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Formulation Design and Functional Characterization of a Novel Fermented Beverage with Antioxidant, Anti-Inflammatory and Antibacterial Properties

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Abstract: The aim of this study was to use different concentrations of lemon juice and honey to improve the formulation of a green tea water kefir (GTWK) beverage by applying a central composite design (CCD). Honey's concentration was 10–50% and lemon juice concentration was 1–5%, these were used as the independent factors, whereas pH, bacteria and yeasts' count, total phenolic content, % DPPH· scavenging activity, and overall acceptability were used as the dependent factors. The optimal concentration of honey and lemon juice for highest microbial count, antioxidant activities and overall acceptability was 42.85% and 1.657%, respectively. The analysis of variance revealed that the model was well-fitting, with R^2 ranging from 87.27% to 96.95%, adj-R^2 ranging from 78.17% to 94.26% and a non-significant lack of fits. The optimized fermented beverage showed antibacterial potential against *Escherichia coli* ATCC 11229, *Staphylococcus aureus* subsp. *aureus* ATCC 6538 and *Salmonella typhimurium* ATCC 14028 strains. The anti-inflammatory activity was evaluated on CaCo-2 and RAW 264.7 cells. According to ELISA assay, a significant decrease ($p < 0.05$) in TNF-α concentration was found after inflammatory stimulation, from 1205.41 ± 55.87 pg/mL to 478.17 ± 69.12 pg/mL.

Keywords: water kefir grain; green tea; antioxidant activity; anti-inflammatory activity; antibacterial activity

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1. Introduction

Fermented beverages that are perceived as natural and promote health and wellness are currently in vogue. In addition, non-dairy probiotic beverages have become increasingly popular for lactose intolerance and vegetarianism. These include water kefir, which is obtained by the fermentation of non-dairy substrates by water kefir grain. Water kefir grain is a symbiotic community of lactic acid bacteria (LAB) and yeasts, contained in a polysaccharide matrix [1]. Key microorganisms within this community include lactobacilli, *Leuconostoc* spp. and *Saccharomyces* spp. [1]. Emerging data have shown that these beverages have many nutraceutical benefits, including antiviral, antimicrobial, anticancer and anti-diabetic [1–3].

Green tea (*Camellia sinensis*) is regarded as one of the most popular ingredients for infusions and dietary supplements. It is recognized for its antioxidant, anti-carcinogenic,

and anti-inflammatory properties, as well as its capacity to enhance oral health and prevent arthritis, diabetes, obesity and cardiovascular disease [4–6].

Green tea contains polyphenols, alkaloids, proteins, lignin, amino acids, caffeine, organic acids, minerals, and chlorophyll [7]. Catechins, the most abundant flavonoids, are renowned for their extensive biological properties and have been associated with anti-inflammatory effects in animal cells [8,9]. Other biologically relevant polyphenols identified in tea leaves include phenolic acids, anthocyanidins and flavonol glycosides [10]. However, high doses of catechins can potentially inhibit iron absorption, leading to anemia. This negative effect may be mitigated by adding lemon juice to the tea, as ascorbic acid in lemon juice enhances iron absorption [11]. Lemon juice also enhances the nutritional value of tea by adding minerals, vitamins, and phenolic components [12], and is also known for its detoxifying properties [13].

While sucrose is the most commonly used sweetener in water kefir production, exploring alternative sweeteners such as honey can enhance the flavor characteristics of the beverage. In addition to being a sweetener, honey is a natural source of prebiotics [14,15] and encompasses a wide range of essential nutrients, including proteins, vitamins, minerals, enzymes, and flavonoids. Emerging data indicate that it has antibacterial, anti-inflammatory, and antioxidant effects [16].

Response surface methodology is widely used in the development of food formulations [17]. Central Composite Design (CCD) is a preferred method for problem modeling and analysis when a response of interest is affected by a number of different variables [18]. It enables the determination of the optimal level for each significant variable. This study aims to develop a novel functional green tea water kefir (GTWK) beverage with enhanced sensory and health-promoting properties through the addition of honey and lemon juice. The effects of different concentrations of honey and lemon juice on microbial growth (LAB and yeasts), pH, acidity, phenolic content, DPPH scavenging activity, and product acceptability were investigated using a CCD model with 13 formulations. Furthermore, the antioxidant, anti-inflammatory, and antimicrobial properties of the optimized formula were also determined.

2. Material and Methods

2.1. Water Kefir Grains

The water kefir grains used in this study were obtained from Tunisian households and preserved by the Laboratory of Ecology and Microbial Technology (LETMi, INSAT). A sugar solution (10% w/v) was inoculated with water kefir grains (5% w/v) and then incubated at 25 °C for 48 h. The propagation of water kefir grains in a honey solution was repeated four times for adaptation. The kefir grains used contained 10^7 CFU/g LAB, including *Leuconostoc citreum* and *Lactococcus lactis* species, and 10^7 CFU/g yeasts, with *Saccharomyces cerevisiae* as the predominant identified species. The LAB were identified using molecular tools by sequencing the 16S rRNA region, while the yeasts were identified by 18S rRNA sequencing [19].

2.2. Raw Material

The green tea (Spipa, Tunis, TUNISIA) was purchased from a local market (Tunis, Tunisia). In total, 8 g of green tea was infused in 1 L of boiling distilled water for 6 min before being filtered to remove particulates.

Eucalyptus honey was obtained from the local market (Tunis, Tunisia). It had the following physicochemical characteristics: moisture content, 15.34%; pH, 4.52; free acidity, 0.9 Meq acid/1000 g; soluble dry matter, 80%; total phenolic compounds (TPC), 68.4 mg

GAE/mL; % DPPH scavenging activity, 91.3. The tea infusion was combined with honey, and the mixture was then pasteurized at 65 °C for 20 min.

Lemon (Lunario variety) was purchased from a local market (Tunis, Tunisia). Fruits were washed and the juice was extracted using a citrus juicer (PC302B10, Moulinex, Alençon, France) and was pasteurized at 65 °C for 20 min. The obtained juice had the following characteristics: pH, 2.31; % DPPH scavenging activity, 94.12; TPC, 47.45 mg GAE/mL; total flavonoid, 15.25 mg quercetin eq/mL; total titratable acidity (TTA), 1.93%.

2.3. Preparation of Green Tea Water Kefir

The water kefir beverage was prepared by adding either sucrose (control) or eucalyptus honey (GTWK) to a green tea infusion, ensuring that the final mixture reached a concentration of 30 °Brix. The beverages were then inoculated with 5% (*w*/*v*) water kefir grains. After inoculation, 250 mL flasks, each containing 100 mL of the prepared beverage, were incubated at 25 °C for 48 h.

2.4. Experimental Design

Green tea infusion, honey, and lemon juice were mixed and inoculated with 5% water kefir culture. The mixtures were incubated for 48 h at 25 °C. An experimental design and statistical analysis were performed following the Response Surface Methodology (RSM) for the optimization of the GTWK formulation using Minitab 19.1.1 statistical software. A central composite design (CCD) with five coded levels (−1.41, −1, 0, 1, and +1.41) was employed, along with five center point replicates, for the two independent variables (honey concentration and lemon juice concentration) (Table 1). Thirteen different beverage formulations, comprising honey concentrations (10–50%) and lemon juice concentrations (1–5%), were tested to identify the optimal combination. Their effects on the microbiological, physicochemical, and consumer acceptance of the GTWK were studied.

Table 1. Independent variables (% honey, % lemon juice) and their levels in the central composite design.

Variable	Parameter (%*w*/*v*)	Ranges and Level				
		−1.41	**−1**	**0**	**1**	**1.41**
X_1	Honey	1.7157	10	30	50	58.28
X_2	Lemon juice	0.171	1	3	5	5.82

The concentrations in the final beverages were calculated using the following formula:

$$m_{CS} \times {}^{\circ}B_{cs} = (m_{cs} + m_{tB}) \times {}^{\circ}B_f$$

where m_{cs} is the mass of the carbon source, $^{\circ}B_{cs}$ is the °Brix concentration of the carbon source, m_{tb} is the mass of the acidified green tea infusion and $^{\circ}B_f$ is the final °Brix to be achieved.

Response variables and independent factors during the beverage formulation optimization process were connected by a second-order polynomial equation (Equation (1)).

$$Y = a_0 + a_1 X_1 + a_2 X_2 + a_{11} X_1^2 + a_{22} X_2^2 + a_{12} X_1 X_2 \quad (1)$$

X_1 and X_2 are independent variables; a_0 is the intercept; a_1 and a_2 are regression coefficients for intercept linear terms; a_{11} and a_{22} are regression coefficients for intercept quadratic terms and a_{12} is the intercept regression coefficient for interaction terms.

The effect of the independent variables on the response variables was analyzed and interpreted based on the following equation:

$$\text{LAB (log CFU/mL)} = 6.393 + 0.449\,X_1 - 0.798\,X_2 - 1.022\,X_1^2 - 0.177\,X_2^2 - 0.906\,X_{12} \quad R^2 = 92.14\% \tag{2}$$

$$\text{Yeasts (log CFU/mL)} = 6.586 + 0.808\,X_1 - 0.294\,X_2 - 0.562\,X_1^2 - 0.064\,X_2^2 - 0.215\,X_{12} \quad R^2 = 93.53\% \tag{3}$$

$$\text{pH} = 3.9940 - 0.1320\,X_1 - 0.2437\,X_2 - 0.1382\,X_1^2 - 0.1382\,X_2^2, \quad R^2 = 93.54\% \tag{4}$$

$$\text{TPC (mg GAE/mL)} = 42.50 + 6.017\,X_1 + 4.775\,X_2 - 3.29\,X_1^2 \quad R^2 = 91.20\% \tag{5}$$

$$\%\ \text{DPPH}^{\cdot}\ \text{scavenging activity} = 92.207 + 2.080\,X_1 + 0.668\,X_2 - 1.287\,X_1^2 - 0.464\,X_2^2 \quad R^2 = 96.65\% \tag{6}$$

$$\text{OA} = 2.712 + 0.2691\,X_1 - 0.097\,X_2 - 0.0033\,X_1^2 \quad R^2 = 92.23\ \% \tag{7}$$

2.5. Determination of Microbial Growth

The number of LAB cells was counted using M17 agar, supplemented with cycloheximide, after 48 h of incubation at 37 °C. Yeast's growth was enumerated on the potato dextrose agar (PDA) supplemented with chloramphenicol, after 48 h of incubation at 30 °C [19].

2.6. pH and Acidity

The pH was measured during fermentation with a pH meter (pH/mV Meter 86502 AZ, Taiwan, China). The total titratable acidity was determined by titrating 10 mL of sample with NaOH solution (0.1N) and expressed as % lactic acid [20].

2.7. Determination of Total Phenolic Compounds

The total phenolic compounds (TPC) were quantified according to the Folin–Ciocalteu method optimized and validated by Musci and Yao [21], with few modifications. To avoid interference from oxidized molecules, phenolic compounds were extracted by liquid–liquid separation in ethyl acetate. The ethyl acetate was then evaporated and the residue was resuspended in an equal volume of aqueous methanol (80% *v*/*v*). Then, 250 µL of the sample was combined with 1 mL of the Folin–Ciocalteu reagent and 1 mL of 10% Na_2CO_3. The prepared solution was then kept in the dark at room temperature for 60 min before the absorbance at 765 nm was measured against a blank sample using a spectrophotometer (model 63200 UV/Vis; Jenway, Essex, UK). The TPC content in tea samples was determined from a standard curve of gallic acid in the range of 0.1 to 2 µg/mL (R^2 = 0.9941). The TPC was expressed as mg gallic acid equivalents/mL of sample (mg GAE/mL).

2.8. Flavonoids Assay

Flavonoids were determined using the method described by Djeridane et al. [22]. The sample (1 mL) was mixed with 1 mL of $AlCl_3$ ethanol (2% *w*/*v*) solution. After a 15 min incubation, the absorbance was measured at 430 nm. The content of flavonoids in tea samples was determined from a standard curve of quercetin in the range of 0.1 to 60 µg/mL (R^2 = 0.9962). The results were expressed as mg quercetin equivalents/mL of sample.

2.9. Determination of Antioxidant Activity

2.9.1. 2,2-Diphenyl-1-picrylhydrazyl Radical Scavenging Assay

The method is based on the neutralization of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical [23]. After adding 500 µL of sample to 2.5 mL of methanolic $DPPH^{\cdot}$ solution

(0.5 mM), the mixture was incubated in the dark for 1 h before measuring the absorbance at 517 nm. Methanolic $DPPH^{\cdot}$ solution absorbance was used as a control.

% $DPPH^{\cdot}$ scavenging activity was calculated using the following formula:

$$\%DPPH^{\cdot}\text{scavenging activity} = \left[\frac{A_{control} - A_{sample}}{A_{control}}\right] \times 100$$

2.9.2. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic Acid) Diammonium Salt Radical Scavenging Assay

The ABTS assay is another method for screening antioxidant activity, which was established using the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) method as described by Delgado-Andrade et al. [24]. The ABTS solution was prepared by mixing 7 mM of ABTS stock solution with 2.5 mM potassium persulfate. The mixture was then stored in a dark room for 16 h prior to use. The absorbance of the ABTS solution was adjusted to 0.700 ± 0.2 at 734 nm by dilution in ethanol.

For analysis, 15 µL of the sample was mixed with 950 µL of fresh ABTS solution, and the absorbance was measured at 734 nm after 6 min.

% $ABTS^{\cdot+}$ scavenging activity was calculated using the following formula:

$$\%ABTS^{\cdot+}\text{ scavenging activity} = \left[\frac{A_{control} - A_{sample}}{A_{control}}\right] \times 100$$

where $A_{control}$ is the measurement of the absorbance of control reaction (ethanol mixed with ABTS) and A_{sample} is the absorbance of the sample.

2.9.3. Iron-Chelating Activity

Iron-chelating activity was determined following the method outlined by Custodio et al. [25]. Briefly, 300 µL of the sample was added to 300 µL of $FeSO_4$. After incubation for 5 min at room temperature, 300 µL of ferrozine was added. The mixture was further incubated for 10 min at room temperature, and the absorbance was then measured at 562 nm.

The iron-chelating activity was calculated using the following formula:

$$\%\text{ Iron-chelating activity} = \left[\frac{A_{control} - A_{sample}}{A_{control}}\right] \times 100$$

where $A_{control}$ is the measurement of the absorbance of control reaction (ethanol mixed with $FeSO_4$ and ferrozine) and A_{sample} is the absorbance of the sample.

2.9.4. Power Reducing Activity

The power-reducing activity was determined according to the method described by Son et al. [26]. Briefly, 0.2 mL of the extract was added to 0.2 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 0.2 mL of 1% potassium ferricyanide. The mixture was then incubated at 50 °C for 20 min.

After incubation, 0.2 mL of 10% trichloroacetic acid was added, and the sample was centrifuged at $8000\times g$ for 10 min at 4 °C. The supernatant was collected and mixed with 0.4 mL of distilled water and 0.1 mL of ferric chloride. After a reaction time of 10 min, the absorbance was measured at 700 nm. HCl–cysteine was utilized as a standard for this assay.

2.10. Anti-Inflammatory Activity of the Optimal Formula

Fermented beverages are known for their potential anti-inflammatory effects, often demonstrated by the reduction in pro-inflammatory cytokines such as tumor necrosis factor- alpha (TNF-α). The anti-inflammatory activity of the optimal formula obtained was determined according to the method described by Zheng et al. [27].

The optimized formula of GTWK was added to the wells containing a co-culture of CaCO-2 cells (cell line derived from a colon carcinoma) and RAW 264.7 cells (macrophage cell line established from a tumor in a male mouse). Positive and negative controls were included. The plate was then incubated for 30 min and 3 h at 37 °C and 5%CO_2.

To induce inflammation, a volume of 1 mL of LPS (inflammatory agent) mixed with nutrient medium (EMEM) was added to all wells except the negative control, and the plaque was further incubated for 30 min and 3 h. At the end of the incubation period, each volume was collected and stored in separate Eppendorf tubes for ELISA assay.

The enzyme-linked immunosorbent assay (ELISA) was performed based on the method of Chun et al. [28] using the commercial kit (Pierce endogen, Rockford, IL, USA) following the manufactory's instructions, briefly, after incubation supernatants were collected for assay. Briefly, the 96 well plates were coated with 50 µL of capture antibody mixed with TNF-α, plates were covered incubated at room temperature for 2 h. After incubation, the plates were washed three times with a prepared washing buffer and a volume of 100 µL of streptavidin was inoculated in wells, plates were placed for incubation at room temperature for 30 min. Plates were rewashed another time and 100 µL of TMB substrate solution was added and placed for incubation at the dark for 30 min. The reaction was stopped by adding 100 µL of stop solution. Finally, the absorbance of samples was read at 450–550 nm with an automated microplate ELISA reader (Lisa Plus, Nuremberg, Germany) and a standard curve of TNF- α was used.

2.11. Consumer Acceptance Test

The consumer acceptance test of different water kefir formulations was carried out with a group of 100 participants, including 45 students from the National Institute of Applied Sciences (TUNISIA), who had received semi-formal training in sensory analysis through practical laboratory sessions. When selecting the consumers, attention was paid to demographic diversity in terms of age and gender. They were also examined for regular consumption of tea, kefir or kombucha. In addition, it was confirmed that participants had no allergies to key ingredients such as honey, lemon or green tea. Participants were between 20 and 58 years old, with an approximately equal gender distribution (58 women and 42 men).

The analysis included several water kefir samples, including a commercial sample, to allow comparison with established products. For comparison, a control sample consisting of green tea water kefir with sugar but without honey or lemon juice was also included. All samples were freshly prepared and stored refrigerated until the time of testing.

Consumers rated color, aroma, sweetness, acidity, mouthfeel (effervescence and body), and overall acceptability (OA) of the samples using a 9-point hedonic scale, where 9 = "Like extremely"; 8 = "Like very much"; 7 = "Like moderately"; 6 = "Like slightly"; 5 = "Neither like nor dislike"; 4 = "Dislike slightly"; 3 = "Dislike moderately"; 2 = "Dislike very much" and 1 = "Dislike extremely".

2.12. Antimicrobial Activity of GTWK

The potential of the optimized formula of GTWK to inhibit several pathogenic bacteria was investigated. The pathogens tested included *Staphylococcus aureus* subsp. *aureus* ATCC 6538, *Escherichia coli* ATCC 11229, *Salmonella typhimurium* ATCC 14028, *Shigella sonnei* ATCC

25931, and *Pseudomonas aeruginosa* ATCC 27853. The agar well diffusion technique was implemented as described by Kaydan et al. [29] with few modifications.

The strains were grown on Mueller–Hinton agar for 24 h at 37 °C. Cells were removed from the agar surface and suspended in sterile physiological solution. The cell density of the bacterial suspensions was reduced to 10^7 CFU/mL. A total of 1 milliliter of cell suspension was mixed with 19 mL of melted Mueller–Hinton agar and poured into a Petri dishes. After three wells (6–8 mm in diameter) were cut from the agar medium, 0.5 mL of crude or NaOH (1N) neutralized GTWK was added to each well. Zones of inhibition were measured in centimeters after inoculated plates were incubated for 24 h at 37 °C. Chloramphenicol (30 µg) was used as a reference control to assess the susceptibility of the strains tested.

2.13. Statistical Analysis

All experiments were carried out in triplicate. All data are reported as the mean ± standard deviation. Statistics were performed using Minitab19.1.1 statistical software and SPSS Statistics 25. Data were subjected to one-way ANOVA; pairwise comparison of treatment means was achieved by Tukey's procedure at $p < 0.05$.

3. Results and Discussion

3.1. Effect of Honey on Microbial Growth, Antioxidant Activity, and Consumer Acceptance

Water kefir was prepared by fermenting kefir grains in a green tea infusion supplemented with either honey or sucrose, adjusted to a final concentration of 30 °Brix. A comparison between the control and the GTWK revealed significant advantages of using honey as the carbon source (Table 2). Specifically, GTWK exhibited enhanced microbial growth, particularly of LAB, which can be attributed to the rich nutritional content and prebiotic properties of honey [14]. These findings align with previous studies demonstrating the positive impact of honey on microbial populations in fermented beverages [15].

Table 2. Changes in microbiology and physico-chemical parameters in the control and GTWK during fermentation.

Parameter	Control			GTWK		
Fermentation Time (h)	**0**	**24**	**48**	**0**	**24**	**48**
LAB count (logUFC/mL)	6.32 ± 0.12	6.5 ± 0.14	6.7 ± 0.32 [a]	6.35 ± 0.24	7.38 ± 0.18	7.66 ± 0.32 [b]
Yeasts count (log UFC/mL)	6.45 ± 0.11	6.83 ± 0.22	7.04±0.08 [a]	6.28±0.18	6.33±0.25	6.87±0.17 [b]
pH	6.312 ± 0.02	5.21 ± 0.15	4.25 ± 0.06 [a]	6.2 ± 0.24	5.32 ± 0.12	4.66 ± 0.04 [b]
Titrable acidity (%)	1.03 ± 0.44	1.27 ± 0.19	1.46 ± 0.11 [a]	1.05 ± 0.76	1.11 ± 0.01	1.25 ± 0.22 [b]
Total phenolic content (mg eq GAE/mL)	40.21 ± 0.39	41.15 ± 0.01	41.97 ± 0.05 [a]	42.35 ± 0.12	43.21 ± 0.1	43.92 ± 0.03 [b]
% $DPPH^{\cdot}$ scavenging activity	88.713 ± 0.07	89.012 ± 0.61	90.065 ± 0.02 [a]	90.52 ± 0.76	93.33 ± 0.14	94.27 ± 0.32 [b]
% $ABTS^{\cdot+}$ scavenging activity	49.19 ± 014	52.11 ± 0.23	55.84 ± 0.05 [a]	58.5 ± 0.12	63.02 ± 0.62	69.2 ± 0.4 [b]
% Iron-chelating activity	45.022 ± 0.19	46.08 ± 0.03	47.73 ± 0.14 [a]	47.92 ± 0.15	51.16 ± 0.43	56.94 ± 0.66 [b]

a, b: means with same superscripts explain that there is no significant difference between the two samples.

During fermentation, the control sample showed a more pronounced decrease in pH and a greater increase in acidity compared to the honey-sweetened version. Moreover, the inclusion of honey significantly improved the antioxidant properties of water kefir ($p < 0.05$), likely due to the presence of phenolic and other bioactive compounds in honey. Honey's diverse phenolic profile is well-documented for its strong antioxidant activity, corroborating earlier research that showed its role in enhancing DNA protection and overall antioxidant capacity in fermented beverages [30].

In terms of consumer acceptance (Figure 1), GTWK stands out due to its distinct aroma, moderate sweetness, and high OA. Its balanced sweetness and low acidity contribute to a

refreshing and well-rounded profile, further enhanced by its unique eucalyptus aroma. In contrast, the control sample has a simpler aroma, milder sweetness, and lower OA. The mouthfeel of the control sample is also less satisfying. Commercial water kefir, which includes a mix of figs, lemons, blueberries, and cane sugar, offers a pleasant mouthfeel and a balanced aroma, but it lacks the depth and richness of GTWK. Its moderate sweetness and stronger acidity make it less preferred in terms of OA when compared to GTWK (p-value < 0.05). These findings align with previous studies, which suggest that honey enhances sweetness and boosts consumer preference for fermented products [31].

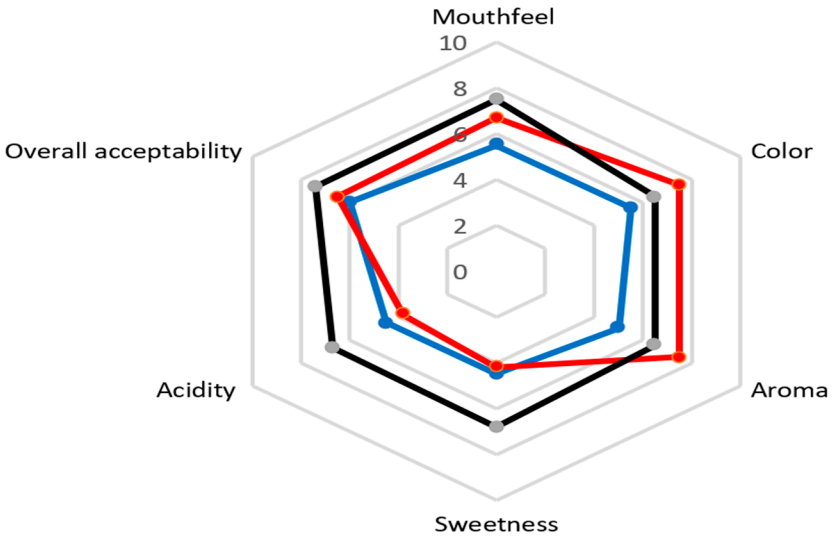


Figure 1. Consumers acceptance of control (■), GTWK (■) and commercial water kefir (■) after fermentation (t = 48 h).

3.2. Study of Independent Factors on GTWK Formulation

3.2.1. Effect of Independent Factors on Microbial Growth

The optimal concentrations of honey and lemon juice were determined using a CCD consisting of 13 experiments, as detailed in Tables 1 and 3. Water kefir grains possess a complex microbiological composition, comprising a symbiotic association of LAB and yeasts. After 48 h of fermentation, the count of LAB and yeasts ranged between 3.17 and 7.36 log CFU/mL, and 4.24 and 7.11 log CFU/mL, respectively. The results indicated that the abundance of the two different microbial groups in the GTWK was equal.

The highest LAB and yeasts counts were obtained with the use of 50% honey and 1% lemon juice, and the lowest were obtained when the honey and lemon juice concentrations were 50% and 5%, respectively. MINITAB statistical software was used to fit the quadratic polynomial equation to the experimental data. Linear, square and interaction coefficients were calculated with a t-statistic to determine their significance (Table 4). The linear effect of honey's concentration was positive, contrary to the quadratic one. However, both were significant for microbial growth (p-value < 0.05). Laureys et al. [32] showed that the biomass growth was affected by nutrient availability. High nutrient concentration positively enhanced biomass growth, but beyond a certain value, no improvement was achieved. The linear effect of lemon juice's concentration, which is negative, was also significant (p-value < 0.01). Increasing the concentration of lemon juice led to a decrease in biomass production due to the low pH obtained. Negative interaction effects were observed for biomass production, but they were significant only for LAB growth.

Table 3. Matrix of the CCD and observed responses for each parameter (Y1: LAB count (log CFU/mL), Y2: yeast count (log CFU/mL), Y3: pH, Y4: TPC (mg GAE/mL), Y5: % DPPH· scavenging activity, Y6: OA score).

Run	X_1 (%*w*/*v* Honey)	X_2 (%*w*/*v* Lemon Juice)	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6
1	50	1	7.24 ± 0.12	7.11 ± 0.03	4.52 ± 0.11	37.2 ± 0.14	94.58 ± 0.11	6.74 ± 0.25
2	1.717	3	3.12 ± 0.07	4.24 ± 0.13	4.51 ± 0.06	23.31 ± 0.25	88.9 ± 0.065	3.21 ± 0.13
3	58.284	3	5.05 ± 0.04	6.86 ± 0.15	4.06 ± 0.09	45.56 ± 0.44	94.22 ± 0.09	5.56 ± 0.07
4	50	5	3.2 ± 0.16	5.98 ± 0.03	3.31 ± 0.10	47.7 ± 0.22	95.03 ± 0.1	5.33 ± 0.14
5	10	5	5.02 ± 0.02	5.01 ± 0.01	4.13 ± 0.22	38.18 ± 0.05	91.17 ± 0.22	4.69 ± 0.11
6	30	5.82	5.01 ± 0.3	6.23 ± 0.01	4.02 ± 0.09	43.14 ± 0.09	94.29 ± 0.09	6.15 ± 0.09
7	10	1	5.06 ± 0.07	5.28 ± 0.01	4.7 ± 0.26	30.05 ± 0.06	89.33 ± 0.26	4.46 ± 0.19
8	30	3	5.92 ± 0.09	6.48 ± 0.02	4.62 ± 0.17	41.68 ± 0.3	94.51 ± 0.17	6.69 ± 0.07
9	30	3	6.08 ± 0.07	6.59 ± 0.010	4.67 ± 0.15	43.17 ± 0.15	94.9 ± 0.15	7.42 ± 0.19
10	30	3	5.93 ± 0.02	6.15 ± 0.07	4.48 ± 0.34	45.02 ± 0.28	93.41 ± 0.34	7.01 ± 0.13
11	30	3	5.9 ± 0.18	6.95 ± 0.02	4.5 ± 0.21	41.73 ± 0.25	94.327 ± 0.21	6.28 ± 0.21
12	30	0.1715	7.36 ± 0.07	6.90 ± 0.01	4.55 ± 0.19	29.3 ± 0.29	92.12 ± 0.19	8.31 ± 0.35
13	30	3	6.05 ± 0.01	6.74 ± 0.004	4.6 ± 0.24	40.89 ± 0.36	93.89 ± 0.24	6.56 ± 0.47

Table 4. Estimated effects and coefficients for Y1 (LAB count), Y2 (yeast count), Y3 (pH), Y4 (TPC), Y5 (% DPPH· scavenging activity) and Y6 (OA score).

Source Title	Y_1		Y_2		Y_3		Y_4		Y_5		Y_6	
	Coeff	Signif.	Coeffi	Signif	Coeffi	Signif	Coeffi	Signif	Coeff	Signif	Coeff	Signif
Model												
a_0	6.393	***	6.586	***	4.574	***	42.5	***	94.207	***	7.58	***
Linear												
a_1	0.449	0.027	0.808	***	−0.132	0.005	6.017	***	2.08	***	0.807	0.005
a_2	−0.789	0.002	−0.294	0.023	−0.2437	***	4.775	0.002	0.668	0.007	−0.574	0.023
Quadratic												
a_{11}	−1.022	0.001	−0.562	0.001	−0.1383	0.005	−3.29	0.018	−1.287	***	−1.371	***
a_{22}	−0.177	0.342	−0.064	0.575	−0.1383	0.005	−2.4	0.06	−0.464	0.045	0.054	0.807
Interaction												
a_{12}	−0.906	0.005	−0.215	0.176	−0.015	0.753	0.59	0.687	−0.349	0.207	−0.45	0.152
R^2	92.14%		93.50%		93.54%		91.20%		95.65%		90.98%	
Adj-R^2	86.53%		88.90%		88.92%		84.92%		94.26%		84.55%	

*** represents $p < 0.001$.

In order to have a strong correlation between the experimental and predicted values, the values of R^2 and adj-R^2 should be close to 1 [33]. The statistical model showed 93.53%, 92.14% correlation with the experimental data for yeast and LAB counts, respectively (Table 4). Furthermore, the values of lack of fit are not significant (Table 5), which means that the model was a good fit to the experimental design.

Table 5. Analysis of variance for Y1 (LAB count), Y2 (yeast count), Y3 (pH), Y4 (TPC), Y5 (% DPPH· scavenging activity) and Y6 (OA score).

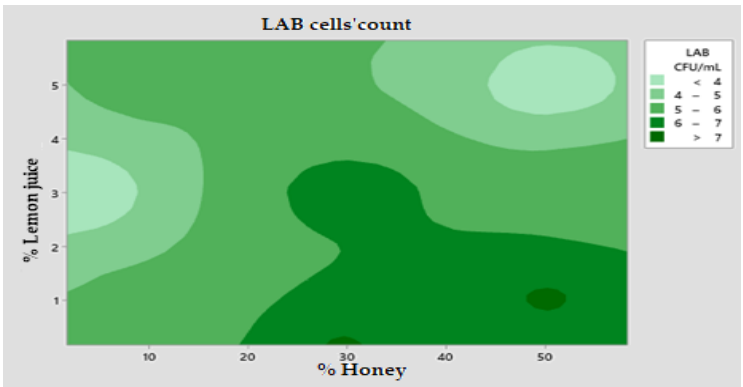
Source	Sum of Squares	Degree of Freedom	Mean of Squares	F-Value	*p*-Value
Y_1 (log CFU/mL)					
Model	17.1504	5	3.4301	16.42	0.001
Linear	6.5901	2	3.295	15.77	0.003
lack of fit	1.1864	3	0.3954	5.73	0.063
Pure error	0.2762	4	0.069		
Total	18.6131	12			
Y_2 (log CFU/mL)					
Model	8.3042	5	1.6608	20.25	***
Linear	5.9199	2	2.9599	36.09	***
Lack of fit	0.2121	3	0.0701	0.78	0.563
Pure error	0.3619	4	0.0907		
Total	8.8783	12			

Table 5. *Cont.*

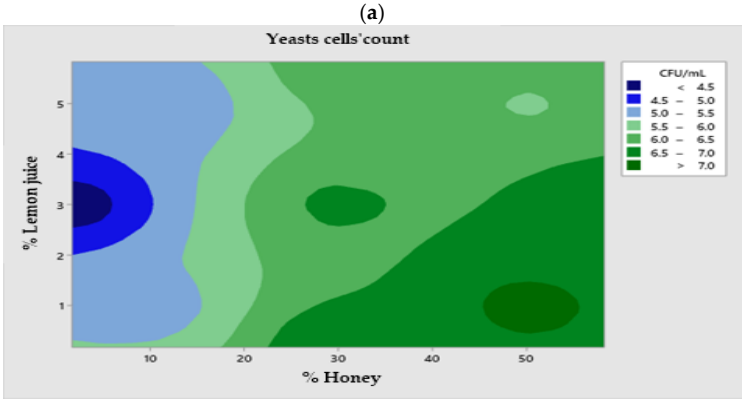
Source	Sum of Squares	Degree of Freedom	Mean of Squares	F-Value	*p*-Value
		Y_3			
Model	0.8507	5	0.1701	20.26	***
Linear	0.6145	2	0.3072	36.59	***
Lack of fit	0.0324	3	0.0108	1.64	0.314
Pure error	0.0263	4			
Total	0.9095	12			
		Y_4 (mg GAE/mL)			
Model	576.3831	5	115.27	14.51	0.001
Linear	472.0681	2	236.03	29.72	***
Lack of fit	44.9411	3	14.981	5.62	0.064
Pure error	10.6572	4	2.664		
Total	631.9811	12			
		Y_5			
Model	50.8092	5	10.162	40.39	***
Linear	38.1832	2	19.092	75.88	***
Lack of fit	0.4389	3	0.1463	0.44	0.735
Pure error	1.3222	4	0.3305		
Total	52.5703	12			
		Y_6			
Model	20.5792	5	4.115	16.63	0.001
Linear	7.1141	2	3.557	14.37	0.003
Lack of fit	0.9647	3	0.3216	1.67	0.308
Pure error	0.7683	4	0.1921		
Total	22.312	12			

*** represents $p < 0.001$.

The estimated response function, the impact of the independent factors (X1 and X2) and the dependent variables (Y_1 and Y_2) are displayed in Figure 2. Microbial growth was higher with honey concentrations between 40 and 45% and lemon juice concentrations between 2 and 2.8%.

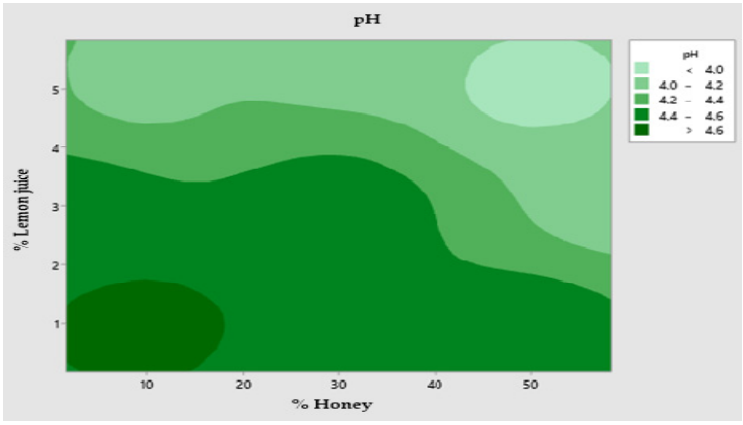


(**a**)



(**b**)

Figure 2. *Cont.*



(**c**)

Figure 2. Two-dimensional contour plots representing the effect of lemon juice and honey concentration on LAB (**a**) and yeasts count (**b**) and pH (**c**).

3.2.2. Effect of Variables on pH

A decrease in pH was associated with LAB and yeast growth during fermentation (Table 3). The decrease was likely due to the production of gas and organic acids. The results showed that the lowest pH (3.31) was observed in the presence of 50% honey and 5% lemon juice. These low pH values have a strong influence on the antimicrobial activity of these products. Indeed, foods have a longer shelf life when the pH is below 4.5 because it inhibits the growth of the pathogenic and spoilage microorganisms [34].

The decrease in pH was related to the increase in the concentrations of honey and lemon juice. In fact, their linear and quadratic terms were negative and significant ($p < 0.05$). However, their interaction term was not significant (Table 4). The lack of fit is non-significant and the R^2 and adj-R^2 values are 93.45% and 88.92%, respectively, which shows the power of the model to fit the experimental data (Tables 4 and 5).

The graphical representation of the data by the main surface plot showed that the optimal value of pH was at a level of 20–40% of the honey concentration and a level of lemon concentration between 1.5 and 2.5% (Figure 2).

3.2.3. Effect of Variables on TPC and Antioxidant Activity

Phenolic compounds are well-known for their antioxidant properties, and the fermentation process can significantly influence their content. Our results showed that both the TPC and DPPH$^{\cdot}$ scavenging activity increased with the rising concentrations of honey and lemon juice, reaching a maximum at a concentration of 50% honey and 5% lemon juice (Table 3). Honey and lemon juice are recognized as sources of natural antioxidants due to their high levels of specific flavonoids [30]. An increase in TPC was observed after 48 h of fermentation, except for run 12 (1.715% honey and 3% lemon juice). Our findings are consistent with several studies, suggesting the capacity of fermentation to improve TPC, thus enhancing the antioxidant activity of fermented foods [35]. The increase in TPC is likely due to the bioconversion and depolymerization of conjugated forms of phenolic compounds into their free forms [36]. However, a decrease in TPC during fermentation has also been reported in some studies, which could be attributed to decarboxylation or hydrogenation by phenolic acid reductases [37].

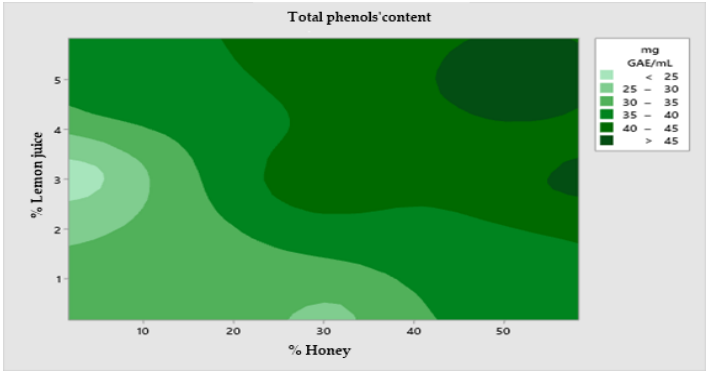
The % DPPH$^{\cdot}$ scavenging activity of GTWK varied between 88.9% and 94.9%. The antioxidant activity is not only due to TPC, but also to synergism between polyphenols and other microbial molecules such as exopolysaccharides [38]. Another possible explanation is that some microorganisms have antioxidant activity. According to De Oliveira et al. [39], *Liquorilactobacillus satsumensis* (formerly *Lactobacillus satsumensis*) and *Saccharomyces cere-*

visiae isolated from a honey-based kefir beverage had a high DNA protection index, which might be attributed to their strong extracellular antioxidant production. Furthermore, it was observed that different concentrations of honey and lemon juice had an influence on the pH of GTWK, which could affect the TPC and their structure, thus affecting their antioxidant activity. Muzolf et al. [40] revealed that the pH of the medium affected the biological activity of green tea catechins.

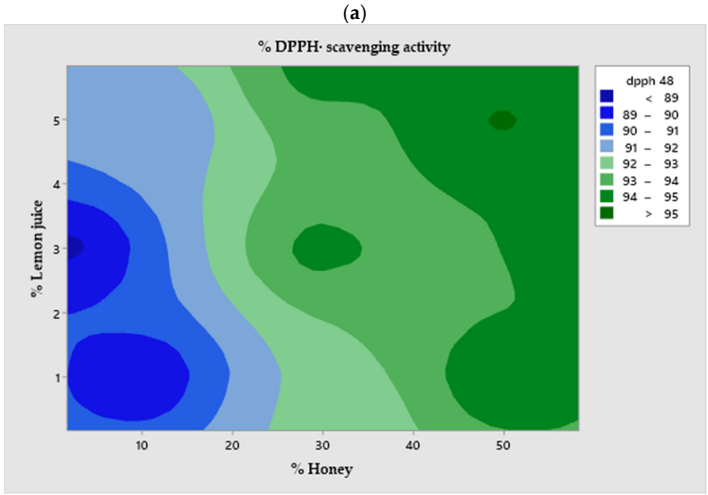
At a linear level, honey and lemon juice had a positive and significant effect on TPC evolution during fermentation (p-value = 0.001 and 0.002, respectively). However, their quadratic effects were negative but only that of honey was significant (p-value = 0.018). The interaction of both factors was not significant (p-value = 0.687). The results obtained by ANOVA are reported in Tables 4 and 5, which indicate that the model is significant (p-value = 0.001). In addition, R^2 (91.2%) and adj-R^2 (84.92%) are close to 1 and the value of lack of fit is not significant estimating that the proposed model fits well with the experimental data.

The results in linear terms confirm the positive influence of the two selected parameters on the antioxidant activity of GTWK (Table 3). Their quadratic effects were found to be negative and significant, but their interaction was not (p-value = 0.207). The results obtained by ANOVA confirm the significance of the model (Table 5). A non-significant lack of fit and R^2 and adj-R^2 close to 1 provide information about the strength of the model.

A three-dimensional response surface was created as a contour plot to explore the interactions between variables and response variation. According to Figure 3, honey concentration between 40 and 45% and lemon concentration between 2 and 2.5% would both exhibit the maximum levels of TPC and DPPH· scavenging activity. These predictive values attest to the beneficial effects of the two components on the chemical properties of GTWK.

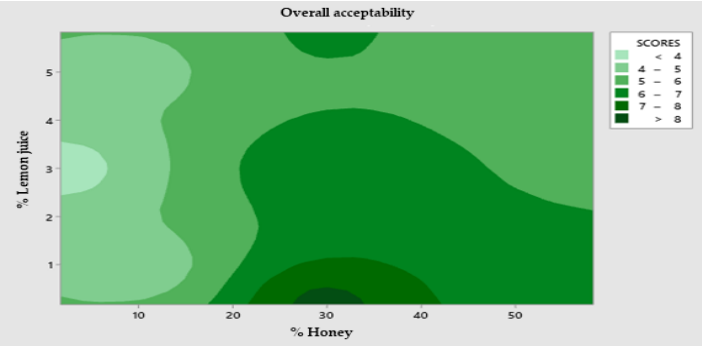


(**a**)



(**b**)

Figure 3. *Cont.*



(**c**)

Figure 3. Two-dimensional contour plots representing the effect of lemon juice and honey's concentration on total phenols content (TPC) (**a**), % $DPPH^{\cdot}$ scavenging activity (**b**) and overall acceptability (OA) score (**c**).

3.2.4. Effect of Variables on Overall Acceptability

Consumers' acceptance plays a significant role in the development of new products. It provides insight into consumer behavior. OA was calculated using the consumers' 9-point hedonic scale evaluations. Table 3 shows the different effects of both factors on OA.

The consumers' acceptance of the 13 formulations revealed significant differences in various sensory attributes, as shown in Table 6. Statistical analysis indicated notable variations across the formulations ($p < 0.05$), with the values followed by different letters in each column highlighting these differences. While mouthfeel scores did not show significant differences, other sensory attributes, such as color, aroma, sweetness, acidity, and overall acceptability, exhibited substantial variation.

Table 6. Consumers' acceptance of the thirteen different beverage formulations.

Formulation	Mouthfeel	Color	Aroma	Sweetness	Acidity	Overall Acceptability
F1	7 ± 0.02^{a}	6 ± 0.12^{a}	7.5 ± 0.2^{a}	8 ± 0.06^{a}	6 ± 0.09^{a}	6.74 ± 0.25^{a}
F2	7 ± 0.11^{a}	7.5 ± 0.14^{b}	5 ± 0.03^{b}	4 ± 0.042^{b}	3 ± 0.02^{b}	3.21 ± 0.13^{b}
F3	7 ± 0.03^{a}	8 ± 0.02^{c}	8.5 ± 0.14^{c}	7.5 ± 0.05^{c}	4.9 ± 0.13^{c}	5.56 ± 0.07^{c}
F4	7 ± 0.061^{a}	7 ± 0.15^{d}	8 ± 0.01^{d}	5 ± 0.11^{d}	2.5 ± 0.11^{d}	5.33 ± 0.14^{d}
F5	7 ± 0.015^{a}	7 ± 0.01^{d}	6 ± 0.11^{e}	5.5 ± 0.13^{e}	2.63 ± 0.04^{e}	4.69 ± 0.11^{e}
F6	7 ± 0.17^{a}	6.5 ± 0.04^{e}	7.5 ± 0.09^{a}	6.5 ± 0.02^{f}	6.7 ± 0.08^{f}	6.15 ± 0.09^{f}
F7	7 ± 0.08^{a}	7 ± 0.71^{d}	6 ± 0.01^{e}	4 ± 0.15^{b}	3 ± 0.02^{b}	4.46 ± 0.19^{g}
F8	7 ± 0.01^{a}	7 ± 0.22^{d}	6.5 ± 0.013^{f}	7 ± 0.08^{g}	5 ± 0.14^{c}	6.69 ± 0.07^{a}
F9	7 ± 0.22^{a}	7 ± 0.16^{d}	6.5 ± 0.02^{f}	7 ± 0.034^{g}	6 ± 0.05^{a}	7.42 ± 0.19^{h}
F10	7 ± 0.31^{a}	7 ± 0.02^{d}	6 ± 0.11^{e}	7 ± 0.01^{g}	5.0 ± 0.03^{c}	7.01 ± 0.13^{i}
F11	7 ± 0.05^{a}	7 ± 0.01^{d}	6 ± 0.02^{e}	7 ± 0.05^{g}	3.9 ± 0.01^{j}	6.28 ± 0.21^{j}
F12	7 ± 0.01^{a}	6 ± 0.36^{a}	7 ± 0.19^{a}	6.5 ± 0.11^{f}	7 ± 0.02^{h}	8.31 ± 0.35^{k}
F13	7 ± 0.013^{a}	6.5 ± 0.01^{e}	6.5 ± 0.01^{f}	7 ± 0.07^{g}	4 ± 0.015^{j}	6.56 ± 0.47^{a}

Means with the same superscripts (a–k) indicate that there is no significant difference between the two samples according to Tukey's test ($p > 0.05$).

For color, formulation F3 stood out with the highest score (8 ± 0.02), while F1 and F12 had the lowest scores (6 ± 0.12 and 6 ± 0.36, respectively). Aroma scores also varied considerably, with F3 achieving the highest score (8.5 ± 0.14) and F2 the lowest (5 ± 0.03). Regarding sweetness, F3 performed best again with a score of 7.5 ± 0.05, while F2 and F7 received the lowest scores (4 ± 0.042 for both). In terms of acidity, F12 showed the highest acidity score (7 ± 0.02), whereas F4 had the lowest (2 ± 0.11). When it came to OA, F12 received the highest OA score (8.31 ± 0.35), while F2 had the lowest (3.21 ± 0.13).

The evaluation showed the importance of balancing honey and lemon juice concentrations to optimize sensory profiles. Formulation F12 received the highest OA score, which was attributed to its well-balanced sweetness, aroma, acidity, and color. In contrast, formulation F7 received the lowest rating, due to an imbalance characterized by insufficient

sweetness and excessive acidity. Formulations containing 30% honey and 3% lemon consistently achieved OA values between 6.5 and 7, reflecting a favorable balance of sweetness, acidity, and aroma. This balance is essential, as it directly influences the unique flavor profile of water kefir. The self-carbonating properties of water kefir, resulting from lactic acid and alcoholic fermentations, contribute to its refreshing taste, effervescent texture, and overall sensory appeal, as highlighted in previous research [41]. Furthermore, higher concentrations of lemon juice led to a lighter color and increased acidity, while higher concentrations of honey (above 30%) enhanced sweetness, aroma complexity, and sensory perception. These findings align with earlier studies, which demonstrate that honey can significantly improve the sensory qualities of fermented products [42].

According to Table 4, the linear effects of honey and lemon juice's concentration on OA are significant (p-value = 0.005 and 0.023). However, only the quadratic effect of honey was significant (p-value < 0.005). The interaction term was also not significant. The significance of the model was completed using ANOVA (Table 5). The application of analysis of variance to the data revealed that the model was significant, as evidenced by the determination of R^2 (92.23%), adj-R^2 (86.69%), and the lack of fit (p-value = 0.308).

The 2D contour plot was used to assess the influence of the predictor variables on the OA (Figure 3). The highest OA score was shown at 42% honey concentration and 2% lemon juice concentration.

3.2.5. Optimization of Independent Variables and Model Validation

Validation tests were carried out to determine the effect of the proportion of ingredients under optimal conditions, specifically 42.85% honey and 1.657% lemon juice. The results are shown in Table 7. The experimental values were slightly higher than the predicted values, except for the TPC, which was slightly lower. This discrepancy could be attributed to variations in experimental conditions or factors not considered by the model. The % $DPPH^{\cdot}$ scavenging activity and the observed OA value were slightly higher than the predicted values, indicating a better radical scavenging ability and a slight improvement in organoleptic evaluation. All validation results are within the 95% confidence interval. These results suggest that the CCD model is reliable and capable of accurately predicting the optimal formulation of GTWK despite minor variations in some parameters.

Table 7. Predicted and experimental responses for optimum conditions.

Response Variables	Experimental Value	Predicted Value
LAB count (log CFU/mL)	7.61 ± 0.11	7.147
Yeasts count (log CFU/mL)	7.35 ± 0.07	7.114
TPC (mg GAE/mL)	40.17 ± 0.08	43.93
% $DPPH^{\cdot}$ scavenging activity	95.81 ± 0.32	93.56
OA score	7.29 ± 0.06	7.319

3.3. Antioxidant Activity Profile of the Optimized GTWK Formula

The antioxidant activity of the optimal formula was evaluated using different methods (Table 8). Fermentation can influence the concentration of polyphenols and other antioxidant compounds. Typically, the fermentation process initially improves antioxidant activity by breaking down complex polyphenols into simpler, more easily absorbed forms. However, extended fermentation can lead to a decrease in total polyphenol content due to their degradation. This makes kefir grain a more advantageous option for tea fermentation than the microbial consortium of kombucha, given that the average fermentation length for kombucha is at least 7 days, while kefir fermentation lasts for a maximum of 48 to 72 h.

Table 8. Antioxidant activity for optimal formula of GTWK (% $ABTS^{\cdot+}$ scavenging activity, % iron-chelating activity and reducing power).

Fermentation Time (h)	% $ABTS^{\cdot+}$ Scavenging Activity	% Iron-Chelating Activity	Reducing Power (µmol HCL-Cystein/L)
0	65.01 ± 0.02	58.03 ± 0.03	259.99 ± 02.25
24	67.34 ± 0.03	60.21 ± 0.04	311.87 ± 3.22
48	73.02 ± 0.11	61.51 ± 0.04	326.87 ± 9.62
p-value	<0.001	<0.001	<0.001

The ABTS assay revealed an increase in free radical scavenging activity, improving from 65% to 73% after 48 h of fermentation. This indicates an enhanced ability to counteract free radicals, a key aspect of antioxidant power that reflects the substance's capacity to neutralize harmful reactive oxygen species. Metal-chelating activity measurements showed an initial percentage of 58.05 ± 0.03% at the start of fermentation, which increased to 61.5 ± 0.044% after 48 h. This increase in metal-chelating activity suggests a greater ability to bind metal ions and prevent oxidative reactions, a critical aspect of reducing oxidative stress. This result is consistent with previous studies, such as Wang et al. [43], which confirmed increased chelating activity during fermentation. Furthermore, the reducing power of GTWK significantly increased from 259.99 ± 2.25 to 326.87 ± 9.62 µmol HCl–cysteine/L after 48 h. These values highlight the increased capacity of the fermented tea to donate electrons and reduce oxidized compounds, which is consistent with the findings of Sabokbar and Khodaiyan [44] who showed a significant increase in reducing activity due to fermentation.

These observed antioxidative activities are attributed to the fermentation process, particularly through the action of kefir bacteria such as *Lactococcus lactis* and *Leuconostoc citreum*, which are known to produce metabolites with antioxidant properties. The antioxidant effects of these microorganisms arise from their production of metabolites such as folate, butyrate, and glutathione, as well as their iron-chelating capabilities through antioxidant enzymes such as superoxide dismutase [45,46].

Exopolysaccharides from *Leuconostoc citreum* have been shown to provide significant protection against oxidative damage by modulating antioxidant enzymes [47]. Similar antioxidant potentials have been observed in *Lactococcus lactis* [48]. In addition, the antioxidant activity of bacteriocin (nisin) and the ability of an exopolysaccharide *from L. lactis* to scavenge radicals and reduce cell damage were also highlighted [49,50]. *Saccharomyces cerevisiae* also contributes to the antioxidant properties of this beverage. Its antioxidant properties include strong metal-chelating activity and free radical scavenging abilities, as reported by Fakruddin et al. [51].

3.4. Anti-Inflammatory Effect of the Optimized GTWK Formula

The anti-inflammatory activity of the optimal formula was evaluated using two types of cell lines: CaCo-2 and RAW 264.7 cells. The ELISA results showed a significant decrease (p-value < 0.05) in TNF-α concentration after the introduction of the inflammatory stimulus, with the amount of proinflammatory cytokines decreasing from 1205.41 ± 55.87 pg/mL to 478.17 ± 69, 12 pg/mL decreased. This result aligns with the findings of Di Cagno et al. [52], who demonstrated that treating inflamed CaCo-2 cells with a fermented plant juice led to a reduction in pro-inflammatory cytokines, highlighting the potential anti-inflammatory properties of lactic acid fermentation. Similarly, Vázquez-Cabral et al. [53] reported that black tea infused and fermented with kombucha significantly decreased TNF-α levels, further supporting the anti-inflammatory effects of fermented beverages.

Recent studies on the biological activities of kefir beverages have also shown their anti-inflammatory effects. Kefir has been found to enhance anti-inflammatory mediators by regulating and decreasing pro-inflammatory cytokines [54]. Vinderola et al. [55] and Carasi et al. [56] demonstrated that exopolysaccharides (EPS) produced by *Lactobacillus kefiranofaciens* isolated from kefir may have an immunomodulatory effect, leading to a reduction in the expression of pro-inflammatory mediators such as IFN-γ, GM-CSF, and IL-1β. Additionally, kefir peptides have shown significant anti-inflammatory potential. Specifically, their administration to inflamed cells at various concentrations effectively reduced the expression of inflammatory mediators such as TNF-α, IL-1β, and IL-4 in lung tissue [57].

3.5. Antimicrobial Activity of the Optimized GTWK Formula

The antimicrobial activity of fermented beverages is a critical indicator of their functional properties. Results in Table 9 show no significant difference between neutralized and crude samples in inhibiting *Escherichia coli* ATCC 11229 (Figure 4) and *Staphylococcus aureus* subsp. *aureus* ATCC 6538, with inhibition zones of 13–14 mm for both strains. This aligns with studies on rice milk kefir, where LAB produced antimicrobial peptides and bacteriocins that disrupt pathogenic cell membranes or metabolic processes [58,59].

Table 9. Antimicrobial activity of optimal formula of GTWK.

Microorganism	Crude Sample of GTWK (Inhibition Halos in mm)	Neutralized Sample of GTWK (Inhibition Halos in mm)	Chloramphenicol (Inhibition Halos in mm)
Echerichia coli ATCC 11229	14 ± 0.013 [a]	13 ± 0.021 [a]	14 ± 0.047 [a]
Staphylococcus aureus subsp. *aureus* ATCC 6538	13 ± 0.004 [b]	13 ± 0.003 [b]	13 ± 0.054 [b]
Shigella sonnei ATCC 25931	n.d.	n.d.	11 ± 0.013
Salmonella thyphimirium ATCC 14028	12 ± 0.06 [a]	n.d.	16 ± 0.019 [b]
Pseudomonas aeruginosa ATCC 27853	n.d.	n.d.	11 ± 0.005

a, b: means with same superscripts explain that there is no significant difference between the two samples in the inhibition of pathogenic bacteria. n.d., not detected.

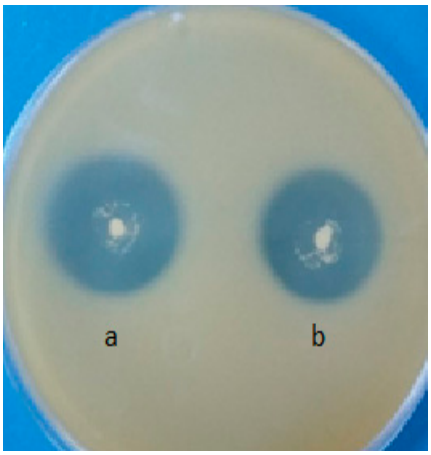


Figure 4. Antimicrobial activity of the GTWK against *Echerichia coli* ATCC 11229 (**a**: crude sample, **b**: neutralized sample).

The observed antimicrobial activity can be attributed to organic acids (e.g., lactic and acetic acid) produced during fermentation, which lower the beverage's pH, creating an acidic environment that disrupts pathogen growth [28]. Specifically, lactic acid interacts with the outer membrane of Gram-negative bacteria, increasing permeability and enabling

other antibacterial compounds to penetrate the cells. Furthermore, at low pH values, weak organic acids are undissociated and can easily penetrate the plasma membrane, causing a drop in intracellular pH and disrupting the transmembrane proton motive force [60].

The addition of lemon juice further amplified this effect. Lemon contains high levels of citric acid, which synergizes with fermentation-derived acids to enhance pH reduction, and bioactive flavonoids, which directly impair microbial membrane integrity and metabolic functions. For example, naringenin—a citrus flavonoid—exerts antibacterial effects against *Staphylococcus aureus* ATCC 6538 by perturbing membrane fatty acid composition and protein conformation [61]. This synergy is evident in the inhibition of *Salmonella Typhimurium* ATCC 14028, which was observed exclusively in the crude sample (12 mm inhibition zone). The loss of activity in the neutralized sample (pH-adjusted) underscores the critical role of lemon-derived citric acid and flavonoids, combined with fermentation acids, in suppressing pH-sensitive pathogens. Notably, the beverage showed no activity against *Shigella sonnei* ATCC 25931 and *Pseudomonas aeruginosa* ATCC 27853, likely due to intrinsic resistance mechanisms in these strains.

4. Conclusions

This study applied RSM to optimize a GTWK beverage formulation, identifying 42.85% honey and 1.657% lemon juice as the ideal combination to enhance fermentation outcomes. The optimized beverage exhibited superior sensory properties and functional performance, including high total phenolic content, antioxidant capacity, and significant anti-inflammatory effects (TNF-α reduction from 1205.41 $\pm$ 55.87 to 478.17 $\pm$ 69.12 pg/mL, $p < 0.05$). It also demonstrated antimicrobial efficacy against *Escherichia coli* ATCC 11229, *Staphylococcus aureus* subsp. *aureus* ATCC 6538 and *Salmonella typhimurium* ATCC 14028. By synergizing honey polyphenols, lemon flavonoids, and fermentation metabolites, the GTWK beverage emerges as a promising functional drink, offering health benefits and appealing taste for holistic nutrition.

Author Contributions: Conceptualization: A.A.; methodology: A.A.; validation: L.A.; analysis: A.A.; investigation: A.A., S.M. and E.G.; writing—original draft preparation: A.A.; reviewing L.A. and P.F.; editing and supervision: L.A. All authors have read and agreed to the published version of the manuscript.

Funding: No funding was received for this study.

Institutional Review Board Statement: Ethical approval was not required as the study consisted solely of standard analytical tests and a sensory evaluation conducted with informed volunteers under non-invasive conditions.

Informed Consent Statement: Ethical review and approval for consumer acceptance analysis were waived for this study because the University of Carthage does not require approval from an Institutional Review Board (or Ethics Committee) regarding consumer acceptance evaluation. All participants involved in the consumer acceptance analysis signed an informed consent form according to the principles of the Declaration of Helsinki before the beginning of the study. Participation in the consumer survey was on a voluntary basis.

Data Availability Statement: All data used to support the research findings are included in this published article.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

Abs	Absorbance
ANOVA	Analysis of variance
CCD	Central composite design
CFU	Colony forming unit
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
GAE	Galic acid equivalent
GTWK	Green tea water kefir beverage
LAB	Lactic acid bacteria
OA	Overall acceptability
PDA	Potato dextrose agar
RSM	Response surface methodology
TPC	Total phenolic compounds
TTA	Total titratable acidity

References

1. Guzel-Seydim, Z.B.; Gökırmaklı, Ç.; Greene, A.K. A comparison of milk kefir and water kefir: Physical, chemical, microbiological and functional properties. *Trends Food Sci. Technol.* **2021**, *113*, 42–53. [CrossRef]
2. Rodrigues, K.L.; Araújo, T.H.; Schneedorf, J.M.; Ferreira, C.S.; Moraes, G.O.I.; Coimbra, R.S.; Rodrigues, M.R. A novel beer fermented by kefir enhances anti-inflammatory and anti-ulcerogenic activities found isolated in its constituents. *J. Funct. Foods* **2016**, *21*, 58–69. [CrossRef]
3. Zamberi, N.R.; Abu, N.; Mohamed, N.E.; Nordin, N.; Keong, Y.S.; Beh, B.K.; Abu Bakar Zakaria, Z.; Nik Abdul Rahman, N.M.; Banu Alitheen, N. The antimetastatic and antiangiogenesis effects of kefir water on murine breast cancer cells. *Integr. Cancer Ther.* **2016**, *15*, 53–66. [CrossRef]
4. McKay, D.L.; Blumberg, J.B. The role of tea in human health: An update. *J. Am. Coll. Nutr.* **2002**, *21*, 1–13. [CrossRef]
5. Chacko, S.M.; Thambi, P.T.; Kuttan, R.; Nishigaki, I. Beneficial effects of green tea: A literature review. *Chin. Med.* **2010**, *5*, 13. [CrossRef] [PubMed]
6. Ferrario, A.; Luna, M.; Rucker, N.; Wong, S.; Gomer, C.J. Pro-apoptotic and anti-inflammatory properties of the green tea constituent epigallocatechin gallate increase photodynamic therapy esponsiveness. *Lasers Surg. Med.* **2011**, *43*, 644. [CrossRef] [PubMed]
7. Reto, M.; Figueira, M.E.; Filipe, H.M.; Almeida, C.M. Chemical composition of green tea (*Camellia sinensis*) infusions commercialized in Portugal. *Plant Foods Hum. Nutr.* **2007**, *62*, 139–144. [CrossRef] [PubMed]
8. Peluso, I.; Serafini, M. Antioxidants from black and green tea: From dietary modulation of oxidative stress to pharmacological mechanisms. *Br. J. Pharmacol.* **2017**, *174*, 1195–1208. [CrossRef] [PubMed]
9. Musial, C.; Kuban-Jankowska, A.; Gorska-Ponikowska, M. Beneficial properties of green tea catechins. *Int. J. Mol. Sci.* **2020**, *21*, 1744. [CrossRef] [PubMed]
10. Xing, L.; Zhang, H.; Qi, R.; Tsao, R.; Mine, Y. Recent advances in the understanding of the health benefits and molecular mechanisms associated with green tea polyphenols. *J. Agric. Food Chem.* **2019**, *67*, 1029–1043. [CrossRef]
11. Zijp, I.M.; Korver, O.; Tijburg, L.B.M. Effect of tea and other dietary factors on iron absorption. *Crit. Rev. Food Sci. Nutr.* **2000**, *40*, 371–398. [CrossRef] [PubMed]
12. Klimek-Szczykutowicz, M.; Szopa, A.; Ekiert, H. Citrus Limon (Lemon) phenomenon-a review of the chemistry, pharmacological properties, applications in the modern pharmaceutical, food, and cosmetics industries, and biotechnological studies. *Plants* **2020**, *9*, 119. [CrossRef] [PubMed]
13. Ramazan, I.; Irfan, S.; Mahmood, S.; Ranjha, M.M.A.N.; Shahzad, J.K.; Jaangla, K.T.; Bin Masood, A.; Mustafa, S. A critical review on nutritional and medicinal importance of lemon. *Acta Sci. Agric.* **2019**, *3*, 95–97. [CrossRef]
14. Mohan, A.; Quek, S.Y.; Gutierrez-Maddox, N.; Gao, Y.; Shu, Q. Effect of honey in improving the gut microbial balance. *Food Qual. Saf.* **2017**, *1*, 107–115. [CrossRef]
15. Zahoor, F.; Sooklim, C.; Songdech, P.; Duangpakdee, O.; Soontorngun, N. Selection of potential yeast probiotics and a cell factory for xylitol or acid production from honeybee samples. *Metabolites* **2021**, *11*, 312. [CrossRef] [PubMed]
16. Mežnarić, S.; Karačonji, I.B.; Crnković, G.; Lesar, A.; Pavlešić, T.; Vučković, D.; Gobin, I. Combined inhibitory effect of fir (Abies Alba Mill) honeydew honey and probiotic bacteria *Lactiplantibacillus plantarum* on the growth of Salmonella enterica Serotype Typhimurium. *Antibiotics* **2022**, *11*, 145. [CrossRef]

17. Latha, S.; Sivaranjani, G.; Dhanasekaran, D. Response Surface Methodology: A nonconventional statistical tool to maximize the throughput of *Streptomyces Species* biomass and their bioactive metabolites. *Crit. Rev. Microbiol.* **2017**, *43*, 567–582. [CrossRef] [PubMed]
18. Myers, R.H.; Montgomery, D.C. *Response Surface Methodology Product and Process Optimization Using Designed Experiments*; John Wiley & Sons: Hoboken, NJ, USA, 2002.
19. Laureys, D.; De Vuyst, L. Microbial Species Diversity, Community Dynamics, and Metabolite Kinetics of Water Kefir Fermentation. *Appl. Environ. Microbiol.* **2014**, *80*, 2564–2572. [CrossRef]
20. Tyl, C.; Sadler, G.D. pH and titratable acidity. In *Food Analysis*; Springer: Berlin/Heidelberg, Germany, 2017; pp. 389–406. [CrossRef]
21. Musci, M.; Yao, S. Optimization and validation of folin-ciocalteu method for the determination of total polyphenol content of pu-erh tea. *Int. J. Food Sci. Nutr.* **2020**, *68*, 913–918. [CrossRef]
22. Djeridane, A.; Yousfi, M.; Nadjemi, B.; Boutassouna, D.; Stocker, P.; Vidal, N. Activity of some algerian medicinal plants extracts containing phenolic compounds. *Food Chem.* **2006**, *97*, 654–660. [CrossRef]
23. Kodama, D.H.; Gonçalves, A.E.D.S.; Lajolo, F.M.; Genovese, M.I. Flavonoids, Total phenolics and antioxidant capacity: Comparison between commercial green tea preparations. *Food Sci. Technol.* **2010**, *30*, 1077–1082. [CrossRef]
24. Delgado-Andrade, C.; Rufián-Henares, J.A.; Morales, F.J. Assessing the antioxidant activity of melanoidins from coffee brews by different antioxidant methods. *J. Agric. Food Chem.* **2005**, *53*, 7832–7836. [CrossRef] [PubMed]
25. Custódio, L.; Justo, T.; Silvestre, L.; Barradas, A.; Duarte, C.V.; Pereira, H.; Barreira, L.; Rauter, A.P.; Alberício, F.; Varela, J. Microalgae of different phyla display antioxidant, metal chelating and acetylcholinesterase inhibitory activities. *Food Chem.* **2012**, *131*, 134–140. [CrossRef]
26. Son, S.H.; Yang, S.J.; Jeon, H.L.; Yu, H.S.; Lee, N.K.; Park, Y.S.; Paik, H.D. Antioxidant and immunostimulatory effect of potential probiotic *Lactobacillus paraplantarum* SC61 isolated from korean traditional fermented food, Jangajji. *Microb. Pathogenesis* **2018**, *125*, 486–492. [CrossRef] [PubMed]
27. Zheng, J.; Ahmad, A.A.; Yang, Y.; Liang, Z.; Shen, W.; Feng, M.; Shen, J.; Lan, X.; Ding, X. *Lactobacillus rhamnosus* CY12 enhances intestinal barrier function by regulating tight junction protein expression, oxidative stress, and inflammation response in lipopolysaccharide-induced Caco-2 cells. *Int. J. Mol. Sci.* **2022**, *23*, 11162. [CrossRef] [PubMed]
28. Chun, S.-C.; Jee, S.Y.; Lee, S.G.; Park, S.J.; Lee, J.R.; Kim, S.C. Anti-inflammatory activity of the methanol extract of moutan cortex in LPS-activated Raw264.7 cells. *J. Evid. Based Complement. Altern. Med.* **2007**, *4*, 327–333. [CrossRef] [PubMed]
29. Kadyan, S.; Rashmi, H.M.; Pradhan, D.; Kumari, A.; Chaudhari, A.; Deshwal, G.K. Effect of Lactic Acid Bacteria and Yeast fermentation on antimicrobial, antioxidative and metabolomic profile of naturally carbonated probiotic whey drink. *LWT* **2021**, *142*, 111059. [CrossRef]
30. Fiorda, F.A.; de Melo Pereira, G.V.; Thomaz-Soccol, V.; Medeiros, A.P.; Rakshit, S.K.; Soccol, C.R. Development of kefir-based probiotic beverages with DNA protection and antioxidant activities using soybean hydrolyzed extract, colostrum and honey. *LWT-Food Sci. Technol.* **2016**, *68*, 690–697. [CrossRef]
31. Padauleng, M.A.; Maruddin, F.; Nur Yuliati, F.; Tashi Wangdi, J.; Ihsan Andi Dagong, M. The physiochemical properties of kefir using honey concentrations. *Canrea J. Food Technol. Nutr. Culin.* **2021**, *4*, 8–16. [CrossRef]
32. Laureys, D.; Aerts, M.; Vandamme, P.; De Vuyst, L. Oxygen and diverse nutrients influence the water kefir fermentation process. *Food Microbiol.* **2018**, *73*, 351–361. [CrossRef] [PubMed]
33. Cruz, A.G.; Faria, J.A.F.; Walter, E.H.M.; Andrade, R.R.; Cavalcanti, R.N.; Oliveira, C.A.F.; Granato, D. Processing optimization of probiotic yogurt containing glucose oxidase using response surface methodology. *J. Dairy Sci.* **2010**, *11*, 5059–5068. [CrossRef] [PubMed]
34. Panda, S.K.; Behera, S.K.; Witness Qaku, X.; Sekar, S.; Ndinteh, D.T.; Nanjundaswamy, H.M.; Ray, R.C.; Kayitesi, E. Quality enhancement of Prickly Pears (*Opuntia* sp.) Juice through probiotic fermentation using *Lactobacillus fermentum*-ATCC 9338. *LWT-Food Sci. Technol.* **2017**, *75*, 453–459. [CrossRef]
35. M'hir, S.; Mejri, A.; Atrous, H.; Ayed, L. Optimization of parameters using response surface methodology to develop a novel kefir-like functional beverage from cheese whey enriched with myrtle juice. *J. Chem.* **2021**, *2021*, 2984470. [CrossRef]
36. Hur, S.J.; Lee, S.Y.; Kim, Y.C.; Choi, I.; Kim, G.B. Effect of fermentation on the antioxidant activity in plant-based foods. *Food Chem.* **2014**, *160*, 346–356. [CrossRef]
37. Filannino, P.; Bai, Y.; Di Cagno, R.; Gobbetti, M.; Gänzle, M.G. Metabolism of phenolic compounds by *Lactobacillus* spp. during fermentation of cherry juice and broccoli puree. *Food Microbiol.* **2015**, *46*, 272–279. [CrossRef] [PubMed]
38. Luang-In, V.; Deeseenthum, S. Exopolysaccharide-producing isolates from thai milk kefir and their antioxidant activities. *Food Sci. Technol.* **2016**, *73*, 592–601. [CrossRef]

39. De Oliveira, C.B.; Fiorda-Mello, F.; De Melo Pereira, G.V.; Thomaz-Soccol, V.; Rakshit, S.K.; De Carvalho, J.C.; Soccol, C.R. In Vitro probiotic properties and DNA protection activity of yeast and Lactic Acid Bacteria isolated from a honey-based kefir beverage. *Foods* **2019**, *8*, 485. [CrossRef] [PubMed]
40. Muzolf, M.; Szymusiak, H.; Gliszczyńska-Świgło, A.; Rietjens, I.M.C.M.; Tyrakowska, B. pH-dependent radical scavenging capacity of green tea catechins. *J. Agric. Food Chem.* **2008**, *56*, 816–823. [CrossRef]
41. Esatbeyoglu, T.; Fischer, A.; Legler, A.D.S.; Oner, M.E.; Wolken, H.F.; Köpsel, M.; Ozogul, Y.; Özyurt, G.; De Biase, D.; Ozogul, F. Physical, chemical, and sensory properties of water kefir produced from *Aronia melanocarpa* juice and pomace. *Food Chem. X* **2023**, *18*, 100683. [CrossRef] [PubMed]
42. Varga, L. Effect of Acacia (*Robinia pseudo-acacia* L.) Honey on the characteristic microflora of yogurt during refrigerated storage. *Int. J. Food Microbiol.* **2006**, *108*, 272–275. [CrossRef] [PubMed]
43. Wang, Y.; Wu, Y.; Wang, Y.; Xu, H.; Mei, X.; Yu, D.; Wang, Y.; Li, W. Antioxidant properties of probiotic bacteria. *Nutrients* **2017**, *9*, 521. [CrossRef]
44. Sabokbar, N.; Khodaiyan, F. Total phenolic content and antioxidant activities of pomegranate juice and whey based novel beverage fermented by kefir grains. *J. Food Sci. Technol.* **2016**, *53*, 739–747. [CrossRef] [PubMed]
45. Azizi, N.F.; Kumar, M.R.; Yeap, S.K.; Abdullah, J.O.; Khalid, M.; Omar, A.R.; Osman, M.A.; Mortadza, S.A.S.; Alitheen, N.B. Kefir and its biological activities. *Foods* **2021**, *10*, 1210. [CrossRef]
46. Kim, S.; Lee, J.Y.; Jeong, Y.; Kang, C.H. Antioxidant activity and probiotic properties of Lactic Acid Bacteria. *Fermentation* **2022**, *8*, 29. [CrossRef]
47. Li, Y.; Xiao, L.; Tian, J.; Wang, X.; Zhang, X.; Fang, Y.; Li, W. Structural characterization, rheological properties and protection of oxidative damage of an exopolysaccharide from *Leuconostoc citreum* 1.2461 Fermented in Soybean Whey. *Foods* **2022**, *11*, 2283. [CrossRef]
48. Krishnamoorthi, R.; Srinivash, M.; Mahalingam, P.U.; Malaikozhundan, B.; Suganya, P.; Gurushankar, K. Antimicrobial, anti-biofilm, antioxidant and cytotoxic effects of bacteriocin by *Lactococcus lactis* Strain CH3 isolated from fermented dairy products-An in vitro and in silico approach. *Int. J. Biol. Macromol.* **2022**, *220*, 291–306. [CrossRef] [PubMed]
49. Pan, D.; Mei, X. Antioxidant activity of an exopolysaccharide purified from *Lactococcus lactis* subsp. *lactis* 12. *Carbohydr. Polym.* **2010**, *80*, 908–914. [CrossRef]
50. Ramalho, J.B.; Soares, M.B.; Spiazzi, C.C.; Bicca, D.F.; Soares, V.M.; Pereira, J.G.; da Silva, W.P.; Sehn, C.P.; Cibin, F.W.S. In vitro probiotic and antioxidant potential of *Lactococcus lactis Subsp. Cremoris LL95* and its effect in mice behaviour. *Nutrients* **2019**, *11*, 901. [CrossRef] [PubMed]
51. Fakruddin, M.; Hossain, M.N.; Ahmed, M.M. Antimicrobial and antioxidant activities of *Saccharomyces cerevisiae* IFST062013, a potential probiotic. *BMC Complement. Altern. Med.* **2017**, *17*, 64. [CrossRef]
52. Di Cagno, R.; Filannino, P.; Vincentini, O.; Cantatore, V.; Cavoski, I.; Gobbetti, M. Fermented *Portulaca oleracea* L. juice: A novel functional beverage with potential ameliorating effects on the intestinal inflammation and epithelial injury. *Nutrients* **2019**, *11*, 248. [CrossRef] [PubMed]
53. Vázquez-Cabral, B.D.; Larrosa-Pérez, M.; Gallegos-Infante, J.A.; Moreno-Jiménez, M.R.; González-Laredo, R.F.; Rutiaga-Quiñones, J.G.; Gamboa-Gómez, C.I.; Rocha-Guzmán, N.E. Oak kombucha protects against oxidative stress and inflammatory processes. *Chem. Biol. Interact.* **2017**, *272*, 1–9. [CrossRef] [PubMed]
54. SaeidiFard, N.; Djafarian, K.; Shab-Bidar, S. Fermented foods and inflammation: A systematic review and meta-analysis of randomized controlled trials. *Clin. Nutr. ESPEN* **2020**, *35*, 30–39. [CrossRef]
55. Vinderola, G.; Perdigón, G.; Duarte, J.; Farnworth, E.; Matar, C. Effects of the oral administration of the exopolysaccharide produced by *Lactobacillus kefiranofaciens* on the gut mucosal immunity. *Cytokine* **2006**, *36*, 254–260. [CrossRef]
56. Carasi, P.; Racedo, S.M.; Jacquot, C.; Romanin, D.E.; Serradell, M.A.; Urdaci, M.C. Impact of kefir derived *Lactobacillus kefiri* on the mucosal immune response and gut microbiota. *J. Immunol. Res.* **2015**, *2015*, 361604. [CrossRef]
57. Chen, Y.H.; Chen, H.L.; Fan, H.C.; Tung, Y.T.; Kuo, C.W.; Tu, M.Y.; Chen, C.M. Anti-inflammatory, antioxidant, and antifibrotic effects of kefir peptides on salt-induced renal vascular damage and dysfunction in aged stroke-prone spontaneously hypertensive rats. *Antioxidants* **2020**, *9*, 790. [CrossRef] [PubMed]
58. Sirirat, D.; Jelena, P. Bacterial inhibition and antioxidant activity of kefir produced from thai jasmine rice milk. *Biotechnology* **2010**, *9*, 332–337. [CrossRef]
59. de Lima, M.D.S.F.; da Silva, R.A.; da Silva, M.F.; da Silva, P.A.B.; Costa, R.M.P.B.; Teixeira, J.A.C.; Porto, A.L.F.; Cavalcanti, M.T.H. Brazilian kefir-fermented sheep's milk, a source of antimicrobial and antioxidant peptides. *Probiotics Antimicrob. Proteins* **2018**, *10*, 446–455. [CrossRef] [PubMed]

60. Alakomi, H.L.; Skyttä, E.; Saarela, M.; Mattila-Sandholm, T.; Latva-Kala, K.; Helander, I.M. Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. *Appl. Environ. Microbiol.* **2000**, *66*, 2001–2005. [CrossRef]
61. Wang, L.H.; Wang, M.S.; Zeng, X.A.; Xu, X.M.; Brennan, C.S. Membrane and genomic DNA dual-targeting of citrus flavonoid naringenin against *Staphylococcus aureus*. *Integr. Biol.* **2017**, *9*, 820–829. [CrossRef] [PubMed]

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