

Article

The Possibility of Using *Lactobacillus plantarum* 299v to Reinforce the Bioactive Properties of Legume-Derived Beverages

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Featured Application: This study investigates the possibility of using the probiotic strain *Lactobacillus plantarum* 299v to ferment legume-derived beverages to enhance their bioactive properties. It has practical relevance in the development of innovative functional foods, nutraceuticals, supplements, or pharmaceuticals. Moreover, this study may be significant for exploring novel plant-based foods and searching for alternative protein sources. The investigations suggest effective approaches to increase the use of pulses, promoting their consumption and significance in the food industry.



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Abstract: There is a global need to explore alternative protein sources and develop new health-promoting plant-based products. This study aimed to produce beverages from organic seeds of chickpeas and green or red lentils and assess the potential of using *L. plantarum* 299v to ferment these beverages. Spectrophotometric methods were used to evaluate the ability of the beverages to neutralize 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH[•]) and chelate Fe²⁺. Total dietary fiber (including soluble and insoluble fractions) and the protein profiles were compared among the tested legume-derived beverages. The observations were focused on reductions in pH levels, total extract content, dietary fiber, ash, and moisture levels in all the fermented legume products. Fermentation contributed to increased antioxidative activity in all the tested products. The strongest ability to chelate Fe²⁺ (58.33 ± 2.35%) was noted in non-fermented green lentil products, while green lentil beverages fermented for 72 h exhibited the highest value of free radical scavenging activity (88.44 ± 4.24 DPPH[•] inhibition %). Chickpea products fermented for 72 h displayed the highest count of viable bacterial cells at 7.0 ± 0.7 × 10⁸ CFU/g, while those fermented for 48 h exhibited the greatest number (2764) of potential biopeptide sequences. These findings highlight the feasibility of using this probiotic strain to enhance the functional properties of legume-based beverages.

Keywords: bioactive peptides; antioxidative activity; probiotic fermentation; legume-based beverages

1. Introduction

Contemporary societies exhibit noticeable shifts in dietary preferences, reflecting evolving culinary trends and nutritional habits. These changes are closely tied to pressing global issues, such as environmental sustainability, public health, and effective food

management. As a result, there is a growing emphasis on exploring alternative protein sources and creating innovative plant-based foods, including analogs and substitutes for meat. Furthermore, there is an urgent demand for the development of new technologies and formulations to facilitate the design and market introduction of novel plant-derived food products. These products are sought to possess health-promoting properties with high protein content, aligning with the evolving needs and preferences of consumers.

Legumes are widely acknowledged as a valuable protein source essential for producing health-promoting foodstuffs and supplements. This significance is particularly underscored in the context of various diet-based therapies and the prevention of endemic and dynamically evolving health issues, such as high blood cholesterol, diabetes, and obesity [1]. The nutritional profile of legumes, characterized by high protein content, low-glycemic carbohydrates, and mineral richness, positions them as promising raw materials capable of addressing current challenges in food production and meeting consumers' expectations [2–5].

Consuming legumes offers benefits in preventing and managing both diabetes and cardiovascular diseases, and incorporating them into the diet of obese individuals provides compounds with pancreatic lipase inhibitory properties, aiding in weight reduction and control [6]. Regular consumption of legumes has been demonstrated to decrease the risk of various civilization diseases, including diabetes, tumors, obesity, and cardiovascular disorders [7–9]. Additionally, pulse-based foodstuffs might be a suitable matrix for active probiotic microflora and may serve as an alternative to traditional dairy products for individuals with lactose intolerance and allergies to milk protein components or those adhering to a vegan diet [10,11].

However, concerns and reluctance among consumers toward pulse-based foodstuffs (especially soy-derived products) have hindered the broader applications of legumes in food manufacturing. This is primarily attributed to their undesirable flavor and aroma, the presence of anti-nutritional factors (ANFs), and digestive discomfort experienced after consuming pulses.

In recent years, fermented soy products have gained popularity; yet, in Europe, fermented products based on legumes, such as lentils and chickpeas, are less prevalent and accessible than soy products. While the effect of legume fermentation on nutritional and functional characteristics has been studied, most investigations have focused on soy-based products and solid-state fermentation processes [12].

Generally, lactic acid bacteria (LAB) are the most abundant microorganisms involved in many fermentation processes of plant-derived raw material contributing to multidirectional changes in physicochemical as well as functional properties that include lowering the pH, inhibiting the growth of putrefactive bacteria, improving the organoleptic properties of food, enhancing the nutrient profile, and optimizing health benefits [13]. LAB not only possess the ability to bio-preserve food but also synthesize additional substances that impact the organoleptic characteristics and properties of food [14,15]. Moreover, certain LAB strains are recognized as probiotics [16]. Beyond lactic acid production, probiotic LAB strains, such as *L. plantarum* 299v, produce a plethora of bioactive metabolites, including organic acids, short-chain fatty acids, carbohydrates, antimicrobial peptides, enzymes, vitamins, cofactors, and immune signaling compounds. Collectively known as postbiotics, these substances exhibit antimicrobial properties and contribute to prolonging shelf life [17].

Consuming specific fermented legumes has been associated with a wide range of health-promoting properties [13]. This includes the decrease in the occurrence and seriousness of chronic conditions like cardiovascular disease, breast and prostate cancer, menopausal symptoms, and bone loss [18–22]. It was indicated that bean extracts fermented with *Lactiplantibacillus plantarum* subsp. *plantarum* may potentially lead to an antihypertensive effect, attributed to the abundant presence of gamma-aminobutyric acid and the action of angiotensin-converting enzyme [22]. Similarly, fermenting pea seeds with *Lactiplantibacillus plantarum* subsp. *plantarum* resulted in the release of potentially antihypertensive peptides during in vitro digestion [23].

Nevertheless, to the best of our knowledge, the lactic acid fermentation of various pulse-derived beverages (especially chickpea products) using selected probiotic bacterial strains remains an area requiring further characterization.

Considering the aforementioned aspects and to address the needs arising from the growing consumer interest in health-promoting foods, this research aimed to produce probiotically fermented pulse-based foodstuffs and assess their potential functional features. Therefore, the objective of this investigation was to produce different variants of organic legume-based beverages subjected to fermentation by the probiotic strain *Lactobacillus plantarum* 299v. This study evaluated selected functional properties, including antioxidant activity and the capacity for metal ion chelation, exhibited by the resulting products. Additionally, the influence of the applied bioprocess on peptide profiles and dietary fiber content was analyzed.

2. Materials and Methods

2.1. Bacterial Strain and Preparation of Inoculum for Fermentation

The probiotic strain *Lactobacillus plantarum* 299v (Sanprobi IBS, Sanum sp. z o.o., Szczecin, Poland) was systematically transferred (2% v/v of inoculum) into fresh MRS broth (BTL, Łódź, Poland) and anaerobically incubated at 37 °C for 18 h. The starter culture used as the inoculum in the fermentation process was prepared according to the method described by Sadat-Mekmene et al. [24], with certain modifications. Briefly, bacterial cell biomass was harvested by centrifugation (8000 × g, 10 min, 4 °C; Eppendorf Centrifuge 5415R, GmbH, Hamburg, Germany) after 18 h of incubation. The resulting pellet was washed twice with a sterile NaCl solution (0.9%) and resuspended (also in a saline solution) to yield a suspension with an optical density (measured using a spectrophotometer Helios Gamma, Thermo Fisher Scientific, Waltham, MA, USA) equivalent to OD₅₅₀ = 0.5 (corresponding to 6 × 10⁸ CFU/mL). This suspension was used for inoculation.

2.2. Preparation of Fermented Legume-Based Beverages

Organic seeds (bearing the EU organic production logo—PL-EKO-01) of chickpeas, red lentils, and green lentils (sourced from SYMBIO Polska S.A., Lublin, Poland) obtained from the local market served as the raw material for producing fermented plant-based beverages.

Each variant of the legume-based beverage was prepared similarly, following the method outlined by Marazza et al. [25], with some adjustments. Initially, 200 g of seeds (chickpeas, red lentils, or green lentils) were washed and soaked in tap water at room temperature for 16 h. Following drainage, the seeds were processed using a food processor designed for soymilk production (SMM200S, Manta, Warszawa, Poland). Subsequently, 1000 mL of fresh water was added to the processor (containing only one type of pulse seeds), and a program for beverage production was initiated. The resulting mixtures were then strained through cheesecloth. The filtered liquids (950 mL) were transferred to sterile beakers, sealed tightly, and pasteurized in a water bath at 85 °C for 10 min. After cooling to approximately 37 °C, the prepared variants of plant-based products were inoculated in sterile conditions with 2% (v/v) of the previously prepared fresh inoculum. Subsequently, they were thoroughly mixed, transferred (in a volume of 25 mL) to sterile glass screw-top bottles, and securely sealed. The fermentation process was conducted at 37 °C for 72 h. Meanwhile, pasteurized uninoculated samples (of each beverage type) were maintained as controls. The pH values of the beverages were measured after pasteurization (before inoculation), immediately after inoculation, and after 48 and 72 h of fermentation with the use of a pH meter (Hanna Instruments HI 221, Hanna Instruments, Warszawa, Poland).

Samples of the fermented beverages were collected after 48 h and 72 h of incubation and promptly subjected to heat treatment (excluding those intended for microbiological analysis) in a water bath at 95 °C for 5 min to deactivate bacteria and inhibit enzyme activity. All the aforementioned procedures were executed in sterile conditions.

2.3. Assay of Dietary Fiber and Total Extract Content

The determination of dietary fiber followed AOAC methods, i.e., the enzymatic-gravimetric method 993.19 for soluble dietary fiber (SDF) and method 991.42 for insoluble dietary fiber (IDF). Additionally, the analysis included the determination of total dietary fiber (TDF), ash content (%), and moisture content (%) in the tested samples.

The values of the refractive index of the beverages were measured using a refractometer (Kruss DR201-95 A.KRÜSS Optronic, GmbH, Hamburg, Germany), and the results were expressed in °Brix. This measurement was conducted in triplicate to ensure the accuracy and reliability of the data.

2.4. Radical Scavenging Activity

The capacities of both fermented and unfermented legume-derived beverages to neutralize DPPH• (1,1-diphenyl-2-picrylhydrazyl) radicals were evaluated following the method outlined by Maleki et al. [26]. A 3.0 mL aliquot of DPPH methanol solution (25 mg/L) was added to tubes containing 1.0 mL of the test sample (0.2 g/mL). The radical scavenging activity, expressed as the percentage inhibition of DPPH• absorbance at 517 nm, was determined using a SmartSpec™Plus Spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) after 30 min of incubation in darkness at room temperature.

The calculation of the % inhibition of DPPH• absorbance at 517 nm was as follows:

$$\text{Inhibition [\%]} = [1 - (\text{At}/\text{Ac})] \times 100 \quad (1)$$

where Ac is the absorbance of the control, while At is the value of absorbance of the analyzed sample (final product obtained after 24 h of fermentation or unfermented beverage). The experiment was carried out in five repetitions for each variant of legume-based beverages.

2.5. Analysis of the Profiles of Potentially Bioactive Peptide Sequences

The analysis of the protein profiles in the tested legume-derived material was conducted using nanoLC-TIMS-MS/MS. Initially, the samples were freeze-dried (Christ Alpha 1-2 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and ground to a fine powder (7000 rpm for 5 min) using a laboratory mill (Retsch GM200, Retsch GmbH, Haan, Germany). The extraction process followed the method described by [27]. Subsequently, the obtained peptides (2 µg) were desalted using SPE (Pierce C18 spin columns from Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol. After drying, they were resuspended in 20 µL of 2% acetonitrile (ACN) with 0.1% formic acid (HCOOH) (Sigma-Aldrich, Saint Louis, MO, USA). For the nanoLC-MS/MS analysis, 1 µL of the sample was injected into the Ultimate3000 nanocapillary chromatography system (Thermo Scientific). Separations were achieved using an Aurora column (25 cm long, 75 µm ID, C18, IonOptics) with a precolumn PepMap100 C18 5 µm (5 mm long, 0.3 mm ID, Thermo Scientific). The linear gradient was formed using 0.1% HCOOH in H₂O (solvent A) and 0.1% HCOOH in ACN (solvent B) at a flow rate of 300 nL/min. The gradient ranged from 2% to 35% B over 90 min, followed by an isocratic hold at 85% B for 7 min. The system was controlled by the Hystar software version 5.0. (Bruker Daltonics, Bremen, Germany), and the chromatographic system was directly coupled to the timsTOFPro 2 (Bruker Daltonics) mass spectrometer. Operating in the positive-ion mode, the instrument conducted scans ranging from 100 to 1700 m/z and ion mobility 1/K0 ranging from 0.6 to 1.6. Fragmentation was performed using the parallel accumulation and serial fragmentation (PASEF) method with standard settings. The acquired mass spectra were analyzed using MaxQuant (version 2.0.1.0). Afterward, the activity of identified amino acid sequences was determined using the method described by Li et al. [28] with PeptideRanker [29], where any peptide predicted to exceed a threshold of 0.5 is considered to exhibit biological activity.

2.6. Determination of the Ability to Chelate Fe²⁺ Ions

The capacity to chelate metal ions (Fe²⁺) in the tested beverages was assessed spectrophotometrically following the method outlined by Maleki et al. [26]. Briefly, 200 µL of each tested beverage sample (0.2 g/mL) was sequentially combined with 9.6 mL of distilled water, 200 µL of 2 mM iron chloride solution (Fe²⁺), and 400 µL of ferrozine (5 mM). After thorough mixing, the samples were incubated for 10 min in darkness at room temperature and then centrifuged (8000× *g* for 10 min at 4 °C; Eppendorf Centrifuge 5415R, GmbH, Hamburg, Germany). The clear supernatants were subjected to spectrophotometric measurements at 562 nm using a SmartSpec™Plus Spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA), with water serving as the blank.

The ability to chelate iron ions (Fe²⁺) was calculated using the following formula:

$$\text{Fe}^{2+} \text{ chelating capacity (\%)} = [1 - (\text{At}/\text{Ac})] \times 100 \quad (2)$$

where Ac is the absorbance of the control, while At is the value of absorbance of the analyzed sample (final product obtained after 24 h of fermentation or unfermented beverage).

2.7. Counting the Viable Cells of the Probiotic Strain in Final Products

The viable cell counts (CFU/mL) in the final products (plant-based beverages collected immediately after 72 h of fermentation) were determined using the pour-plate method on MRS agar, following the protocol outlined by Zhao and Shah [30]. The plates were then incubated in anaerobic conditions at 37 °C for 72 h. The experiment was conducted in triplicate to ensure the reliability and consistency of the results.

2.8. Statistical Analysis

Statistical analyses were conducted using the Statistica 13.1 software package (StatSoft, Krakow, Poland). Significant differences among the mean values of the measured parameters were determined using analysis of variance (ANOVA) followed by the Tukey test ($p < 0.05$). Hierarchical cluster analysis (HCA) was employed to explore the similarity between the examined products (regarding the tested physicochemical parameters). Clustering was performed using the Ward method with a distance matrix (similarity matrix) formed based on Euclidean distance.

The presence of statistically significant differences between the mean values of the analyzed parameters was verified using the non-parametric Kruskal–Wallis test. Correlations between the analyzed properties were determined using the Pearson linear correlation test with XLSTAT software (version 2021.2.1, Addinsoft, New York, NY, USA). All results are expressed as mean values ± standard deviation.

3. Results and Discussion

3.1. Determination of the Physicochemical Properties of Legume-Derived Beverages

The fermentation of plant-based materials constitutes a multifaceted bioprocess, yielding diverse metabolites that exert an influence on the physicochemical characteristics and functional attributes of the resultant products [31]. A principal alteration within the food matrix during this transformative process was manifested as fluctuations in the initial pH level. Across all the beverage variants, the most substantial reduction in pH occurred after 48 h of incubation (Table 1). The lowest pH value (4.82 ± 0.013) was observed in the final products derived from chickpeas (Ch) obtained after 72 h of fermentation (final products). Conversely, beverages derived from green lentils (GL) exhibited the highest pH levels after both 48 h and 72 h of incubation.

Table 1. Comparison of changes in pH and total extract content among tested beverages.

Tested Parameter	Raw Material	Type of Beverage				Values of Pearson Correlation (r) **
		Unfermented		Fermented		
		Before Pasteurization	Control *	48 h	72 h	
pH	Ch	7.57 ± 0.005 ^{dB}	7.53 ± 0.005 ^{cB}	4.89 ± 0.012 ^{bA}	4.82 ± 0.013 ^{aA}	−0.878
	RL	7.68 ± 0.010 ^{cC}	7.58 ± 0.013 ^{bC}	5.32 ± 0.005 ^{aB}	5.31 ± 0.010 ^{aB}	−0.867
	GL	7.17 ± 0.005 ^{cA}	7.08 ± 0.005 ^{bA}	5.55 ± 0.005 ^{aC}	5.54 ± 0.024 ^{aC}	−0.869
TEC [°Brix]	Ch	5.62 ± 0.08 ^{dB}	5.30 ± 0.07 ^{cA}	4.12 ± 0.08 ^{bAB}	2.26 ± 0.28 ^{aB}	−0.942
	RL	5.74 ± 0.05 ^{dC}	5.32 ± 0.11 ^{cA}	4.14 ± 0.11 ^{bB}	1.86 ± 0.17 ^{aA}	−0.929
	GL	5.38 ± 0.04 ^{cA}	5.24 ± 0.09 ^{cA}	3.96 ± 0.11 ^{bA}	2.14 ± 0.24 ^{aAB}	−0.952

Explanatory notes: Ch—beverage variant obtained from chickpea; RL—beverage variant obtained from red lentil; GL—beverage variant obtained from green lentil; TEC—total extract content; * control—unfermented beverage after pasteurization. Values are expressed as the mean ($n = 3$) ± standard deviation. The mean values in the same row followed by different lowercase letters (a–d) indicate significant differences ($p < 0.05$) between the values of the tested parameter recorded for the selected variant of beverage in all the tested time intervals, whereas uppercase letters (A–C) denote significant differences ($p < 0.05$) between the values of the tested parameter noted among all the tested products in the selected period. ** Values of Pearson correlation marked in bold indicate significance (at $p < 0.05$) between the fermentation time of the beverage variant and the analyzed parameter.

The obtained results correspond to the findings described by Verni et al. [32]. The authors analyzed seven strains of *Lactobacillus* as potential starter cultures for the fermentation of beverages produced from lentil grains. Notably, their outcomes revealed that all the examined strains exhibited the capacity to ferment the pulse-derived substrate within 24 h. However, it was observed that only three strains, namely, *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus fermentum* DSM 20052, and *Lactobacillus paracasei* subsp. *paracasei* DSM 20312, exhibited both the desired growth kinetics and the capability to effectively reduce the pH level.

It is noteworthy that the final pH of fermented legume-derived raw material is contingent not solely upon the acidification capacity of lactic acid bacteria (via organic acid production). Rather, it is also influenced by intricate biochemical transformations and the activity of bacterial proteolytic systems, which liberate alkalizing compounds from native proteins, thereby modulating the pH level [33].

The probiotic fermentation applied in this study significantly decreased the total extract content (TEC) of all the legume-based beverages (Table 1). The most pronounced changes, compared to the control, were observed in the final products (after 72 h of fermentation) of the RL variant. The highest TEC (2.26 ± 0.279 °Brix) was noted in the final products derived from chickpeas. The findings suggest that the applied pasteurization process may also have contributed to the reduced values of the measured parameter in the beverages, except for the GL variant (Table 1).

The decline in TEC may be attributed to bacterial metabolic activity, which facilitates the conversion of carbohydrates into lactic acid (associated also with decreasing pH). Interestingly, the capacity of certain lactic acid bacteria to synthesize and excrete extracellular polysaccharides (exopolysaccharides, EPSs) may also contribute to the reduction in TEC levels in fermented products [34], since EPSs augment the viscosity of fermented products through interactions with proteins and carbohydrates, consequently leading to a decrease in TEC. Since *L. plantarum* has been documented as a producer of exopolysaccharides [35], it might be expected that its EPSs were also produced during the fermentation of the legume-based beverages, thereby contributing to the modification in the TEC levels in the products. Moreover, the variations observed in the analyzed parameters among the different beverage variants may be linked to the differential preferences of the tested strain in metabolizing specific constituents present in diverse legume raw materials. This was proved by Sebastian et al. [36] in an investigation examining soy beverages fermented by probiotic strains of *S. thermophilus* and *L. bulgaricus*.

The present results reveal that the probiotic fermentation also led to a reduction in the fiber content in the legume-based beverages (Table 2). This is consistent with the findings elucidated by Liang et al. [37], who noted that microorganisms participating in fermentation can degrade fiber and induce alterations in its constituent fractions.

Table 2. Comparison of the contents of dietary fiber, ash, and moisture between the variants of legume-based beverages.

Legume-Based Beverage	IDF [% d.m.]	SDF [% d.m.]	TDF [% d.m.]	Ash [% d.m.]	Moisture [%]
Ch-0	17.35 ± 0.05 ^{cC}	7.76 ± 0.04 ^{cB}	25.11 ± 0.03 ^{cC}	3.69 ± 0.05 ^{aC}	1.29 ± 0.06 ^{bA}
Ch-48	16.74 ± 0.01 ^{bC}	6.68 ± 0.09 ^{bC}	23.42 ± 0.12 ^{bC}	3.66 ± 0.03 ^{aC}	1.15 ± 0.02 ^{aA}
Ch-72	16.19 ± 0.03 ^{aC}	6.03 ± 0.01 ^{aC}	22.21 ± 0.04 ^{aC}	3.64 ± 0.02 ^{aC}	1.20 ± 0.03 ^{abB}
RL-0	14.89 ± 0.06 ^{cB}	6.86 ± 0.02 ^{cC}	21.75 ± 0.07 ^{cB}	2.75 ± 0.01 ^{bB}	1.15 ± 0.09 ^{abA}
RL-48	14.28 ± 0.01 ^{bB}	6.09 ± 0.02 ^{bA}	20.36 ± 0.04 ^{bB}	2.70 ± 0.01 ^{abB}	1.32 ± 0.07 ^{bB}
RL-72	13.71 ± 0.02 ^{aB}	5.50 ± 0.09 ^{aA}	19.22 ± 0.11 ^{aB}	2.64 ± 0.04 ^{aB}	1.11 ± 0.03 ^{aA}
GL-0	13.11 ± 0.09 ^{aA}	6.30 ± 0.02 ^{cA}	20.62 ± 0.04 ^{aA}	2.61 ± 0.03 ^{aA}	1.53 ± 0.01 ^{bB}
GL-48	13.69 ± 0.05 ^{bA}	5.41 ± 0.01 ^{bB}	19.10 ± 0.06 ^{bA}	2.55 ± 0.08 ^{aa}	1.35 ± 0.09 ^{aB}
GL-72	13.11 ± 0.09 ^{aA}	5.10 ± 0.02 ^{aB}	18.21 ± 0.11 ^{aA}	2.54 ± 0.02 ^{aa}	1.40 ± 0.03 ^{abC}

Explanatory notes: Ch—beverage variant obtained from chickpea; RL—beverage variant obtained from red lentil; GL—beverage variant obtained from green lentil; control products (unfermented) are marked with “0”, whereas fermented beverages are labeled with 48 (fermented for 48 h) or 72 (fermented for 72 h). Values are expressed as the mean ($n = 3$) ± standard deviation. The mean values in the same column followed by different lowercase letters (a–c) indicate significant differences ($p < 0.05$) in the values of the tested parameter recorded for one beverage variant (Ch(0-72), RL(0-72), or GL(0-72)) in the analyzed periods (0 h, 48 h, and 72 h), whereas uppercase letters (A–C) denote significant differences ($p < 0.05$) between the means recorded for the beverage variants (Ch, RL, and GL) in the determined time (0 h, 48 h, or 72 h). SDF—soluble dietary fiber; IDF—insoluble dietary fiber; TDF—total dietary fiber; d.m.—dry mass.

In general, the beverages derived from chickpeas exhibited the highest contents of IDF, SDF, and TDF at every stage of fermentation. The sole exception was observed in the control variant of red lentils (RL-0), which had SDF content comparable to that in the chickpea products (Table 2).

The reduction in the IDF, SDF, and TDF levels across all the fermented beverage variants may also be attributed to the enzymatic activity of bacteria, including cellulases, α -galactosidases, and others. These enzymes facilitate the degradation of dietary fiber, leading to the conversion of IDF into soluble fractions, which are more susceptible to further modifications [38]. These findings are congruent with those reported by Chitra et al. [39], who demonstrated that the fermentation process resulted in a considerable decrease in the total dietary fiber content in chickpea dhal (from 161.2 to 82.4 g/kg).

Furthermore, across all the beverage variants, a discernible negative correlation between the duration of probiotic fermentation and the contents of IDF, SDF, TDF, and ash was observed (Table 3). Additionally, a similar correlation between the fermentation time and the moisture content was noted in the case of products derived from both chickpeas and green lentils.

Table 3. The correlations between the fermentation time of the beverage variants and the analyzed parameters.

Legume-Based Beverage	IDF [% d.m.]	SDF [% d.m.]	TDF [% d.m.]	Ash [% d.m.]	Moisture [%]
Ch	−0.985	−0.997	−0.994	−0.601	−0.655
RL	−0.984	−0.991	−0.989	−0.882	0.009
GL	−0.981	−0.996	−0.997	−0.577	−0.722

Explanatory notes: Ch—beverage variant obtained from chickpea; RL—beverage variant obtained from red lentil; GL—beverage variant obtained from green lentil; SDF—soluble dietary fiber; IDF—insoluble dietary fiber; TDF—total dietary fiber; d.m.—dry mass. Values of Pearson correlation marked in bold indicate significance (at $p < 0.05$) between the fermentation time of the beverage variant and the analyzed parameter.

3.2. Profiles of Potentially Bioactive Peptide Sequences

Among the various types of beverages, significant differences in both the numbers and amino acid sequences of peptide profiles were observed (Table 4, Supplementary Materials: Table S1 and Spreadsheets 1–3).

Table 4. Sequences of potential biopeptides detected in lentil-based beverages.

Sequence	Molecular Mass [Da]	Variant of Lentil Beverage						Probability of Peptide Bioactivity *
		RL-0	RL-48	RL-72	GL-0	GL-48	GL-72	
CLQRIFK	963.53	+	+	+	—	—	—	0.631
HPTFILCSRK	1257.67	+	+	+	—	—	—	0.572
MEMFDLEFMRR	1503.67	+	+	+	—	—	—	0.754
SLGSNTPINMIR	1301.68	—	—	—	+	+	+	0.606

Explanatory notes: RL—beverage variant obtained from red lentil; GL—beverage variant obtained from green lentil; control products (unfermented) are marked with “0”, whereas fermented products are labeled with 48 (fermented for 48 h) or 72 (fermented for 72 h); “+” indicates the presence of the sequence in the determined beverage variants, whereas “—” means that the sequence was not detected in the tested beverages. * Predicted probability that the peptide will be bioactive (value exceeding the threshold of 0.5 is considered to exhibit biological activity) calculated in PeptideRanker [17].

Remarkably, beverages derived from chickpeas exhibited the highest count of identified peptides (2764) including the largest number of sequences with potential bioactivities, particularly evident in products fermented for 48 h, as detailed in Supplementary Materials Table S1. In contrast, only three potential biosequences (HPTFILCSRK, CLQRIFK, and MEMFDLEFMRR) were identified in both fermented and unfermented beverages derived from red lentils. In turn, only one potential biopeptide (SLGSNTPINMIR) was detected in the beverages produced from green lentils (Table 4).

The present results correspond to those reported by Li et al. [28] revealing that *Lactobacillus* strains are suitable for the production of biopeptides in fermented plant-based food matrices (the potential activity of each peptide was identified using the PeptideRanker tool as well). In broccoli fermented by *L. plantarum* A3 and *L. rhamnosus* ATCC7469, the authors detected 2060 and 1707 amino acid sequences with a score exceeding 0.5, respectively. These results underscore the feasibility of using the tested lactic acid bacteria to enrich plant-based foods in situ with health-promoting components, thereby conferring their functional properties akin to diverse foodstuffs.

3.3. Determination of Antioxidant Activity and Ability to Chelate Fe²⁺

The accumulation of free radicals is widely recognized as a major factor in the pathophysiology of numerous civilization diseases and various health disorders attributable to the adverse effects of oxidative stress [40]. Therefore, the mitigation of the detrimental effects of these reactive molecules necessitates the provision of sufficient quantities of antioxidant compounds to bolster the body’s inherent defense mechanisms against long-term oxidative stress conditions. It has been proposed that the fermentation process exerts a positive influence on the free radical scavenging properties of plant-derived food products [41]. This is in agreement with the present results indicating that the fermentation of the legume-based beverage variants significantly enhanced their antioxidant properties (Table 5). The final products (beverages fermented for 72 h) exhibited significantly higher abilities to neutralize free radicals than their unfermented counterparts.

Table 5. Comparison of radical scavenging activity and ability to chelate Fe²⁺ in the control and final products.

Tested Beverage	Radical Scavenging Activity (DPPH• Inhibition %)	Fe ²⁺ Chelating Capacity (%)
Ch-0	25.31 ± 6.18 ^a	23.12 ± 4.65 ^{ab}
Ch-72	88.07 ± 1.04 ^d	28.13 ± 6.98 ^b
GL-0	40.40 ± 5.68 ^b	58.33 ± 2.35 ^c
GL-72	88.44 ± 4.24 ^d	27.49 ± 4.02 ^{ab}
RL-0	60.07 ± 3.99 ^c	22.20 ± 2.25 ^a
RL-72	86.28 ± 4.14 ^d	24.29 ± 5.50 ^{ab}

Explanatory notes: Ch—beverage variant obtained from chickpea; RL—beverage variant obtained from red lentil; GL—beverage variant obtained from green lentil; “-0” indicates the control (unfermented) beverage, while “-72” refers to products obtained after 72 h of fermentation (final product). Values expressed as mean values ± mean standard deviation ($\bar{x} \pm s/SD$; $n = 6$) followed by different lowercase letters (in the same column) are significantly different ($p < 0.05$ level); the tested maximum concentration was 0.2 g/mL.

Among all the control variants, RL-0 exhibited the strongest antioxidant properties (60.07 ± 3.99% of DPPH• inhibition), while the lowest levels of DPPH• inhibition were observed in Ch-0 (25.31 ± 6.18%). However, the chickpea-derived beverages were distinguished from all the final products by the most substantial increase (3.5-fold) in the tested bioactivity, reaching up to 88.33 ± 0.92% of DPPH• inhibition (Table 5).

The findings presented herein are consistent with those reported by de Oliveira Silva et al. [31], who observed that the fermentation process contributed to enhancing the antioxidant activity (up to 206%) and improving the nutritional quality of soybean products. Moreover, the present results (Table 5) align with the findings shown by Sebastian et al. [36], who examined the capacity of soya beverages fermented with probiotic strains of *L. bulgaricus* and *S. thermophilus* to neutralize free radicals. They reported a three-fold increase in the antioxidant activity of fermented products (expressed as a percentage of DPPH radical inhibition capacity) compared to unfermented control samples. Similar outcomes were also described by Li et al. [28] in their analyses of soya beverages fermented with *L. plantarum* YS-1 or *L. bulgaricus* strains. Soya products fermented by *L. plantarum* exhibited a strong ability to inhibit free radicals (80.3%), comparable to the values demonstrated by the final products (Ch-72, RL-72, and GL-72) fermented by *L. plantarum* 299v.

The augmentation of the antioxidant activity observed in fermented legume products has been attributed to the rise in both the content and accessibility of phenolic compounds [20]. These components undergo conversion during fermentation into liberated and highly biologically active forms capable of effective neutralization of free radicals. Additionally, the antioxidative properties inherent in fermented plant-based products are linked to the presence of viable lactic bacterial cells and their repertoire of enzymatic and non-enzymatic mechanisms. These biochemical reactions function to impede the generation and activity of reactive oxygen species [42].

It is pertinent to note that the accumulation of heavy metals and certain metal ions in the body can have adverse effects on human health. Specifically, Fe²⁺ ions, under the conditions of oxidative stress, play a role in catalyzing the Fenton reaction, thereby contributing to the formation of highly reactive hydroxyl radicals that exert prooxidative effects on lipids, potentially leading to atherosclerotic lesions [43]. Consequently, ensuring a diet rich in compounds capable of binding and inactivating such metal ions exerting a negative impact on human health is considered a crucial strategy. Therefore, the investigation also evaluated the ability of the legume-derived beverages to chelate Fe²⁺ ions.

The results (Table 5) indicated a slight increase in the ability of the beverages derived from chickpeas and red lentils to chelate iron (II) ions following the fermentation process. However, studies conducted by Liu et al. [44] and Maleki et al. [26] did not corroborate a significant effect of fermentation on the ability of plant-based beverages to chelate iron (II). It is proposed that the observed variability in the measured bioactivity may be attributed to variations in the content of phenolic compounds present in the raw plant material [42]. This

may account for the differences noted in the values across the different variants of the tested beverages. Furthermore, the diverse biochemical transformations occurring during fermentation may have contributed to the alterations in the profiles of polyphenols, consequently influencing the capacity of the beverages to chelate Fe^{2+} . Nevertheless, the final products derived from chickpeas and red lentils had higher Fe^{2+} chelating capacity ($28.13 \pm 6.98\%$ and $24.29 \pm 5.50\%$, respectively) compared to their respective control variants. Interestingly, the highest value of the measured activity was recorded for GL-0 ($58.33 \pm 2.35\%$). This effect is likely associated with the adverse impact of pasteurization and the fermentation process on the content and stability of chlorophyll (particularly notable in the material derived from green lentils compared to the other legume products). Chlorophyll exhibits strong antioxidative and chelating activity [45], which is also associated with molecular characteristics. Since magnesium is located in the central porphyrin ring of this natural pigment [46], bivalent metals such as iron, copper, or zinc can displace it from the active center, forming complexes influencing the ability of chlorophyll to chelate Fe^{2+} ions [47]. Also, the fermentation process leading to a reduction in pH levels promotes the displacement of magnesium ions by hydrogen. This results in the conversion of chlorophyll to pheophytin accompanied by a change in the color of the final product and a diminished ability to chelate Fe^{2+} [46]. These mechanisms probably substantially influenced the capacity of the fermented green lentil products to chelate Fe^{2+} ions.

Differences were observed in the results between the Fe^{2+} chelating capacity and DPPH• inhibition % (free radical scavenging activity). In one of the tests, the results for the beverage variants differed, while in the other, the differences in the obtained values were relatively small (Table 5). These discrepancies may arise due to variations in the mechanisms of antioxidant activity assessed with each method. The Fe^{2+} chelating capacity assay primarily measures the ability of antioxidants to reduce ferric ions, reflecting their capacity for electron donation, while the DPPH assay evaluates the ability of antioxidants to scavenge free radicals by hydrogen atom transfer [48]. Moreover, several factors might contribute to discrepancies between these methods in fermented legume-based products. Fermentation might alter the composition and concentration of antioxidant compounds, affecting their reactivity in different assays. Additionally, the presence of fermentation by-products or microbial metabolites could influence the antioxidant activity measured by each assay [48].

To assess the similarity between the tested products concerning both antioxidative activity and the capacity to chelate Fe^{2+} , the hierarchical cluster analysis (HCA) was carried out (Figure 1). Grouping was performed using the Ward method with a distance matrix (similarity matrix) constructed based on Euclidean distance.

The results revealed that the tested products could be categorized into three distinct groups based on the similarity of both analyzed characteristics (at a bond level of 1.5). The first group comprised all the fermented beverages, distinguished by their high efficacy in inhibiting free radicals (over 80% DPPH inhibition). However, their average chelating activities did not differ significantly (Table 5). In contrast, the unfermented beverages derived from chickpeas and the unfermented products from red lentils formed the second cluster. These variants exhibited the lowest average chelating activity. In turn, the unfermented beverages obtained from green lentils (GL-0), characterized by high Fe^{2+} chelating activity and relatively low antioxidant activity, constituted the third group of products (Figure 1 and Table 5).

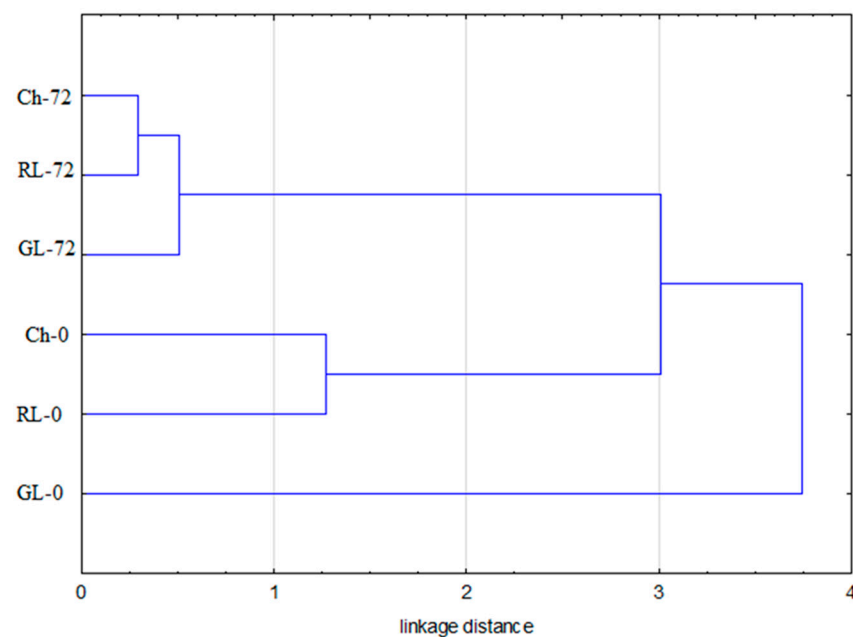


Figure 1. Dendrogram showing the results of hierarchical cluster analyses of similarities of products in terms of the antioxidative activity and Fe^{2+} chelating capacity. Explanatory notes: Ch—beverage variant obtained from chickpea; RL—beverage variant obtained from red lentil; GL—beverage variant obtained from green lentil; “-0” indicates the control (unfermented) beverage, while “-72” refers to products obtained after 72 h of fermentation (final product). The tested maximum concentration was 0.2 g/mL.

3.4. Determination of the Viable Cells of the Probiotic Strain

The highest level of viable bacterial cells ($7.0 \pm 0.7 \times 10^8$ CFU/g) was recorded for the final product prepared from chickpeas (Ch-72), while the lowest count of *L. plantarum* 299v ($1.8 \pm 0.4 \times 10^8$ CFU/g) was noted in GL-72. Also, a higher number of viable cells of the probiotic strain in the fermented beverages was noted in the products characterized by lower pH levels (Tables 1 and 6).

Table 6. Comparison of changes in pH and total extract content among the tested legume-derived beverages.

Tested Final Product	Survival (CFU/g)
Ch-72	$7.0 \pm 0.7 \times 10^8$ ^c
RL-72	$3.1 \pm 0.5 \times 10^8$ ^b
GL-72	$1.8 \pm 0.4 \times 10^8$ ^a

Explanatory notes: Ch-72—beverage obtained from chickpea after 72 h of fermentation; RL-72—beverage obtained from red lentil after 72 h of fermentation; GL-72—beverage variant obtained from green lentil after 72 h of fermentation; the mean values ($n = 3$) $\pm$ standard deviation followed by different lowercase letters (a–c) indicate a significant difference ($p < 0.05$) between the final products.

Given the broad spectrum of health-promoting properties attributed to the tested strain, extensively documented in clinical research, it is reasonable to anticipate that the obtained final products may exhibit characteristics akin to functional foods. The findings suggest that the fermented beverages could serve as a carrier for the probiotic strain, as they adhere to the FAO/WHO stipulation that the concentration of probiotic bacteria in a 100 g portion should be at least 10^{-6} CFU/g [49]. However, it is also indicated that fermented products should contain a higher count of live bacterial cells, ranging from 10^{-7} to 10^{-9} CFU/mL or g of product, and should be consumed daily to elicit a probiotic effect [50]. While all the final products in this study met these recommendations, it is imperative to conduct further in-depth investigations to validate their health-promoting effects.

4. Conclusions

The results indicate the potential of the probiotic strain *Lactobacillus plantarum* 299v as a starter culture suitable for the fermentation of legume-based beverages to improve the physicochemical properties and functionality of final products. The changes in the values of the parameters of the beverages were dependent on the type of raw material. The applied bioprocess enhanced the free radical scavenging activity, especially in the products obtained from chickpeas and green lentils. Furthermore, the chickpea-derived beverages fermented for 48 h exhibited the highest amount of potentially biologically active peptides. Also, the findings suggest that the analyzed plant-based products may serve as a conveyance medium for the probiotic strain. Nevertheless, the probiotic potential of obtained beverages has to be thoroughly examined. The promising outcomes of this preliminary investigation encourage further scrutiny to refine the technology for developing novel health-promoting organic legume-derived beverages fermented by *L. plantarum* 299v. The present findings can be a base for further studies leading to the development of innovative substitutes for dairy analogs. Nevertheless, a wide range of analyses determining the characteristics of these products (including assessment of product stability, organoleptic evaluation, and investigation of the health-enhancing effects on the human body) are highly required.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app14125187/s1>, Table S1: The sequences of potential biologically active peptides detected in chickpea beverages; Spreadsheets 1–3: Peptide sequences identified in tested legume material: Spreadsheet 1—Peptides sequences identified in green lentil material; Spreadsheet 2—Peptides sequences identified in red lentil material; Spreadsheet 3—Peptides sequences identified in chickpea material. Supplementary Files Spreadsheets 1–3 [Data set]. Zenodo. <https://doi.org/10.5281/zenodo.11214881>.

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