phosphorous and potassium compared to controls (micronutrient values for control—whole cow milk were taken from Indian food composition tables, NIN (ICMR) [51]) (Table 6). Fermentation also increases mineral bioavailability [52]. These differences in nutritional parameters highlight the impact of fermentation on the nutrient profile of milk, enhancing certain minerals while significantly altering carbohydrate content.

Table 6. Micronutrient analysis of HCMK compared to control.

Variables	HCMK	Control *	*p*-Value
Calcium (mg/100 mL)	129.3 ± 0.4	118 ± 2.9	<0.001
Iron (mg/100 mL)	2.99 ± 0.09	0.15 ± 0.02	<0.001
Zinc (mg/100 mL)	3.91 ± 0.26	0.33 ± 0.03	<0.001
Magnesium (mg/100 mL)	44.1 ± 1.61	8.28 ± 1.38	<0.001
Manganese (mg/100 mL)	0.21 ± 0.02	0.01 ± 0.00	<0.001
Phosphorous (mg/100 mL)	28.8 ± 1.30	96.56 ± 9.10	<0.001
Potassium (mg/100 mL)	107 ± 1.10	115 ± 4.1	0.005
Sodium (mg/100 mL)	59.4 ± 0.82	25.46 ± 2.86	<0.001

* Values are referenced from Indian food composition tables, NIN (ICMR) [51].

3.5. Microbial Analysis of HCMK

The TBC of HCMK 2.08 × 10^8 CFU/mL was substantially higher compared to the control, 4.62 × 10^4 CFU/mL (Figure 4). The enhanced bacterial load of kefir is attributed to the fermentation process through which lactic acid bacteria, acetic acid bacteria, and other beneficial microorganisms proliferate, contributing to the probiotic nature of kefir [15]. Similarly, the yeast count in HCMK also increased significantly to 3.65 × 10^6 CFU/mL (Figure 4), indicating the presence of yeast strains responsible for fermentation and the development of characteristic flavor along with texture in HCMK.

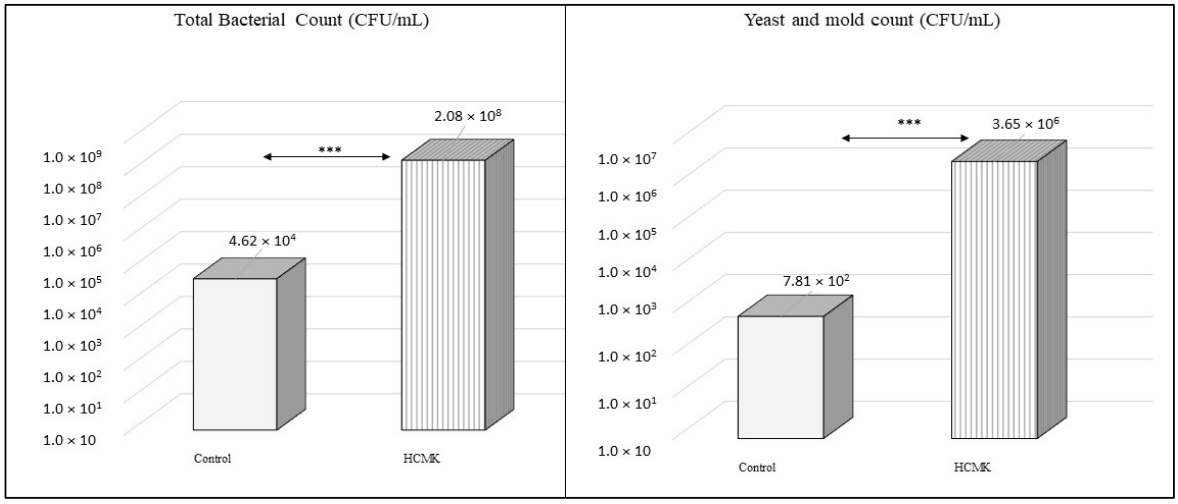

Figure 4. Microbial analysis of HCMK compared to control; *** $p < 0.001$ indicates a significant difference.

Even though the kefir drink was prepared in homemade conditions, it meets the standard guidelines of the Codex Alimentarius standards for fermented milk by the FAO, as it contains a TBC of more than 10^7 CFU/mL and yeast count of more than 10^4 CFU/mL [50].

Coliform bacteria and mold growth were absent in both samples, indicating the hygienic quality of HCMK and control. This absence of coliform count is attributed to the antimicrobial activity of kefir grains against *E. coli* [2,18]. The microbial analysis in this study was consistent with the findings of previous reports indicating a TBC of 10^6–10^8 CFU/mL and yeast count of 10^5–10^7 CFU/mL in cow milk kefir [37,40,41,46,53]. Overall, the microbial analysis highlighted the symbiotic association of beneficial bacteria and yeast in homemade milk kefir, contributing to its probiotic properties.

3.6. GC-MS Analysis of HCMK

The GC-MS chromatogram of HCMK, as shown in Figure 5, highlights the different identified compounds with varying retention times and peak intensities. Table 7 provides a detailed list of the 15 identified compounds, peak area percentage, and retention times of the HCMK sample. The main compounds identified during the analysis were melezitose (34.27%), 6-oxa- bicyclo [3.1.0] hexan-3- one (19.01%), acetate, [3-(acetyloxy) (9.7%), 4H-pyran-4-one, 2,3- dihydro-3,5-dihydroxy-6- methyl (6.38%), and propane, 2,2-difluoro (5.81%) (Table 7).

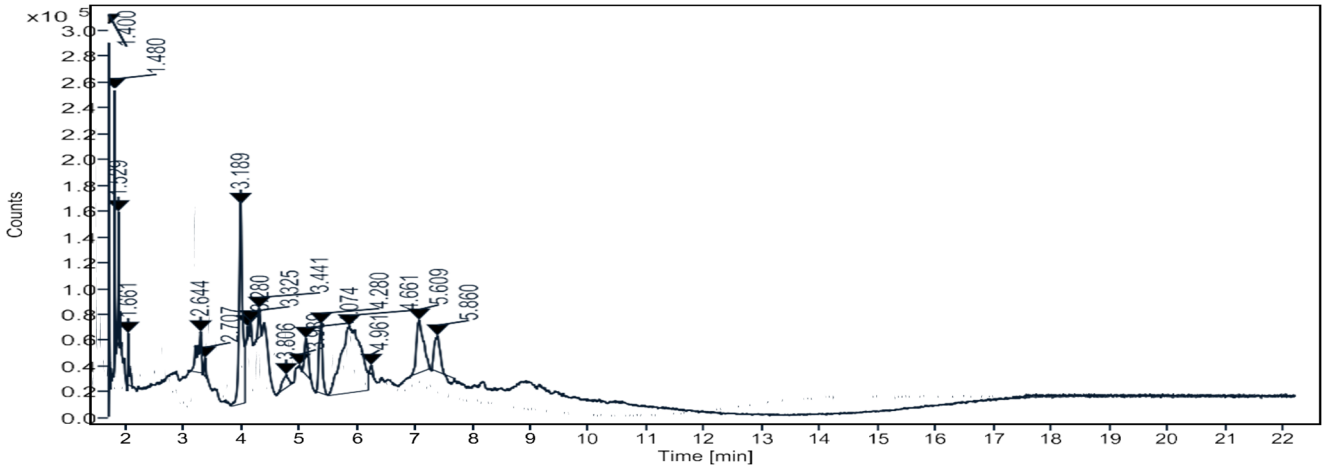

Figure 5. GCMS chromatogram of HCMK.

Table 7. Bioactive constituents recognized in HCMK.

Peak No.	Retention Time (min)	Area	Area (%)	Compound Name	Molecular Formula
1	1.48	163,268.909	5.81	Propane, 2,2-difluoro-	$C_3H_6F_2$
2	1.529	67,745.031	2.41	Methanamine, N- methoxy-	C_2H_7NO
3	1.661	37,226.914	1.32	Phosphorylethanolamine	$C_2H_8NO_4P$
4	2.707	19,334.348	0.69	2-Furanmethanol	$C_5H_6O_2$
5	3.189	534,255.475	19.01	6-Oxa- bicyclo [3.1.0]hexan-3- one	C_5H_8O
6	3.28	7784.616	0.28	Pentanoic acid, heptyl ester	$C_{12}H_{24}O_2$
7	3.325	15,857.547	0.54	cis-3-Methyl-2-n- propylthiophane	$C_8H_{16}S$
8	3.441	39,485.769	1.4	o-Acetyl-L-serine	$C_5H_9NO_4$
9	3.989	1470.105	0.05	3-cis-Methoxy-5-cis-methyl-1R-cyclohexanol	$C_8H_{16}O_2$
10	4.074	103,697.594	3.69	sec-Butyl nitrite	$C_4H_9NO_2$
11	4.28	179,275.616	6.38	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6- methyl-	$C_6H_8O_4$
12	4.661	963,262.59	34.27	Melezitose	$C_{18}H_{32}O_{16}$
13	4.961	15,365.821	0.55	2-Aminooxy-4- methylvaleric acid, methyl ester	$C_9H_{17}NO_4$
14	5.609	272,762.556	9.7	Acetate, [3-(acetyloxy)-	$C_{10}H_{18}O_5$
15	5.86	125,246.91	4.46	2,4:3,5-Dimethylene-I- iditol	$C_8H_{14}O_6$

Intriguingly, 2-furanmethanol, melezitose, and 4H-pyran-4-one have been reported to possess antioxidant and anti-inflammatory properties, thereby offering therapeutic applications [54–56]. Furthermore, melezitose is a trisaccharide known for its prebiotic properties that promote gut health [56]. The compounds methanamine, N-methoxy, and 6-oxa- bicyclo [3.1.0] hexan-3- one, play a crucial role as precursors in the organic synthesis of different pharmaceutical drugs [57,58]. Phosphorylethanolamine, o-acetyl-L-serine, 2-aminooxy-4- methylvaleric acid, and methyl ester identified in HCMK are involved in protein synthesis, cellular signaling, and membrane function [59,60]. Sec-butyl nitrite is an alkyl nitrite compound that has been studied for its potential cardioprotective effects [61]. 2,4:3,5-dimethylene-I-iditol, a cyclic sugar alcohol compound, has been studied for its potential antimicrobial activity against *Candida albicans*, *Candida glabrata*, *Staphylococcus aureus,* and *E. coli* strains [62]. The hydrophobic and positively charged ester groups detected in this study are consistent with the study by Al-Mohammadi et al., who also reported the presence of ester groups in the kefir drink [2]. The presence of these diverse bioactive compounds in HCMK has been suggested as a nutraceutical, offering a promising source of natural home-based drinks with beneficial health effects. In contrast, reports have further shown the presence of various other bioactive compounds in cow milk kefir, which remained undetected in our study. This was potentially due to several factors, including the variety of kefir grain, environmental conditions, and sample preparation techniques, indicating variability in the detection of bioactive compounds [2,63].

3.7. Bioactive Compounds Analysis of HCMK

3.7.1. Total Phenolic Content of HCMK

The total phenolic content in HCMK was determined at 18.1 ± 0.13 mgGAE/100 mL, which was almost two-fold higher than that of the control at 9.82 ± 0.21 mgGAE/100 mL ($p < 0.001$) (Figure 6. Phenolic compounds are known for their antioxidant properties and are often associated with various health benefits [64]. The higher levels of phenolic compounds in HCMK suggest that fermentation contributes to the release or synthesis of these beneficial compounds, thereby enhancing the overall antioxidant capacity of the product [65]. These results highlight the potential health-promoting properties of homemade milk kefir, particularly its antioxidant activity and phenolic content, which may offer protective effects against oxidative stress and contribute to overall health and well-being.

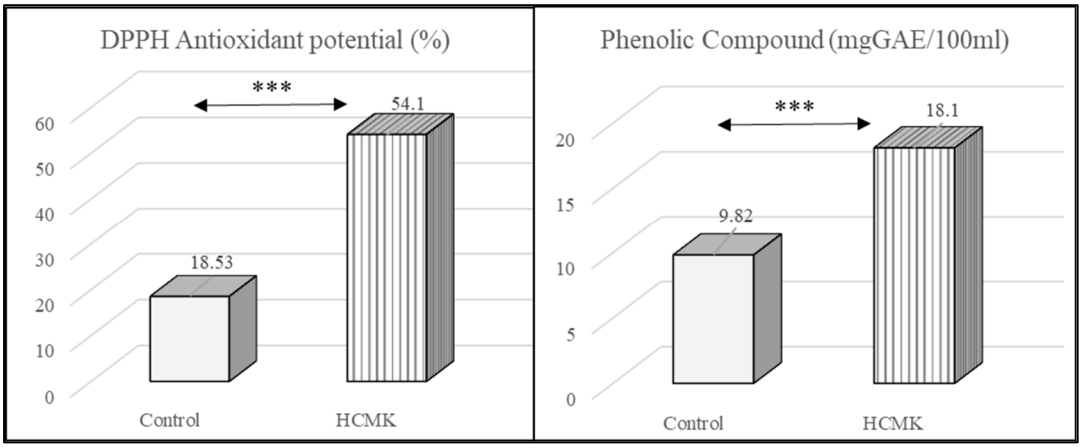

Figure 6. Antioxidant efficacy of HCMK compared to control; *** $p < 0.001$ indicates a significant difference.

3.7.2. Antioxidant Efficacy of HCMK

HCMK exhibited significantly higher DPPH antioxidant potential in comparison to the control (54.1 ± 0.25% vs. 18.42 ± 0.38%, $p < 0.001$) (Figures 6 and 7). The IC_{50} value for the DPPH radical scavenging activity of HCMK was lower than that of the control

(11.47 µg/mL vs. 14.1 µg/mL, $p < 0.001$), indicating higher antioxidant activity of HCMK (Table 8). This substantial increase in the antioxidant activity of HCMK can be attributed to the fermentation process, which causes an increase in the concentrations of bioactive compounds such as 2-furanmethanol, melezitose, and 4H-pyran-4-one with antioxidant potential determined by GC-MS analysis [54–56].

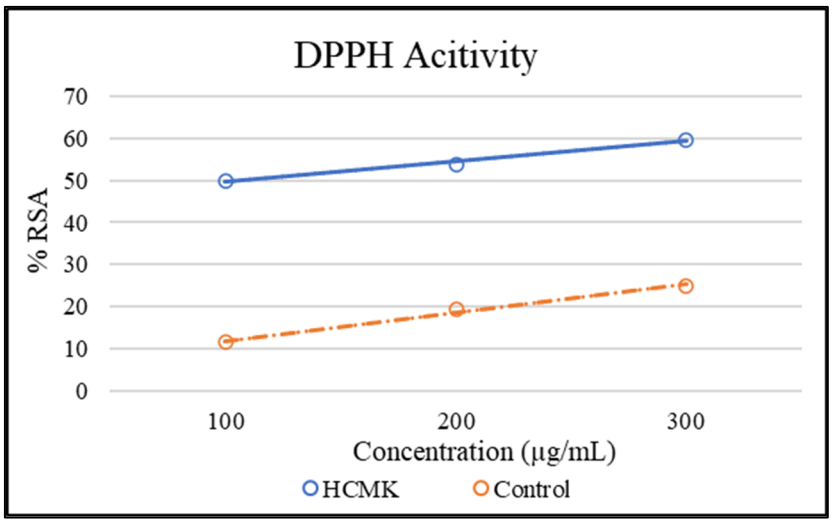

Figure 7. DPPH radical scavenging activity % of the HCMK and control; % radical scavenging activity (RSA).

Table 8. IC_{50} values (µg/mL) of the HCMK and control.

Samples	IC_{50} (µg/mL)	*p*-Value
HCMK	11.47 ± 0.5	<0.001
Control	14.1 ± 0.2	

4. Conclusions

The current research provides a detailed chemical, microbial, nutritional, and antioxidant profile of HCMK, showing its composite and versatile nature. Optimized parameters (3% kefir grains and a 24 h fermentation time) enhanced its sensory and microbial properties while maintaining balanced acidity. Microbial analysis revealed a rich diversity of beneficial microorganisms, supporting the probiotic nature of HCMK. Nutritional analysis showed that the drink is a good source of protein, calcium, and iron. Additionally, HCMK exhibited the presence of a diverse group of bioactive compounds, such as phenols, esters, unsaturated fatty acids, polyalkenes, and aromatic aldehydes, which are associated with possible therapeutic properties, including antimicrobial, anti-inflammatory, and antioxidant effects. Therefore, it is important to focus future research on investigating the long-term stability and the influence of different varieties of kefir grains on the properties of HCMK. Additionally, the development of this traditional probiotic dairy food product for use in the formulation of various synbiotic drinks, kefir candies, or gummies is warranted. Overall, this study sheds light on the inherent variability in home-based conditions and individual preparation practices and views HCMK as a promising nutritious and health-promoting drink in the food and pharmaceutical industries.

Author Contributions: M.C. contributed to conceptualization, formal analysis, investigation, data curation, writing—original draft preparation, and visualization. R.S. and R.K.T. contributed equally to validation, data curation, writing—review, project administration, supervision, and editing. S.C. and A.A. contributed to data curation, formal analysis, and investigation. K.S.: validation, editing, and supervision. All authors have read and agreed to the published version of the manuscript.

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