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Phytochemical and Structural Changes of Chickpea Beverage Prepared Using Ultrasound-Assisted Fermentation with Optimized Ultrasound Parameters Modelled by Response Surface Methodology

Nana Adwoa Nkuma Johnson [1,*], John-Nelson Ekumah [1,2], Selorm Yao-Say Solomon Adade [1,3], Yanshu Li [1], Garba Betchem [1], Eliasu Issaka [4] and Yongkun Ma [1,*]

[1] Department of Food Science and Engineering, School of Food and Biological Engineering, Jiangsu University, 301 Xuefu Road, Zhenjiang 202013, China; nelsonjokum@ujs.edu.cn (J.-N.E.); sysadade@st.ug.edu.gh (S.Y.-S.S.A.); sysadade@gmail.com (Y.L.); garbabetchem1@gmail.com (G.B.)
[2] Department of Nutrition and Food Science, College of Basic and Applied Sciences, University of Ghana, Accra P.O. Box LG 134, Ghana
[3] Department of Nutrition and Dietetics, Ho Teaching Hospital, P.O. Box MA 374, Ho 00233, Ghana
[4] School of Environmental Science and Engineering, Jiangsu University, 301 Xuefu Road, Zhenjiang 202013, China; qemmy17@gmail.com
* Correspondence: nana.anjohnson@yahoo.com (N.A.N.J.); mayongkun@ujs.edu.cn (Y.M.)

Abstract: To improve the quality of fermented chickpea beverages, a highly nutritious substitute for dairy, the Box-Behnken design and the response surface methodology were used to obtain optimized ultrasonic parameters for producing ultrasound-assisted fermented chickpea beverages. The determining parameters were the lactic acid, reducing sugar content, and the cell viability of the treated product. The most significant parameters obtained were frequency and treatment duration, while power density was relatively insignificant. The optimum fermentation parameters obtained were a treatment start time of 3 h, treatment duration of 80 min, frequency of 27.5 kHz, and power density of 100 W/L with optimum yields of 0.23096 mg/mL, 2.92898 mg/mL, and 0.488189 for reducing sugar, lactic acid, and cell viability index, respectively, with desirability above 0.95. Further analysis of the ultrasound treatment's effect on the product's structure showed the ultrasound-assisted fermented chickpea beverage was more structurally stable and homogenous, with even distribution of macromolecules present.

Keywords: chickpeas; fermentation; ultrasound-assisted fermentation; Box-Behnken design; response surface methodology

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1. Introduction

Ultrasound is a form of vibrational energy generated by ultrasonic transducers, which convert electrical energy to vibrational sound energy [1]. It provides various benefits in food processing, including shortened process time, increased precision and reproducibility, cheaper costs, improved quality of the product, and the elimination of some downstream purification processes, such as ultrafiltration, filtration, and centrifugation, to mention a few [2,3]. This technology also allows for extending the shelf life of food with minimal temperature involvement, thereby minimally affecting the food's nutritional properties, texture, color, taste, and aroma, ensuring that the treated products have similar characteristics to those of fresh food [4]. Many studies have been conducted over the last few years on the potential of ultrasound to obtain food with greater nutritional value and better organoleptic properties by increasing total polyphenols and anthocyanins in red wines [5], improving and extracting chlorogenic acid in soybeans [6], and treating plant-based protein extracts [7] among others. Its applications in food science range from homogenization, aiding the even

dispersion of components in a mixture, to filtration, assisting in the purification of fluids. In food biotechnology, ultrasound has been used to manipulate cell growth and disperse clusters of substances [8].

Food processing has evolved, and new methods of increasing process efficiency are continuously being sought out and created, and one of these processes is fermentation. The fermentation process, which is the breakdown of complex organic compounds into simpler or single units by microbes [9], is nonthermal [10]. Fermentation employs temperatures near room temperature, which have no damaging effect on the nutritional content of the products, which are usually heat sensitive [10]. Fermentation is key in forming numerous food products, from simple chemical compounds to more complicated macromolecules. The fermentation sector has been at the forefront of evaluating new technologies to increase the overall efficiency of bioprocesses while also increasing yield and product quality [3,11]. Food manufacturers must implement innovative food fermentation technologies to meet consumer needs, ensure the creation of higher-quality safe products, and meet the demand for more efficient energy procedures [11]. Ultrasound processing, a nonthermal technology included in the "Green Food Processing" concept proposed by the authors of [12], refers to technologies that allow for food processing with lower consumption of energy and water, thereby obtaining processing methods that are more sustainable and environmentally friendly. In this regard, ultrasound-assisted fermentation, according to Ref. [11], may be described as the use of appropriate ultrasonic treatment protocols to promote the growth and proliferation of microbial cells while also effectively increasing the overall efficiency of the fermentation process. Appropriate applications of ultrasound, such as low-intensity ultrasonic waves, have been employed in food processing to accelerate biological processes without harming microorganisms, which have successfully enhanced fermentation processes and resulted in higher-quality products during fermentation [13]. Low-intensity treatments coupled with shorter treatment times also control or minimize the heating effect of ultrasound. High-power ultrasound has also been used in the food industry, where a liquid or gaseous medium spreads ultrasonic waves to provide a nonthermal method of processing that can result in improved product quality and reduced processing time [14]. Some examples of this application include emulsion formation, microbial inactivation, ultrasound-assisted extraction of bioactive compounds, and energy transfers, such as freezing and drying [15]. Most of these applications benefit from the technology because of the permanent changes it makes in the medium through which it travels [16].

Cavitation is the main mechanism by which ultrasound is used in fermentation technology. When ultrasonic vibrations move through a medium, the resulting pressure changes cause microscopic gas or vapor bubbles to form, expand, migrate, and quickly implode [17]. While many researchers have focused on the application of ultrasound in fermentation [18–21], no work has been performed with regard to producing a chickpea beverage with ultrasound-assisted fermentation technology. Chickpeas are a leguminous crop with high protein content. They can be processed into a homogenous mixture by blending the peas with water. This beverage serves as an alternative source of calcium, protein, and other essential nutrients without causing digestive discomfort for individuals with lactose intolerance or those following a vegan or plant-based diet. Fermentation has been utilized to enhance the quality of chickpea beverages. Lactic acid bacteria and yeasts mostly used in fermented chickpea beverages (FCB) [22] help maintain a healthy gut microbiome by acting as probiotics and may improve digestion, enhance the immune system, and provide protection against harmful pathogens [23]. FCB are an improved version of chickpea beverages rich in essential nutrients, including proteins, dietary fiber, and various vitamins, and minerals. The fermentation process effectively breaks down phytic acid, which typically hinders the absorption of nutrients, such as iron and zinc. Consequently, this process increases the bioavailability of these nutrients, making them easier for the body to absorb and utilize [24]. Fermentation of chickpea beverages also improves the bioactive contents of the beverage [25]. This study aimed to explore the potential of enhancing the quality of fermented chickpea beverages (FCB) through the

novel integration of ultrasound-assisted fermentation. Leveraging the advantages of FCB, we hypothesized that ultrasound technology could influence carbohydrate utilization and the physical structure of the final product. We utilized the Box-Behnken design and the response surface methodology to determine and optimize the most significant ultrasonic parameters, such as power density, treatment commencement, frequency, and treatment duration. In addition to this, we also sought to understand the impact of this novel treatment on the structural and phytochemical composition of the FCB to ensure that the inherent nutritional benefits of the beverage were maintained.

2. Materials and Methods

2.1. Chickpea Milk Preparation

Fresh Desi *Cicer arietinum* (chickpea) was purchased from the Mulei County Xinjiang agricultural and sideline processing plant, packaged in plastic bags, and stored at room temperature (25 °C) away from moisture. The chickpea beverage was prepared with slight modifications, as the authors of [26] demonstrated. Briefly, 200 g of chickpeas was weighed and soaked overnight with 0.5% $NaHCO_3$. The soaked chickpeas were dehulled and thoroughly washed three times to ensure the absence of any residual husks. The dehulled chickpeas were blanched in 0.5% $NaHCO_3$ solution (1:3; original dry chickpea seeds: 0.5% $NaHCO_3$ solution) for 30 min at 100 °C. The blanched chickpeas were again washed three times in clean tap water. The rinsed chickpeas were blended on high speed in a Silver Crest commercial blender for 5 min with sufficient hot distilled water at 60 °C. The total volume of water used for the milk preparation was 1200 mL, making the chickpeas-to-water ratio 1:6 (g/mL). However, about half of the total water used was incorporated during blending with the remaining water heated to 80 °C and added after blending. The blending process was continued for another 5 min after adding the remaining water, thus creating a smooth chickpea slurry. The obtained chickpea beverage was sterilized in a water bath at 60 °C for 30 min and dispersed into sterile containers (500 mL each) for storage, ensuring a hygienic and adequately prepared chickpea slurry.

2.2. Experimental Design

In order to study the impact of ultrasound on the organic properties of the fermented chickpea beverage, a one-factor-at-a-time experiment was employed to determine the best ultrasonic conditions. Subsequently, using the obtained average parameters that were significant after the one-factor-at-a-time experiment, the Box-Behnken design was employed to obtain an optimized sample. The optimized samples were then compared to determine the effect of ultrasound parameters on the microstructure of the fermented beverage. The fermentation procedure applied to the chickpea slurry for this research followed the optimal fermentation parameters identified in a prior study, which is currently under review. The lactic acid bacteria used, *Lacticaseibacillus paracasei* S2601-8, was grown in MRS broth after being isolated from a bacteria compound purchased from Danyang Yihe Food Co., Ltd., Danyang, China. These optimal conditions were a solid content (A) of 16%, an inoculum size (B) of 3%, a fermentation duration (C) of 16 h, and a controlled temperature (D) set at 44.5 °C. Fermentation was performed in an optical shaker at 200 rpm. In our previous paper [27], a graphical illustration of this process is available.

Screening of ultrasonic parameters using an ultrasonic water bath for the fermentation of chickpea beverages was performed using a single-factor experiment. The experiment required that all physical parameters be held constant while only one parameter was varied at a time to isolate the impact of each parameter. The ultrasonic parameters were the commencement time for treatment, treatment duration, frequency, and power density. The values for each parameter were as follows; commencement times of 0, 3, 6, 9, 12, 15, and 18 h (according to the organism's growth curve), treatment duration of 20, 30, 40, 60, 80, 100, and 120 min, frequency of 20, 28, 35, 40, 50, and 60 kHz (according to what was available in the lab), and power density of 40, 60, 80, 100, 120, and 140 W/L.

Subsequently, the response surface methodology (RSM) in the Design-Expert statistical software, Version 13.0.5.0 was used to optimize the ultrasonic parameters obtained. The effect of the various parameters on the fermentation process was examined using BBD, with lactic acid concentration, reducing sugar content, and cell viability after treatment as the dependent variables. BBD was used to optimize the four critical parameters: start time, treatment duration, frequency, and power density. Based on early experiments using the following parameters' values—start time of 0 and 6 h, treatment duration of 60 and 100 min, frequency of 20 and 35 kHz, and power density of 80 and 120 W/L—a BBD of four-factor-three-level (comprising 29 experimental runs: 24 factorial and 5 center point runs) was used. A second-order polynomial model was constructed to link the association of each element to the response. The equation was:

$$Y = \beta_o + \sum_{(i=1)}^{3} \beta_i X_i + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j + \sum_{(i=1)}^{3} \beta_{ii} X_i^2 \quad (1)$$

where Y = predicted response variable, β_o = intercepts, β_i = linear regression coefficients, β_{ii} = second-order regression coefficients, and β_{ij} = interaction regression coefficients—all estimated by the model—and X_i and X_j = values of the independent variables. The total desirability index (DI) serves as the foundation for selecting the optimum parameters based on the equation:

$$DI = [\prod_{i=1}^{3} d_i(y_i)]^{1/3} \quad (2)$$

where d_i = desirability index (0–1) of the dependent variable, y_i = dependent variables.

2.3. Organic Properties

2.3.1. Reducing Sugars Content

The DNSA method was used to measure the reducing sugars, according to Ref. [28], using the solution of 3,5-dinitrosalicylic acid (DNSA) reagent containing phenol, with some modifications. To prepare the DNSA reagent, 5.3 g of DNSA, 9.9 g of sodium hydroxide, 4.15 g of sodium metabisulfite, and 153 g of Rochelle salts (sodium potassium tartrate) were dissolved in distilled water, after which 3.8 mL phenol was added to the mixture and brought to a final volume of 500 mL with distilled water.

The standard curve for glucose (Supplementary Figure S1A), which was used to determine the reducing sugar content, was obtained by preparing standard glucose solutions of 3.7 mM, 4.7 mM, 5.7 mM, 7.7 mM, 9.7 mM, 10.7 mM, and 12.7 mM concentrations. In each reaction combination, 3 mL of the DNSA reagent was added to a corresponding reducing sugar solution. The mixture was then placed in a water bath for 15 min and then cooled to room temperature in an ice water bath. The absorbance of the sample was taken at 540 nm using a spectrophotometer.

Subsequently, 10 mL of fermented beverage was centrifuged using Ruijiang RJ-TDL-50A at 4000 rpm for 20 min, with the temperature set to 25 °C. After centrifugation, 0.4 mL of the sample was taken and diluted with 5 mL of distilled water. A total of 1.5 mL of the diluted sample was collected and then added to 3 mL of the DNSA reagent, placed in a water bath for 15 minutes, and then cooled to room temperature in an ice water bath. The absorbance of the sample was taken at 540 nm using a spectrophotometer.

2.3.2. Lactic Acid Content

Lactic acid determination was performed according to Ref. [29]. A solution of iron (III) chloride (0.2%) was prepared by dissolving 0.3 g iron (III) chloride in a 100-mL volumetric flask to the mark with water and stirred to the complete dissolution of the salt. The solution was kept at room temperature 25 ± 5 °C.

Briefly, 1.2 g of lactic acid with the known concentration was placed in a 10 mL volumetric flask and diluted with water to form a stock solution with the x concentration of lactic acid. The stock solution was used to prepare twelve series of lactic acid solutions using twofold dilutions. Subsequently, a solution of lactic acid (100 µL) of a corresponding

concentration was added to 4 mL of 0.2% solution of iron (III) chloride, stirred, and left to sit for about 15 min. The reference solution contained 4 mL of 0.2% iron (III) chloride. This served as the standard curve, as seen in Supplementary Figure S1B.

For lactic acid content, 10 mL of fermented beverage was centrifuged using Ruijiang RJ-TDL-50A at 4000 rpm for 20 min at 25 °C. The supernatant was separated from the sediment and put through a tenfold dilution with deionized water. Subsequently, 100 µL solution of the second dilution factor of each sample was added to 4 mL of 0.2% solution of iron (III) chloride, stirred, and allowed to sit for about 15 min to obtain a stable color. The absorbance of the obtained solutions was measured at 390 nm. The concentration of lactic acid was calculated using the calibration curve (Supplementary Figure S1B), taking into account the 10-fold dilution of the test sample.

2.4. Cell Viability

The viability proportion index of the cells that went through ultrasonication was determined following the methodology published by the authors of [30], with some minor adjustments. Briefly, on MRS agar containing 0.15% (*w*/*v*) of bile, the cell survival of ultrasonically treated LAB strains was enumerated after being incubated at 37 °C for 24 h. Calculations were made as follows to determine the viability proportion index (VPI) of *Lacticaseibacillus paracasei*:

$$\text{VPI} = \frac{\text{Final cell population } \left(\log_{10}\text{cfu mL}^{-1}\right)}{\text{Initial cell population } \left(\log_{10}\text{cfu mL}^{-1}\right)} \quad (3)$$

2.5. Phytochemical Analysis Using HPLC

The sample extraction was carried out using a Superclean LC-18 cartridge (Supelco, Bellefonte, PA, USA) after it was conditioned with 10 mL methanol and deionized water. The samples (5 mL), each with pH adjusted to 2 using a 7.2 M HCL, were separately dispensed into the Supelclean LC-18 cartridge. The phenolic acids, flavonols, and anthocyanins were separately eluted using 3.0 mL each of methanol, acetonitrile (20%, pH 2.5), and methanolic HCl (0.01%), respectively. The eluents were transferred into dark sample vials and stored, awaiting filtration and subsequent instrumental analysis.

Phytochemical testing was conducted according to Refs. [25,27] with some modifications. Quickly, 2 g of the chickpea sample was weighed into a 10 mL centrifuge tube containing 5 mL of 70% methanol solution, oscillated and mixed, ultrasonicated for 30 min, and centrifuged. The supernatant was collected and put in a liquid phase vial for testing, using Agilent 1100 and triple quadrupole mass spectrometer API4000 with Agilent Poroshell 120 EC-C18 2.7 µm column (3 × 50 mm). The conditions set for the chromatography were as follows: mobile phase A: 0.5% formic acid in water; mobile phase C: acetonitrile solution; flow rate: 0.6 mL/min; injection volume: 10 µL; and column temperature: 35 °C.

The mass spectrometric detection was conducted with the multiple reaction monitoring (MRM) detection conditions set as follows: spray voltage 4.5–5.5 kv; desolvation temperature 500 °C; desolvation gas (N2) 1000 L/h. The mass spectrometry scanning conditions were also set as follows; ESI+ mode: spray voltage 5.5 kv, desolvation temperature 500 °C, desolvation gas (N_2) 1000 L/h; and ESI-mode: spray voltage 4.5 kv, desolvation temperature 500 °C, desolvation gas (N_2) 1000 L/h.

2.6. Fourier Transform Infrared (FTIR) Spectroscopy Analysis

The FTIR spectroscopy approach described by the authors of [31] was utilized with minor changes to identify the chemical structure of unfermented and fermented materials. With a mortar and pestle (both made of agate), 4 mg of freeze-dried fermented and unfermented (control) chickpea beverages were ground separately and well combined with 200 mg of dried spectroscopic grade KBr (at 105 °C for 24 h) powder. The resulting mixture was compressed with a hydraulic machine (10 t) in 1–2 mm thick transparent

glass-like pellets. The pellets were scanned at a resolution of 4 cm^{-1} in a wavenumber range of 4000–400 cm^{-1}, with 128 scans using the Nicolet IS50 equipment (Thermo Nicolet Corporation, Waltham, MA, USA). The blank (KBr pellet without test samples) utilized in the parameter setup was provided as reference spectra.

2.7. Statistical Analysis

Design-Expert software version 13.0.5.0 (STAT-EASE, Inc., Minneapolis, MN, USA) was employed in the experimental design and statistical optimization process. The ANOVA and lack of fit statistics, the plot of predicted and actual variables, coefficient of variation (CV), and coefficient of determination (R^2) were used to assess the model's adequacy at $p < 0.05$, 0.01, and 0.001 levels of significance. OriginPro version 2019b was used to create the graphs. Each sample was analyzed in triplicate, with results represented as mean ± standard deviation. A multiple T-test was applied to compare paired variables, while Tukey's test was used to assess the mean difference with a significance level set at $p < 0.05$.

3. Results and Discussion

3.1. Model Fitting and Diagnostics for Chickpea Beverage Fermentation Parameters

The results of 29 different experiments obtained from BBD (Table 1) showed that the quality of the product made by fermenting chickpea beverages was affected by the variables of the fermentation process. These factors included the timing of when the treatment started, its total duration, its frequency, and its power density. The statistics of the best fit were utilized in order to develop an accurate model. The fact that the R^2 values for the models using BBD ranged from 98.72% to 99.99%, which is a high number, demonstrated that the models were accurate, as seen in Supplementary Table S1. This range also demonstrated that the models could explain more than 90% of the differences in the variability of the responses. The close similarity between the R^2 value and the modified R^2 value was evidence of the accuracy of the model's predictions [32,33]. The adjusted R^2 values were greater than 99.50%, indicating the removal of nominal terms in their models and an excellent correlation between the independent variables [34,35]. The ANOVA results also suggested a positive correlation between response and predictors (p-value < 0.001), confirming the model's rigidity and strong predictive ability and the significance of the quadratic equations. Furthermore, the lack of fits' p-value was not significant ($p > 0.05$) relative to pure error supporting the goodness of fit of the models (Supplementary Table S1), indicating the credibility and good fitness of their models, further affirming the quality of the models [35,36].

3.2. Influence of Preliminary Ultrasonic Parameters on Reducing Sugar, Lactic Acid, and Cell Viability Index of Lacticaseibacillus paracasei-Fermented Chickpea Beverages

Ultrasonic technology has become a popular method for food processing due to its ability to improve the quality of various food products. It is becoming increasingly popular in food processing as an eco-friendly and nonthermal method for enhancing food quality. Fermentation and ultrasound are known to improve the quality characteristics of food products nonthermally [16,25]. It is essential to determine the effectiveness of the fermentation process based on certain characteristics of the organism and the sample. According to Ref. [37], lactic acid is a naturally occurring acid that is produced by bacteria during fermentation and contributes to the flavor and texture of lactic acid-fermented beverages. Reducing sugars are simple sugars that bacteria can ferment. They provide energy for the bacteria during fermentation. Based on this, it is essential to determine the effectiveness of *Lacticaseibacillus paracasei* S2601-8 in fermenting chickpea beverages, which are also high in carbohydrates. Again, the organism's ability to survive during the ultrasonication process is essential. In light of this, ultrasonic treatment was introduced to improve the quality characteristics of *Lacticaseibacillus paracasei* FCB. It was determined that the best time to start the ultrasonic treatment was after 3 h of fermentation (Figure 1A). It was also observed that

this period fell within the organism's exponential growth phase. The exponential phase is the most important and noticeable phase of the reproductive process for microorganisms. Suppose the exponential phase lasts for a considerable amount of time. It indicates that the LAB can ferment and create a significant quantity of desired products and hydrolyze the sample's components [38]. According to Ref. [7], ultrasonication is a process capable of breaking down the cell wall of plant products, increasing the solubility of proteins and improving the digestibility of carbohydrates and the availability of bioactive compounds.

Table 1. Box-Behnken design matrix with experimental design and data for ultrasound-assisted chickpea milk fermentation.

Run	Independent Variables				Dependent Variables		
	Start Time (h) A	Treatment Duration (min) B	Frequency (kHz) C	Power Density (°C) D	Reducing Sugar (mg/mL) Y_1	Lactic Acid (mg/mL) Y_2	Cell Viability Index Y_3
1	3	80	27.5	100	0.23	2.90	0.49
2	3	100	27.5	120	0.25	3.31	0.53
3	6	80	27.5	120	0.21	2.76	0.48
4	3	80	35	80	0.34	2.27	0.50
5	3	80	20	120	0.28	2.64	0.49
6	0	60	27.5	100	0.29	2.69	0.44
7	3	80	35	120	0.25	2.87	0.49
8	3	60	27.5	80	0.25	3.49	0.57
9	3	100	20	100	0.17	2.75	0.44
10	0	80	27.5	120	0.42	3.05	0.49
11	0	80	35	100	0.38	2.55	0.45
12	6	80	27.5	80	0.37	3.06	0.53
13	3	60	35	100	0.26	2.73	0.48
14	6	80	35	100	0.30	2.25	0.46
15	3	80	27.5	100	0.23	2.92	0.49
16	3	60	20	100	0.21	3.17	0.51
17	3	80	20	80	0.17	3.13	0.48
18	3	60	27.5	120	0.21	3.16	0.51
19	3	100	27.5	80	0.17	2.82	0.48
20	3	80	27.5	100	0.23	2.95	0.49
21	3	80	27.5	100	0.23	2.90	0.49
22	6	60	27.5	100	0.27	3.32	0.55
23	0	80	27.5	80	0.23	2.68	0.44
24	6	100	27.5	100	0.24	2.70	0.46
25	3	100	35	100	0.25	2.60	0.50
26	3	80	27.5	100	0.23	2.95	0.49
27	0	80	20	100	0.27	2.49	0.40
28	0	100	27.5	100	0.28	2.99	0.48
29	6	80	20	100	0.29	2.89	0.48

This means that while LAB are hydrolyzing carbohydrates, ultrasonication assists by increasing the accessibility of these carbohydrates within the chickpeas. This process may facilitate more efficient utilization of carbohydrates by LAB.

The duration of treatment was also investigated. A study by the authors of [39] discovered that knowing the optimal ultrasonic treatment time is essential to avoid any damage caused by the ultrasound as it will be irreversible. This damage applies to every substance in the sample—in this case, the chickpea and LAB. It is, therefore, essential to know how long the treatment must last before it disrupts the microbial cells. The organic properties and cell viability of the ultrasonicated FCB were determined for ultrasonic treatment time. Treatment duration has been known to significantly affect the production of compounds present in a sample. This study observed a steady increase in lactic acid and a decrease in the reducing sugar content as treatment time increased, as seen in Figure 1B.

This is similar to observations made by the authors of [40–42] who noted that increasing the duration of treatment improved the lactic acid yield while decreasing the reducing sugar content. The authors of [43] also used treatment times ranging from 10 to 60 min and observed that the lactic acid content increased with increasing ultrasonic treatment time while the reducing sugar content decreased. The findings correlate with the observation of the highest lactic acid concentration obtained with a treatment time of 80 min, which is longer than the time observed by the authors of [43]. However, a further increase in treatment duration (above 80 min, as in Figure 1B) resulted in a decline in the lactic acid concentration and an increase in the reducing sugar content. Ref. [44] also reported that longer ultrasonic treatment times could improve the quality of food products requiring more reducing sugars and less lactic acid. This change could also be attributed to the observation by the authors of [13]. Increasing the treatment duration may have negatively affected the microbial cells present, thus reducing their metabolic activities.

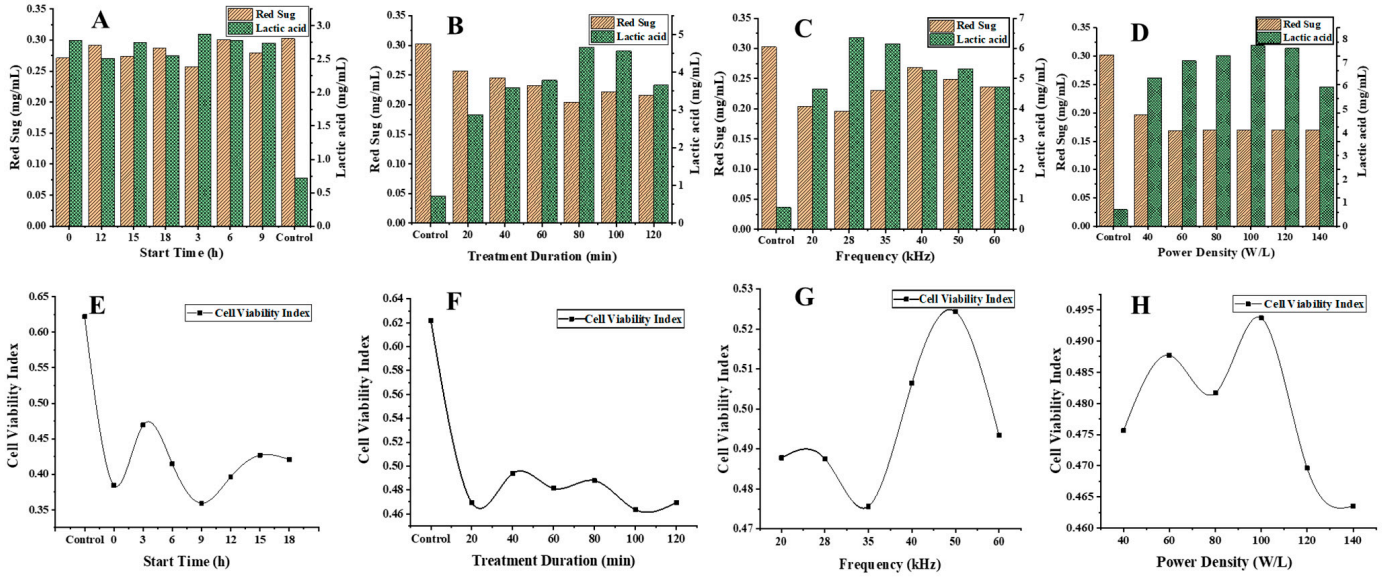

Figure 1. Effect of preliminary ultrasonic parameters, namely, start time (**A**,**E**), treatment duration (**B**,**F**), frequency (**C**,**G**), and power density (**D**,**H**) on reducing sugar and lactic acid concentrations (**A**–**D**) and cell viability (**E**–**H**) of *Lacticaseibacillus paracasei* fermented chickpea beverages.

Different frequencies can be used to optimize the fermentation process and produce desired products. The frequency of the ultrasonic waves used during the ultrasonic-aided fermentation process is an extremely important aspect because of its enormous effect on the end product. It also affects the fermentation substrates and the microorganisms involved. According to Ref. [13], high-frequency waves could be used for cell disruption and increased extraction of metabolites from microorganisms. In contrast, low-frequency waves are used to enhance microbial growth and improve product yield. Frequency is also an important ultrasonic parameter that can improve the production of certain enzymes, such as amylase, protease, and lipase, which can benefit food processing. Again, the authors of [45] found that the frequency of ultrasonic waves significantly affected the fermentation process when using frequencies between 20 and 40 kHz. The study observed a significant increase in fermentation rate, while lower frequencies had little effect. Additionally, the team found that the frequency of the ultrasonic waves affected the amount of biomass produced, with higher frequencies producing more biomass than lower frequencies.

Furthermore, the team discovered that using ultrasound to enhance the fermentation process had a significant positive effect on the quality of the fermentation product, with a conclusion that the frequency of ultrasound waves was an important factor when considering ultrasound to enhance the fermentation process. This study observed similar trends. The highest lactic acid concentrations were obtained at a frequency of 28 kHz,

with a lactic acid concentration of 6.36 mg/mL (Figure 1C). Subsequent increases in the treatment frequency decreased the lactic acid concentration. The reducing sugar content also increased as the frequency increased. The highest cell viability index was observed at a frequency of 50 kHz.

Increasing power density increased the lactic acid concentration and decreased the reducing sugar concentration (Figure 1D). However, the lactic acid concentration decreased after the power density was increased beyond 100 W/L. Studies conducted by the authors of [46] also indicated an increase in the lactic acid concentration when ultrasonic power density was increased while the reducing sugar content decreased. However, the highest power density applied (140 W/L) exhibited the lowest lactic acid content. This suggests that power density should be carefully chosen for the most desirable fermentation results. The effect of all the parameters on the cell viability is represented in Figure 1E–H. The application of ultrasound reduced the cell viability index of the beverages.

3.3. Optimization of Parameters by RSM Using BBD

3.3.1. Effect of Ultrasonic Parameters on the Reducing Sugar Concentrations of *Lacticaseibacillus paracasei*-Fermented Chickpea Beverage

The field of research and development in food processing is constantly improving its capabilities to improve the quality of various foods. One such capability explored involves ultrasound, a widely researched technology with impressive potential applications. Ultrasound technology is an innovative tool capable of performing various complex functions, such as regulating the reducing sugar content in food items. It is essential to know how the four key ultrasonic parameters—commencement time, treatment duration, frequency, and power density—affect achieving the desired accuracy and goals when using ultrasonic technology to produce chickpea beverages.

The results showed that all the parameters were significant with regard to the reducing sugar concentration (Supplementary Table S1). Reducing sugars were negatively correlated with the duration of treatment. These findings are similar to those documented by the authors of [40,43,47] who, in their various studies, observed that increasing treatment duration decreased the reducing sugar content in cucurbits fruit juice, grains, and Chinese liquor, respectively. The decrease in reducing sugar content when treatment duration is increased could result from the thermal degradation of sugars over a prolonged exposure period, as stipulated by the authors of [48].

In contrast, it was observed that increasing frequency and power density increased the reducing sugar content, as seen in Ref. [49]. Ref. [2] stated that the power density of ultrasonic waves also affected the reducing sugar concentrations due to the higher cavitation intensity, which enhanced mass transfer and accelerated the process of hydrolysis, ultimately increasing the yield of reducing sugars. However, according to Ref. [48], excessively high power may cause the thermal degradation of sugars and reduce the yield. The relationship between the various parameters and their effect on reducing sugars is seen in Figure 2. The highest reducing sugar concentration recorded was 0.42 mg/mL, which was achieved at treatment duration of 80 min, frequency of 27.5 kHz, and power density of 120 W/L. The relationship is observed in the second-order polynomial regression equation below:

$$\text{Reducing Sugar} = -0.380709 + 0.134243\,A + 0.000452667\,B + 0.0211365\,C + 0.00105458\,D - 8.25 \times 10^{-5}\,AB - 0.00107667\,AC - 0.00145125\,AD + 6.01667 \times 10^{-5}\,BC + 7.30625 \times 10^{-5}\,BD - 0.000321\,CD + 0.00707426\,A^2 - 6.05792 \times 10^{-5}\,B^2 + 0.000252326\,C^2 + 3.29833 \times 10^{-5}\,D^2$$

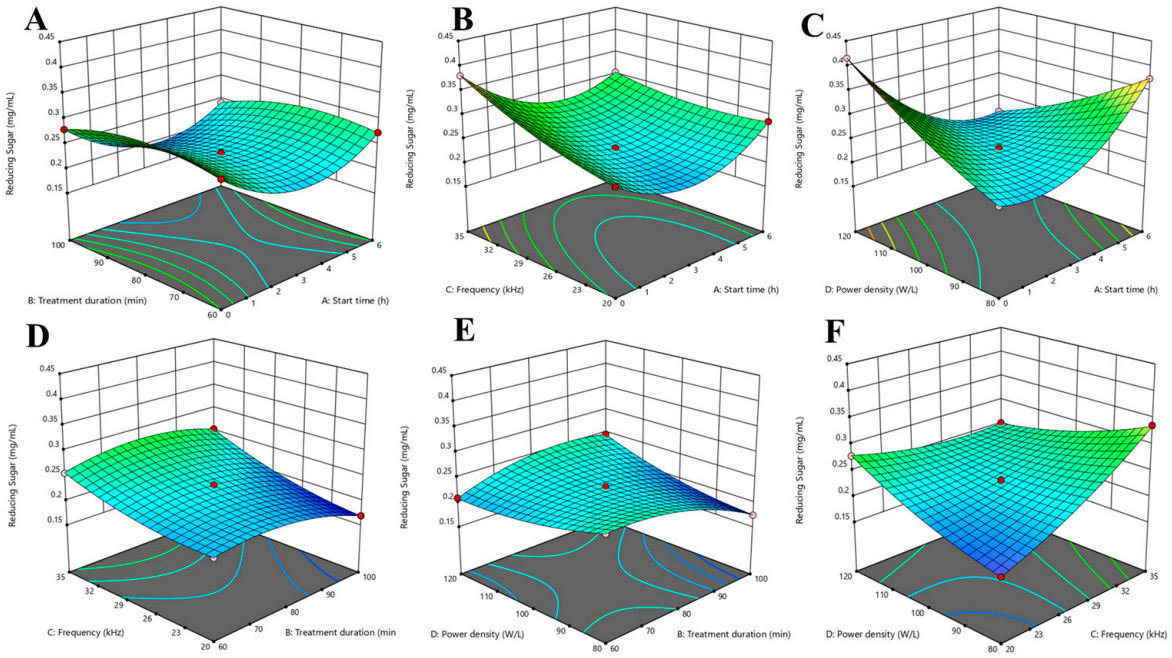

Figure 2. Contour and response surface plots showing the interactive influence of start time and treatment duration (**A**), start time and frequency (**B**), start time and power density (**C**), frequency and treatment duration (**D**), power density and treatment duration (**E**), and power density and frequency (**F**) on reducing sugar content in ultrasound-assisted *Lacticaseibacillus paracasei*-fermented chickpea beverage.

3.3.2. Effect of Ultrasonic Parameters on the Lactic Acid Content in *Lacticaseibacillus paracasei*-Fermented Chickpea Beverage

Ultrasound parameters employed in this study significantly affected the lactic acid content of the treated beverages but not power density. Treatment duration and frequency, however, were negatively correlated with the production of lactic acid obtained after ultrasound-assisted fermentation and were the most significant ultrasonic parameters that influenced lactic acid production. Again, the interactions of the ultrasonic parameters (Figure 3) played a major role in the concentration of lactic acid obtained at the end of the experiment. The results obtained by the authors of [44,50,51] showed that the lactic acid content decreased significantly with increasing ultrasonic treatment duration, and the conclusion was that reducing ultrasonic wave exposure time preserved the integrity of the microbial cells, thus avoiding the destruction of viable cells that could undergo glycolysis. It is essential to note that damage caused due to prolonged treatment time applies to every substance in the sample. Increasing frequency during ultrasonic-assisted fermentation was observed to decrease the production of lactic acid. The authors of [3] found that frequencies of 20 and 30 kHz significantly affected lactic acid, with the highest lactic acid production occurring at 20 kHz in a study they conducted. They also found that an increase in UAF frequency from 20 to 30 kHz caused a decrease in lactic acid production. Similar results were observed by the authors of [52], who also observed that when frequencies of 20 kHz, 40 kHz, and 60 kHz were applied during ultrasound-assisted fermentation, the lactic acid content was significantly higher at 40 kHz compared with 20 kHz and 60 kHz. Concerning this study, the highest lactic acid content of 3.49 mg/mL was observed at a frequency of 27.5 kHz. Lactic acid concentrations observed above the frequency of 27.5 kHz were all below 2.90 mg/mL. Power density, though not a significant parameter, was positively correlated with lactic acid production through ultrasound-assisted fermentation. The authors of [50] stated that higher power density levels increased the cavitation intensity caused by ultrasonic waves, which improved the lactose conversion rate to lactic acid. The observation above aligns with the results obtained by the authors of [46,53], who, in

their various studies, also observed that increased power density caused the lactic acid concentration to increase. Ref. [54] also stated that it was essential to consider the optimal power intensity level applied to a sample to prevent the degradation of lactic acid or other undesirable effects. The simplified model showed a highly significant effect ($p < 0.0001$) of the four fermentation parameters for lactic acid yield, with the highest lactic acid content of 3.49 mg/mL observed at a start time of 3 h, exposure duration of 60 min, frequency of 27.5 kHz and power intensity of 80 W/L. The second-order polynomial regression equation obtained from BBD was as follows:

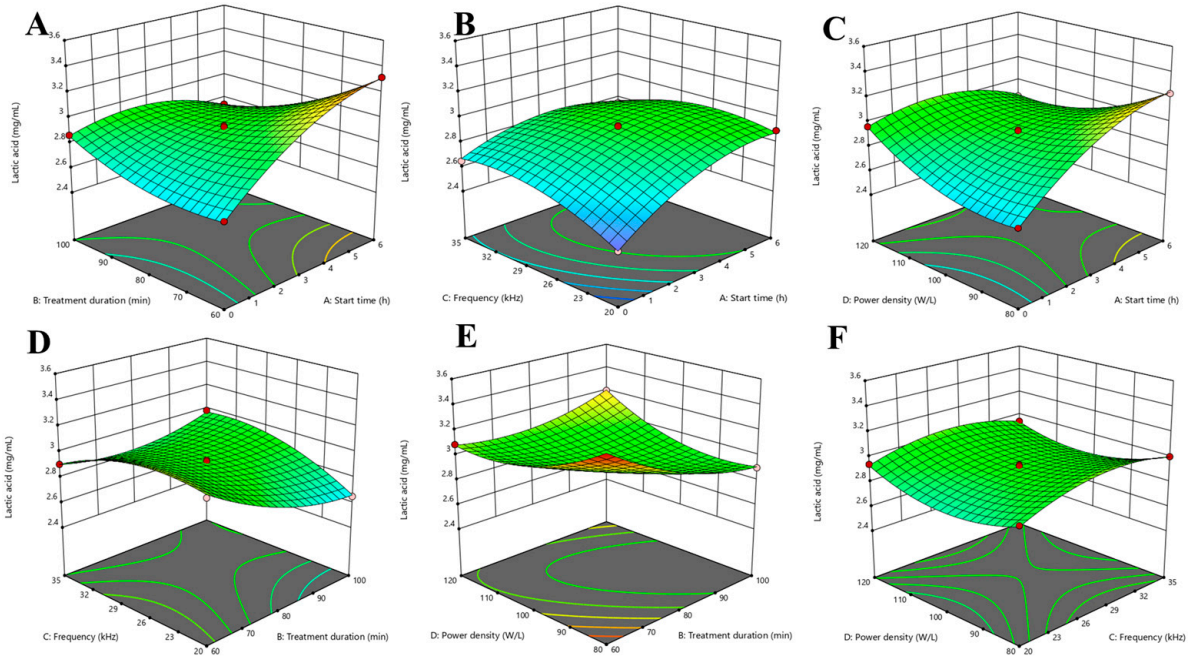

Figure 3. Contour and response surface plots showing the interactive influence of start time and treatment duration (**A**), start time and frequency (**B**), start time and power density (**C**), frequency and treatment duration (**D**), power density and treatment duration (**E**), and power density and frequency (**F**) on lactic acid content of ultrasound-assisted *Lacticaseibacillus paracasei*-fermented chickpea beverage.

$$\text{Lac Acid} = 12.6209 + 0.901432\,A - 0.120728\,B + 0.045109\,C - 0.128478\,D - 0.00383837\,AB - 0.00763633\,AC - 0.00281417\,AD + 0.0004794\,BC + 0.000514394\,BD + 0.00182302\,CD - 0.0147576\,A^2 + 0.000386541\,B^2 - 0.00478162\,C^2 + 0.00023536\,D^2$$

3.3.3. Effect of Ultrasonic Parameters on the Cell Viability of *Lacticaseibacillus paracasei* in Fermented Chickpea Beverages

The effect of ultrasonic parameters on cell viability was observed, with the highest cell viability index observed (0.57) obtained at a treatment commencement time of 3 h, a treatment duration of 60 min at a frequency of 27.5 kHz with a power density of 80 kHz. Treatment duration and power density had a negative correlation with cell viability. According to Refs. [55,56], the power of ultrasound waves can significantly impact cell viability, where lower power density levels may enhance cell growth by increasing mass transfer rates and substrate availability. Higher power density levels can lead to excessive cell disruption and reduced viability. However, ultrasonic power density did not significantly affect the cell viability indices obtained. Treatment duration, on the other hand, had a significant effect on cell viability. The treatment duration refers to the exposure time of ultrasound waves to a sample, and it plays a crucial role in determining the effects of exposure duration on microbial cells. Shorter exposure times may stimulate growth by promoting the mass transfer and nutrient uptake of the microbial cells after the treatment loosens the cell walls of the sample [57].

In contrast, prolonged exposure, according to Ref. [58], could result in cell damage and reduced viability due to increased stress on the microbial cells after exposure. Higher frequencies between 1–2 MHz have been reported to have milder effects on cells and may promote growth, although the efficacy depends on the specific microorganism and fermentation process [55]. The authors of [45] also reported that increasing the frequency of ultrasonic waves positively affected the amount of biomass produced, with higher frequencies producing more biomass than lower frequencies. However, it is important to note that the beneficial effects of high-frequency ultrasound on microbial cell growth and fermentation efficiency are not universal and can vary based on the specific organism, growth conditions, and fermentation process. The interactions between the various factors are shown in Figure 4, while the model obtained for the cell viability index followed the regression equation;

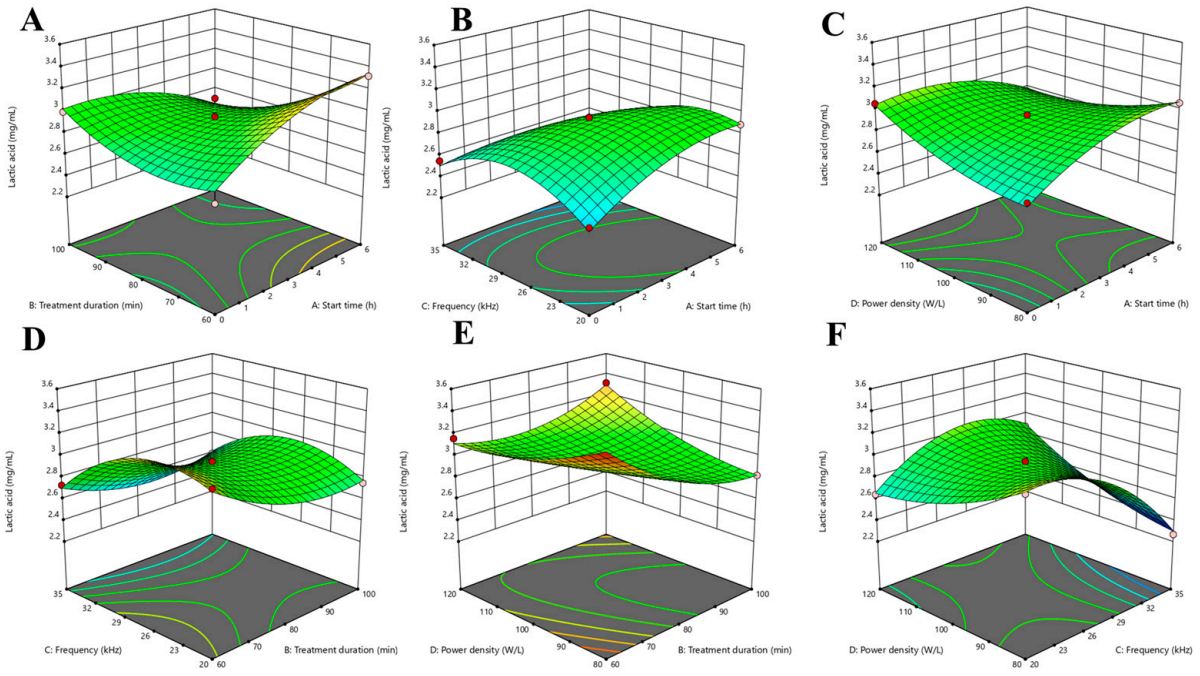

Figure 4. Contour and response surface plots showing the interactive influence of start time and treatment duration (**A**), start time and frequency (**B**), start time and power density (**C**), frequency and treatment duration (**D**), power density and treatment duration (**E**), and power density and frequency (**F**) on cell viability of ultrasound-assisted *Lacticaseibacillus paracasei*-fermented chickpea beverage.

$$\text{Cell viability index} = 1.44421 + 0.131575\,A - 0.0154011\,B + 0.0119736\,C - 0.0138517\,D - 0.000535567\,AB - 0.0007542\,AC - 0.000462325\,AD + 0.000139147\,BC + 6.44519 \times 10^{-5}\,BD - 2.03133 \times 10^{-5}\,CD - 0.00244094\,A^2 + 3.73842 \times 10^{-5}\,B^2 - 0.00032723\,C^2 + 5.31261 \times 10^{-5}\,D^2$$

3.4. Optimization and Verification of Model for Producing Ultrasound-Assisted Chickpea Beverage Fermentation

The desirability function technique was used to obtain an optimal value for each predictor by considering all fermentation parameters. The main goal of this method was to estimate the levels of independent variables to obtain maximum lactic acid yield, reducing sugar content and cell viability indices from the ultrasonic parameters. According to Refs. [32,36], the desirability function takes in a set of inputs and produces a score between 0 and 1 based on which settings have the most desirable sets of replies. The score ranges from 0 to 1, with 0 being the least desirable and 1 being the most desirable. The optimal values for response parameters in this study were used with the desire function approach to determine the best possible fermentation conditions. Optimal ultrasonic conditions

for *Lacticaseibacillus paracasei* in chickpea drinks that yielded maximum lactic acid and reduced sugar concentrations and enhanced cell viability were identified. The desirability function determined the optimum fermentation parameters to be treatment start time (A) = 3 h, treatment duration (B) = 80 min, frequency (C) = 27.5 kHz, and power density (D) = 100 W/L. The optimum yields predicted for reducing sugar, lactic acid, and cell viability index were 0.23 mg/mL, 2.93 mg/mL, and 0.49, respectively (Table 2). The desirability of obtaining these outcomes was greater than 0.94, which is sufficiently high and within the acceptable range (≥0.6), as indicated by Ref. [36].

Table 2. Confirmation of optimized parameters with a confidence interval of 95%.

Analysis	Predicted Mean	Predicted Median	Std Dev	SE Pred	95% PI Low	Observed Mean	95% PI High
Reducing Sugar	0.23	0.23	0.001	0.0007	0.23	0.23	0.23
Lactic acid	2.93	2.93	0.050	0.0300	2.85	2.93	2.99
Cell viability index	0.49	0.49	0.001	0.0010	0.49	0.49	0.49

Verification tests were performed with the obtained parameters after optimization to confirm the models' adequacy, dependability, and reproducibility. The optimum yields obtained after the verification tests produced reducing sugar, lactic acid, and cell viability index of 0.23 mg/mL, 2.93 mg/mL, and 0.49, respectively, as seen in Table 2. The relative percentage errors obtained for the predicted and experimental values were less than 5%, confirming the resilience of the models at a 95% confidence interval [32,59,60].

3.5. Influence of Ultrasound-Assisted Fermentation on the Phytochemicals Contents of Chickpea Beverages

According to Ref. [61], phytochemicals, also known as phytonutrients, are naturally occurring compounds that may be discovered in plants and provide various benefits to human health. Chickpeas contain many phytochemicals, the most notable of which are polyphenols. According to Ref. [62], the concentration of polyphenols that may be found in chickpeas can alter depending on several circumstances. These factors include the chickpea's genotype, the production conditions, and the processing procedures utilized.

In this study, the impact of chickpea beverage fermentation with ultrasound assistance was compared with that of fermented beverage fermentation. Ref. [27] compares the unfermented, fermented, and ultrasound-assisted fermented chickpea beverage (UAFCB) samples. The results shown in Supplementary Table S2 indicate that total polyphenol derivative content was high compared with the other phytochemicals. Polyphenols come in seven varieties, with phenolic acids being the most prevalent. The phytochemical profile of a new product may vary depending on how it is processed. One form of processing that might alter the amount of polyphenols in chickpeas is fermentation and ultrasound treatment.

According to Ref. [3], ultrasound-assisted fermentation is emerging as a potentially beneficial way to enhance the levels of bioactive chemicals present in a range of foods, including chickpeas. The authors of [63,64] also opined that phytochemicals attached to their polysaccharide subunits were less easily released. However, the authors of [65] opined that ultrasonication's chemical bond dissociation effect enhanced the bond cleavage of phenolic acids into their aglycon derivatives, releasing the phenolic compound into the samples as observed; consequently, ultrasound-assisted fermentation could lead to a higher release of bound polyphenol. According to findings from earlier studies by the authors of [9], combining ultrasound processing with fermentation results in a sizeable rise in both the total polyphenolic content (TPC) and the antioxidant activity of a wide range of food items, such as grape pomace, and soybean. The current study provided evidence that supported this finding.

Supplementary Table S2 and Figure 5A indicate that no significant differences between salicylic acid, caffeic acid, tanshinone IIA, epicatechin gallate, and glycyrrhetinic acid were

found in either sample ($p > 0.05$). Although these fluctuations do not lead to any discernible alteration, the authors of [66] suggested that these polyphenolic chemicals' pH changes during fermentation may be the primary origin. Despite the various opinions on the effect of ultrasound on enhancing the phytochemical content of products, though the application of ultrasound had a significant impact on some of the samples' phytochemical composition, there was no discernible difference between the FCB and UAFCB in terms of their overall phytochemical composition. It is plausible to conclude that fermentation significantly contributed to the observed increase in the overall amount of phytochemicals.

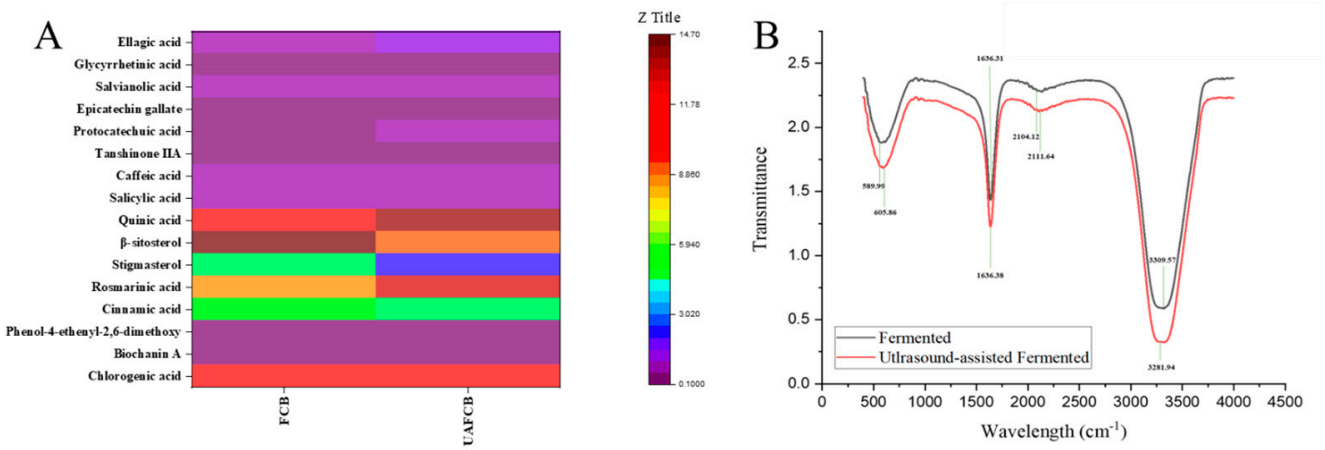

Figure 5. Multivariate heatmap showing the varying phytochemical contents (**A**) and an FTIR image depicting the chemical changes (**B**) of fermented and ultrasound-assisted fermented chickpea beverages.

3.6. Influence of Ultrasonication on the Chemical Structure of Fermented Chickpea Beverage Using FTIR

Chickpea fermentation results in beverages high in protein, fiber, and bioactive components, making them appealing to health-minded drinkers [67]. Their nutritional and functional qualities are improved through fermentation, with lactic acid bacteria playing a pivotal role, as described by Ref. [68]. The authors of [69] noted that ultrasonication makes fermented chickpea drinks more stable and uniform by changing the structure of the proteins and making it easier for the particles to spread out. In turn, this changes the chemical structure of the drink. The molecular structure changes in food systems can be examined using the potent analytical technique known as FTIR spectroscopy [70]. This explains the effects of fermentation and ultrasonication on these beverages. Ultrasonication has been shown to cause changes in the amide I and amide II regions of FTIR spectra, which are related to protein secondary structures [71]. According to Ref. [72], ultrasonication could induce changes in protein conformations, leading to higher protein unfolding. This can be observed in UAFCB in Figure 5B. The wide range between 3500 and 3000 cm^{-1} characterized aromatic compounds and also amines that were found in amino acids, peptides, proteins, alkaloids, DNA, and RNA because of amine (NH_2) groups [26,73]. The range indicated above is consistent with the peak values of 3309.57 cm^{-1} in the fermented sample and 3289.14 cm^{-1} in the ultrasound-assisted fermented sample. These changes in protein structures can significantly impact the beverage's overall structural integrity, enhancing its stability and functional properties. Furthermore, ultrasonication has been reported to affect carbohydrate and lipid components in FCB [3].

FTIR spectra samples showed changes in the fingerprint region of 211.64 cm^{-1} and 2104.12 cm^{-1} for the alkyne regions of the ultrasound-assisted fermented beverages and that of the fermented beverages, respectively, while indicating alterations in the hydrogen bonding of polysaccharides and the presence of lipid components as observed by the peaks of 1636.38 cm^{-1} and 1636.31 cm^{-1} for the USAF beverage and the fermented beverage formed in the region designated for C=O ester groups—all within the ranges indicated by Ref. [73]. These modifications could further influence the fermented chickpea beverage's

structural integrity and overall quality. Again, the peaks observed at 589.99 cm^{-1} for the unfermented sample shifted significantly to 605.86 cm^{-1} for the fermented sample, which is indicative of aliphatic iodo compounds in the samples. This is consistent with the peaks that were observed by the authors of [73] in the aliphatic iodo compounds, in C-I stretch between 500 and 600 cm^{-1}. It can be deduced from the presence of a peak in the aliphatic iodo compound stretch that chickpeas contain iodine compounds as an essential part of their nutritional profile. Since ultrasonication can considerably impact the structural integrity of FCB, FTIR spectroscopy can show changes in protein, carbohydrate, and lipid components. These alterations can increase the fermented chickpea beverage's stability, homogeneity, and functional qualities, making ultrasonication a viable technique for improving the quality of such goods. Ref. [27] provides a comparative analysis of the unfermented, fermented, and ultrasound-assisted fermented chickpea beverage (UAFCB) samples.

4. Conclusions

This study has successfully demonstrated the efficacy of ultrasound technology in enhancing the quality of fermented chickpea beverages. The ultrasound-assisted fermentation process significantly increased lactic acid (0.23 mg/mL) and reduced sugar concentrations (2.93 mg/mL). The optimum parameters identified were a start time of 3 h, treatment duration of 80 min, frequency of 27.5 kHz, and power density of 100 W/L. While phytochemical content remained consistent across fermented and ultrasound-assisted samples, FTIR analysis indicated notable shifts in organic component structures, underscoring the profound impact of ultrasound in the fermentation process. Overall, ultrasound application presents a promising avenue for improving fermented beverage quality, warranting further investigation into fine-tuning fermentation duration to mitigate potential nutritional degradation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/beverages9030062/s1, Figure S1: Standard curves for reducing sugar (A), and lactic acid (B). Table S1: Analysis of variance, regression analysis, and optimal conditions for chickpea milk fermentation; Table S2: Phytochemical content of chickpea beverage samples.

Author Contributions: Conceptualization, N.A.N.J. and Y.M.; Methodology, N.A.N.J. and G.B.; Software, J.-N.E. and G.B.; Formal analysis, E.I.; Investigation, N.A.N.J., Y.L., G.B. and E.I.; Resources, Y.M.; Data curation, N.A.N.J., S.Y.-S.S.A., J.-N.E., Y.L. and E.I.; Writing—original draft, N.A.N.J. and S.Y.-S.S.A.; Writing—review & editing, J.-N.E. and Y.M.; Supervision, Y.M.; Funding acquisition, Y.M. All authors have read and agreed to the published version of the manuscript.

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

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