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Effect of probiotic bacterial species on the bioactivity of a fermented spelt-based beverage

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ABSTRACT

Background: Spelt grain, due to its valuable chemical composition, can serve as a promising substrate for functional beverages. Probiotic microorganisms are capable of hydrolyzing bound phenolics and synthesizing new phenolic compounds during fermentation. Fermentation of spelt with probiotic bacteria increases the concentration of free phenolic compounds and organic acids, thereby enhancing the bioactivity of the beverage.

Objective: The aim of this study was to evaluate the ability of the strains *B. adolescentis*, *P. freudenreichii*, *L. casei*, *L. plantarum*, and *L. fermentum* to accumulate phenolic compounds and organic acids during fermentation and to ensure high antioxidant activity in a spelt-based beverage.

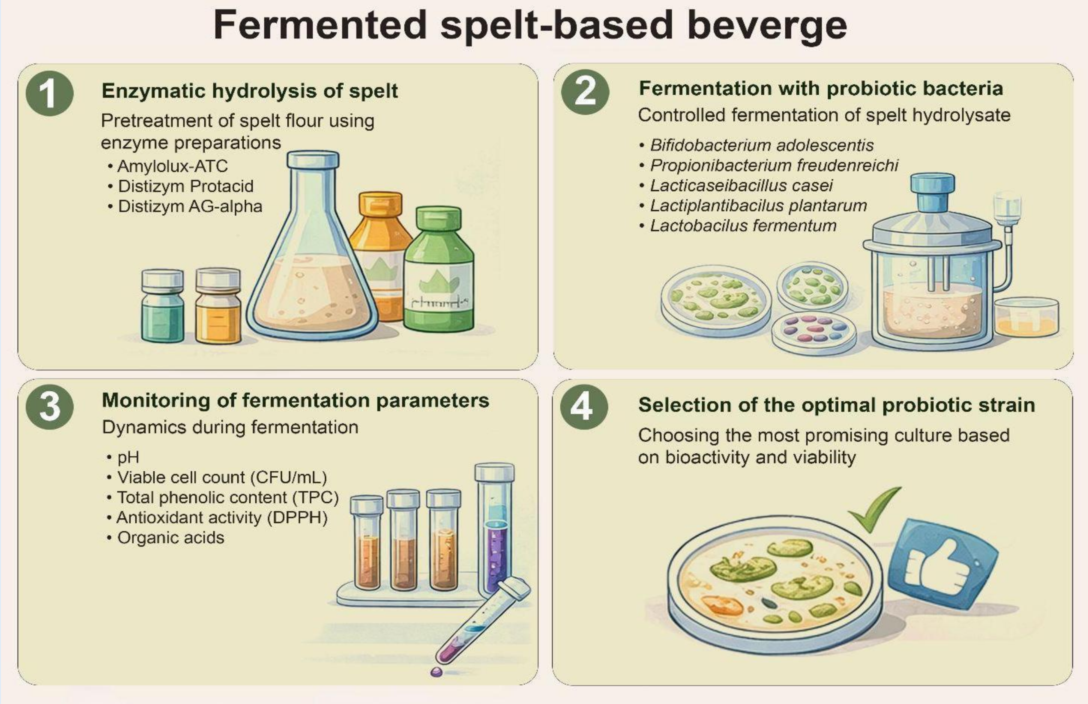
Methods: The spelt substrate was obtained by enzymatic hydrolysis of flour derived from *Triticum dicoccum* grains and subsequently fermented using the probiotic strains *B. adolescentis* AC1531, *P. freudenreichii* B9654, *L. casei* B32, *L. plantarum* B4, and *L. fermentum* B28. The content of proteins, sugars, starch, and free amino nitrogen in the spelt substrate was determined using the Kjeldahl method, high-performance liquid chromatography (HPLC), polarimetry, and spectrophotometry. The number of probiotic bacteria was monitored by plating on MRS agar and corn–lactose medium. Total phenolic content was determined using the Folin–Ciocalteu method, while organic acids were analyzed by HPLC. Antioxidant activity was evaluated by measuring the ability of the samples to scavenge the DPPH radical; results were expressed as mg ascorbic acid equivalents (AAE) and as percentage inhibition of DPPH.

Results: The study demonstrated that, for all tested bacterial strains, the highest levels of total phenolic compounds and antioxidant activity (AOA) were observed at pH 4.5 ± 0.1. The greatest accumulation of phenolic compounds was observed during fermentation with *L. fermentum* B28 and *L. plantarum* B4 (115.5 and 104.5 mg GAE/L, respectively). The highest antioxidant activity was recorded in samples fermented with *L. fermentum* B28, *L. plantarum* B4, and *B. adolescentis* AC1531 (69.4, 67.4, and 66.1% DPPH inhibition; 122.3, 110.8, and 106.9 mg AAE/L, respectively). The most intensive accumulation of organic acids occurred during the period of pH reduction to 4.5 ± 0.1. Fermentation with *B. adolescentis* AC1531 resulted in high concentrations of acetic, citric, and succinic acids. Fermentation with *L. plantarum* B4 and *L. fermentum* B28 resulted in simultaneously high concentrations of lactic, acetic, propionic, succinic, formic, and citric acids. Changes in antioxidant activity across all samples correlated with phenolic compound accumulation and were not associated with acid production.

Conclusion: *L. plantarum* B4, *L. fermentum* B28, and *B. adolescentis* AC1531 demonstrated the highest levels of phenolic compounds, physiologically valuable organic acids, and antioxidant activity during fermentation. These strains can therefore be recommended as promising starter cultures to produce a bioactive spelt-based beverage.

Novelty of the Study: This study evaluates the bioactivity of a spelt-based beverage after fermentation with probiotic bacteria and demonstrates quantitative improvements in the levels of bioavailable phenolic compounds and physiologically valuable organic acids (propionic, acetic, succinic, and formic acids) when specific strains were used (*L. plantarum* B4, *L. fermentum* B28, and *B. adolescentis* AC 1531).

Keywords: spelt; bioactive spelt beverage; fermented cereal beverage; *Bifidobacterium adolescentis* AC1531; *Propionibacterium freudenreichii* B9654; *Lacticaseibacillus casei* B32; *Lactiplantibacillus plantarum* B4; *Limosilactobacillus fermentum* B28, phenolic compounds, antioxidant activity, short-chain fatty acids



Graphical Abstract: Effect of probiotic bacterial species on the bioactivity of a fermented spelt-based beverage

INTRODUCTION

The demand for plant-based products continues to grow worldwide. Fermented plant-based alternatives are at the center of these megatrends. Fermentation of plant substrates by probiotic bacteria enhances the functional and sensory properties of products, improves their digestibility, and extends shelf life [1-5].

Cereal raw materials represent an attractive substrate to produce alternative fermented beverages. Currently, oats and rice are most used as raw materials for the production of cereal-based alternative beverages [5-6]. Spelt is characterized by a relatively high protein content with a valuable amino acid profile and good digestibility. Its grains are rich in dietary fiber, organic acids, trace elements (iron, zinc, and manganese), vitamins E and B-group vitamins, and phenolic compounds. Owing to this valuable chemical composition, spelt grain can be considered a promising raw material to produce functional beverages [7].

However, a considerable proportion of bioactive compounds in spelt, as in other cereals, occurs in bound forms. According to previous studies [8-11], bound phenolics, such as glycosides and esterified derivatives, constitute the dominant fraction in cereals. These bound phenolic compounds are esterified to lignin or linked through ester bonds with arabinoxylans in cereal cell walls and interact with carbohydrates and proteins. As a result, they exhibit low bioavailability but can be released through enzymatic hydrolysis [9-11].

Probiotic bacteria possess glycosidases and esterases capable of hydrolyzing bound phenolics and releasing phenolic aglycones [12-15]. Some microorganisms can also biotransform released phenolic compounds into new phenolic derivatives [12-15]. In addition to lactic acid, probiotic microorganisms can produce other organic acids, including acetic, propionic, and succinic acids. Therefore, fermentation of a spelt-based substrate by probiotic bacteria may lead to increased concentrations of bioactive phenolic compounds and organic acids, resulting in enhanced antioxidant activity of the beverage [7,16].

Probiotic bacteria of various species possess unique enzymatic systems and therefore differ in their ability to release and modify phenolic compounds, as well as in the composition of the organic acids they synthesize. Therefore, the aim of the present study was to investigate the ability of probiotic bacteria *Bifidobacterium adolescentis*, *Propionibacterium freudenreichii*, *Lacticaseibacillus casei*, *Lactiplantibacillus plantarum*, and *Limosilactobacillus fermentum* to accumulate bioavailable phenolic compounds and organic acids during fermentation of a spelt-based substrate and to enhance the antioxidant activity of the resulting beverage.

MATERIAL AND METHODS

The probiotic cultures *Bifidobacterium adolescentis* AC1531 (*B. adolescentis*) and *Propionibacterium freudenreichii* B9654 (*P. freudenreichii*) were obtained from the All-Russian Collection of Industrial Microorganisms. *Lacticaseibacillus casei* B32 (*L. casei*), *Lactiplantibacillus plantarum* B4 (*L. plantarum*), and *Limosilactobacillus fermentum* B28 (*L. fermentum*) were obtained from the microbial culture collection of the St. Petersburg Branch of the Research Institute of the Baking Industry. These strains were used for fermentation of the spelt-based substrate. The cultures were inoculated into the sterile spelt substrate at a level of 5% (v/v). The initial cell concentration was approximately 6 lg CFU/cm³. Fermentation was carried out at 37 ± 1 °C for *B. adolescentis* and 30 ± 1 °C for the other bacterial species.

Preparation of the Spelt-based Substrate: The spelt-based substrate was prepared by enzymatic hydrolysis of flour (particle size modulus 1.16) obtained from *Triticum dicoccum* grain (cv. Runo). Flour and water were mixed at a 1:5 ratio for hydrolysis. The following enzyme preparations were used: α-amylase (AmiloLux-ATS, Sibbiopharm, Russia), protease (Distizym Protacid, Erbslöh, Germany), and glucoamylase (Distizym AG-alpha, Erbslöh, Germany). The enzymes were added at the following dosages: 4 U/g starch, 0.2 U/g flour, and 2 U/g starch, respectively. Hydrolysis with α-amylase and protease was performed at 50 °C for 150 min, followed by hydrolysis with glucoamylase at 60 °C for 30 min. The hydrolysis parameters were selected based on previous studies [17]. The resulting spelt substrate contained 2.6 % protein, 0.5 % fat, 85.48 mg/100 g free amino nitrogen, 23.66 g/dm³ maltose, and 9.90 g/dm³ glucose.

Bacterial Counts: Bacterial counts were determined by plating serial dilutions of the fermented spelt substrate on solid culture media. MRS agar was used for lactic acid bacteria, whereas corn–lactose medium (NPC Biocompass LLC, Uglich, Russia) was used for bifidobacteria and propionic acid bacteria. The plates were incubated in an aerostat (model AE-01, NIKI MLT LLC, St. Petersburg, Russia) for 72 h. After incubation, colonies were counted and expressed as colony-forming units (CFU).

The Specific Growth Rate: The specific growth rate of the bacteria was calculated using the following equation:

$$\mu = lnX - lnX_0 / \tau - \tau_0,$$

where X_0 is the number of CFU/mL at time τ_0, and X is the number of CFU/mL at time τ. Time τ_0 corresponded to 3 h after inoculation of the cultures into the spelt substrate, whereas τ corresponded to 6 h after inoculation.

Changes in pH During Fermentation: Changes in pH during fermentation were monitored hourly using a pH meter Expert-001 (Ekoniks-Expert Ltd., Moscow, Russia).

Total Phenolic Content (TPC): TPC was determined using the Folin–Ciocalteu method on a PE-5400UV spectrophotometer (ECROS LLC, St. Petersburg, Russia) at a wavelength of 765 nm. Gallic acid (Acros Organics, Brussels, Belgium) was used as the standard. Calibration solutions with concentrations ranging from 10 to 100 mg/L were prepared. To each solution, Folin–Ciocalteu reagent (LenReaktiv JSC, St. Petersburg, Russia), distilled water, and sodium carbonate were added. The reaction mixtures were incubated in the dark for 30 min, after which absorbance was measured, and a calibration curve was constructed. Samples of the fermenting spelt substrate were centrifuged for 2 min at 3000 g using an ELMI CM-50 centrifuge (model M.5830, Riga, Latvia). The clear supernatant was analyzed using the same procedure as the gallic acid standards. The results were expressed as mg gallic acid equivalents (GAE) per liter of sample.

Organic Acids: Organic acids were analyzed using high-performance liquid chromatography (HPLC) on a Shimadzu Prominence system (Shimadzu Europa GmbH, Duisburg, Germany) equipped with a UV detector set at 210 nm. Chromatographic separation was performed on a Hitachi 2614 column packed with ion-exchange resin at a temperature of 55 °C. The mobile phase consisted of 10 mM perchloric acid, delivered at a flow rate of 0.8 mL/min.

Antioxidant Activity (AOA): AOA was determined using the DPPH radical scavenging assay on a PE-5400UV spectrophotometer (ECROS LLC, St. Petersburg, Russia) at a wavelength of 517 nm. The results were expressed as percentage inhibition of DPPH radicals (%) and as mg ascorbic acid equivalents (AAE) per liter of sample. The analyzed samples were centrifuged for 2 min at 3000 g using a Centrifuge ELMI CM-50 (model M.5830, Riga, Latvia). The DPPH reagent (Himedia, Mumbai, India) was dissolved in 100 mL of ethanol to obtain a solution with an absorbance of 0.70–0.80. A mixture consisting of 1 mL of clear supernatant and 2 mL of DPPH solution was incubated in the dark for 30 min, after which the absorbance was measured.

The percentage of DPPH inhibition was calculated using the following equation:

$$\text{DPPH \% inhibition} = (1 - A_0 / A_1) *100,$$

where A_0 is the absorbance of the DPPH solution with the sample, and A_1 is the absorbance of the DPPH solution without the sample (control).

To determine antioxidant activity expressed as ascorbic acid equivalents, a calibration curve was constructed using ascorbic acid (Component-Reaktiv, Moscow, Russia). A stock solution was prepared by dissolving 1 g of ascorbic acid in 100 mL of distilled water. Aliquots of 1, 2, 4, 6, 8, and 10 mL of the stock solution were transferred into test tubes, and 2 mL of DPPH solution in ethanol was added to each tube. A seventh tube containing only 2 mL of DPPH solution served as the control. The mixtures were incubated in the dark for 30 min, after which absorbance was measured. A calibration curve was constructed in MS Excel 2010 by plotting the concentration of ascorbic acid (mg/L) against the absorbance values of the reaction mixtures. The resulting regression equation was $y = 727.56x + 3.1093$ ($R^2 = 0.9947$). Antioxidant activity expressed as AAE was calculated using this equation.

Statistical Analysis: Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, San Diego, USA). Differences between samples were evaluated using the Bonferroni (Dunn) t-test at a significance level of $P < 0.05$. All experiments were performed in triplicate (n = 3), and the results were expressed as mean ± standard deviation.

RESULT AND DISCUSSION

Cell Growth and pH Changes: High bacterial growth rates and acidification activity reduce fermentation time and accelerate bioactive compound accumulation, while also decreasing the risk of microbial contamination [18-20]. The accumulation of viable cells (VCC) during fermentation of the spelt-based substrate by the studied bacteria is presented in Fig. 1, while the changes in pH are shown in Fig. 2.

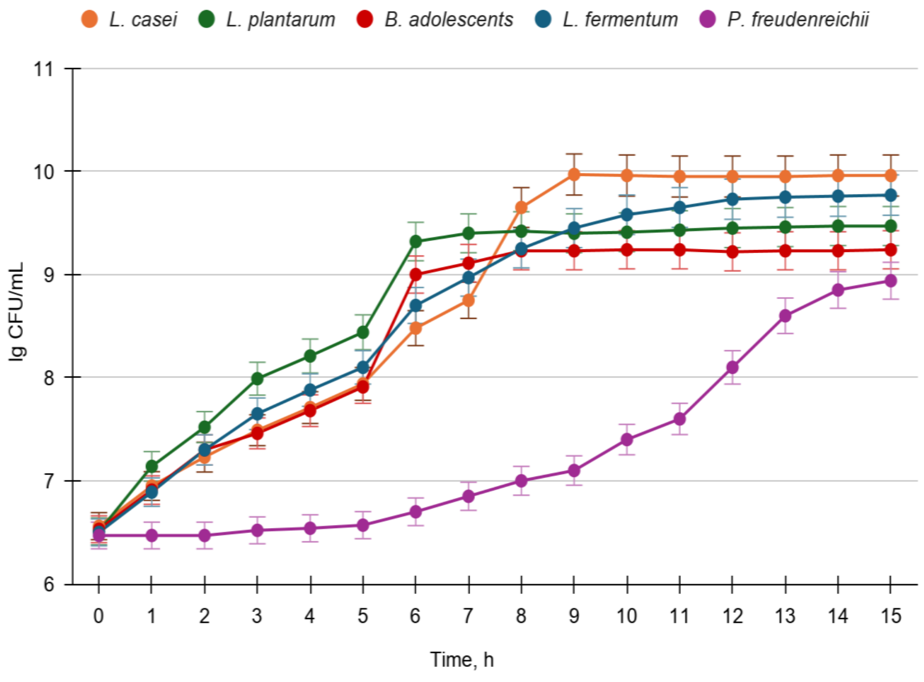


Figure 1. Growth curves of bacterial cells in the spelt-based substrate.

The highest VCC was observed for *L. casei*, reaching 9.97 ± 0.12 lg CFU/mL after 9 h of fermentation. *P. freudenreichii* exhibited the slowest growth in the spelt substrate, with VCC reaching 8.71 ± 0.10 lg CFU/mL after 15 h of fermentation. For *L. fermentum*, the maximum VCC reached 9.73 ± 0.13 lg CFU/mL after 12 h, while *L. plantarum* achieved 9.42 ± 0.11 lg CFU/mL after 7 h, and *B. adolescentis* reached 9.23 ± 0.12 lg CFU/mL after 8 h. Notably, *L.plantarum* and *B.adolescentis* reached the VCC level of 9 lg CFU/mL, which is typically required for starter cultures, within 6 h of fermentation, faster than the other studied strains. The specific growth rates in the spelt-based beverage substrate were 1.182 for *B. adolescentis*, 1.021 for *L. plantarum*, 0.805 for *L. fermentum*, 0.760 for *L. casei*, and 0.138 for *P. freudenreichii*

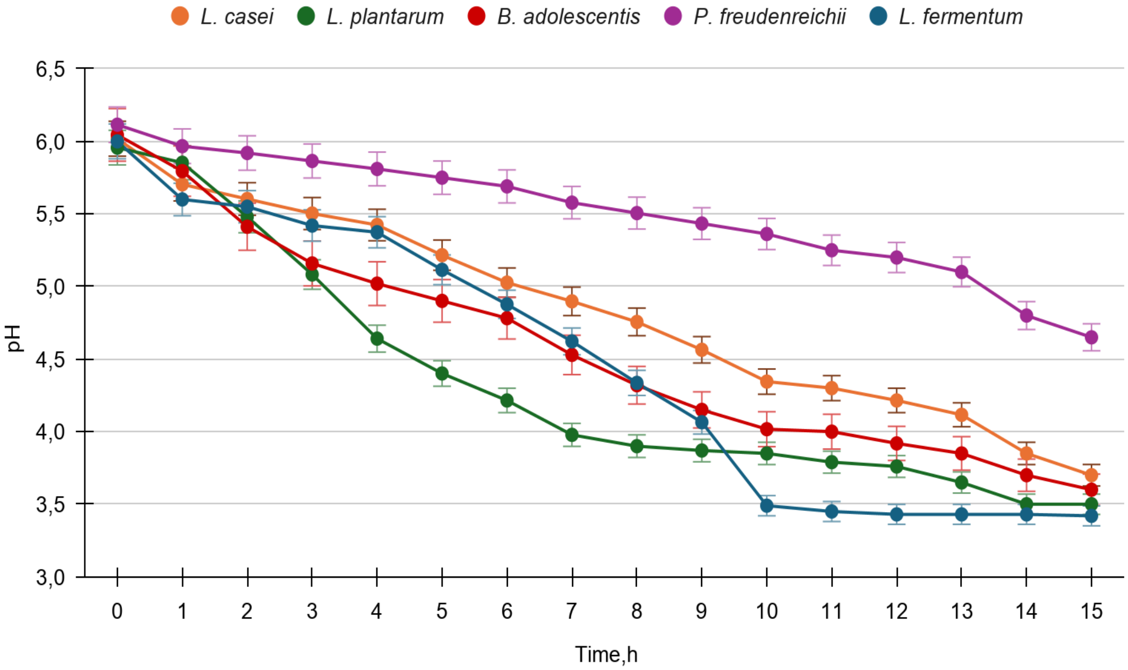


Figure 2. Changes in pH during fermentation of the spelt-based substrate.

During 15 h of fermentation, the pH of the spelt substrate decreased from 5.96–6.12 to 3.42–4.65. This decrease can be explained by the accumulation of organic acids in the substrate. Rapid pH reduction decreases the risk of undesirable microbial contamination and shortens fermentation time [18,20]. The highest acidification activity was observed for *L. plantarum*, whereas the lowest was recorded for *P. freudenreichii*. The preferred pH for fermented beverages is approximately 4.5 ± 0.1 [19,21]. This value was reached after 5 h in samples fermented with *L. plantarum*, and after approximately 7 h in samples with *B. adolescentis* and *L. fermentum*. In samples fermented with *L. casei* , pH 4.5 ± 0.1 was reached after 9 h. In contrast, *P. freudenreichii* reduced the pH only to 4.65 after 15 h of fermentation, failing to reach the desired level.

Accumulation of Phenolic Compounds: Fermentation of the spelt-based beverage substrate with all studied bacterial strains resulted in a significant increase in TPC. The phenolic concentration increased from 65.4 mg GAE/L in the non-fermented substrate to 90.9–115.5 mg GAE/L, corresponding to an increase of approximately 39–76 %. Changes in TPC during fermentation of the spelt substrate by probiotic bacteria are presented in Fig. 3.

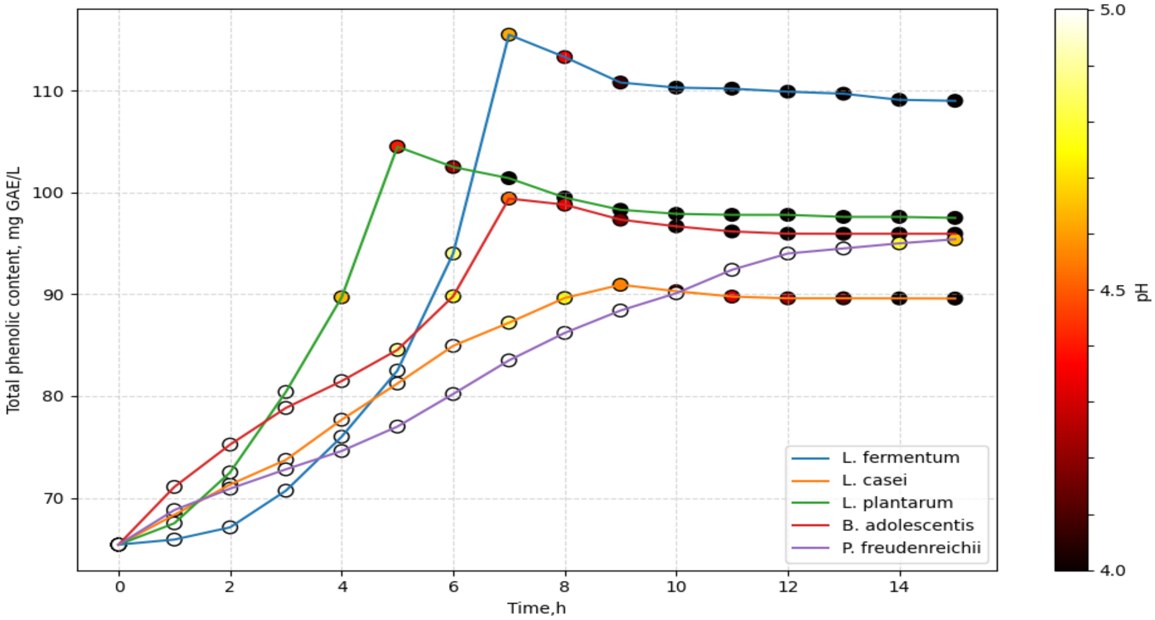


Figure 3. Accumulation of phenolic compounds during fermentation of the spelt-based substrate.

The most pronounced increase in TPC was observed during the first 5–9 h of fermentation, which likely corresponds to the period of active accumulation of viable cells (VCC). After reaching the maximum level, TPC slightly decreased and subsequently stabilized, forming a plateau. Similar patterns were obtained during the fermentation of plant substrates [22-23]. The increase in TPC may be due to the hydrolysis of glycosides in the raw material, the release of phenols bound to the grain cell walls, and the depolymerization of complex polyphenols by bacterial enzymes. The subsequent decrease in TPC results from partial degradation and bacterial transformation. The subsequent decrease in TPC is explained by partial degradation or biotransformation of phenolic compounds by microbial metabolism [24-28].

Our results showed that the highest phenolic concentrations were observed in samples fermented with *Limosilactobacillus fermentum* after 7 h and with *Lactiplantibacillus plantarum* after 5 h of fermentation, reaching 115.5 and 104.5 mg GAE/L, respectively. In samples fermented with *Bifidobacterium adolescentis*, the maximum TPC reached 99.4 mg GAE/L after 7 h, whereas in samples fermented with *Propionibacterium freudenreichii* the maximum value was 95.4 mg GAE/L after 15 h. The lowest accumulation of phenolic compounds was observed in samples fermented with *Lacticaseibacillus casei* (90.9 mg GAE/L after 9 h).

The maximum TPC values corresponded to a pH level of approximately 4.5 ± 0.1. As the pH further decreased, phenolic concentrations declined slightly by 2–6%, and after reaching pH 4.0 ± 0.1, they remained almost unchanged. This can be explained by the active secretion of bacterial enzymes during the logarithmic growth phase and differences in their optimal pH for the release and transformation of phenols.

In contrast, samples fermented with *P. freudenreichii* did not reach pH 4.5 ± 0.1 within 15 h of fermentation, and TPC continued to increase throughout the experiment. The dependence of maximum TPC values and their subsequent decline on the bacterial species used for fermentation has also been reported in previous studies [21, 23-26,28].

Accumulation of Organic Acids: The functional properties of organic acids have been widely described in previous studies [29-31]. The concentrations of organic acids in fermented samples at pH 5.0 ± 0.1, 4.5 ± 0.1, and 4.0 ± 0.1 are presented in Fig. 4.

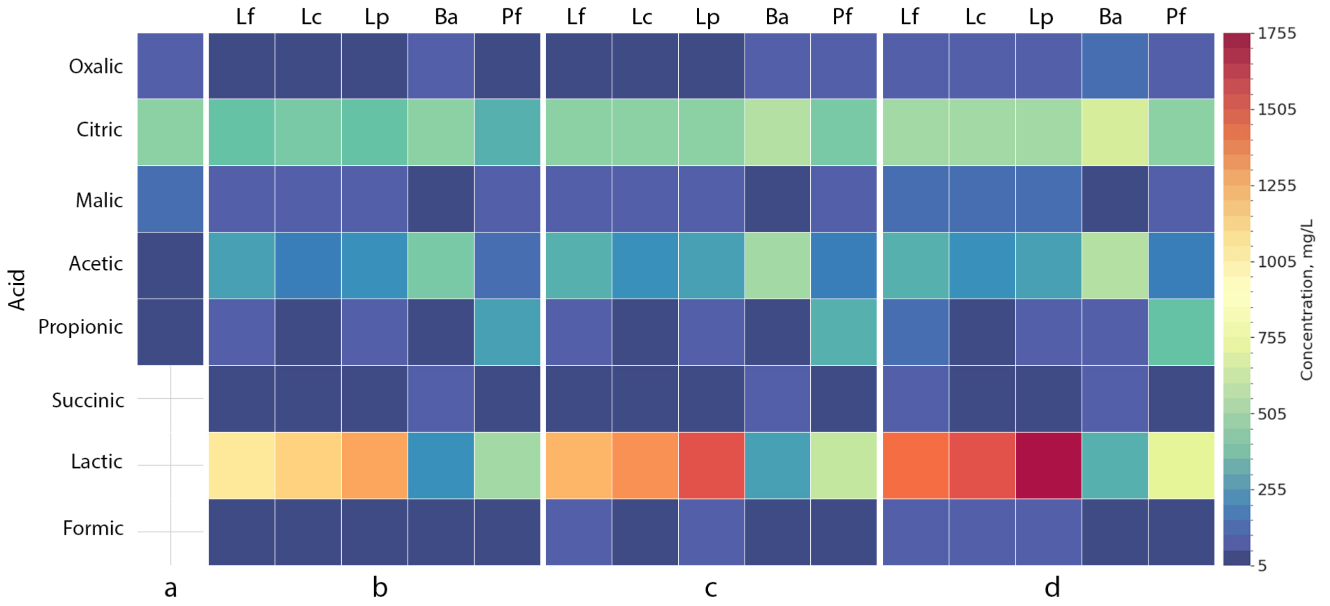


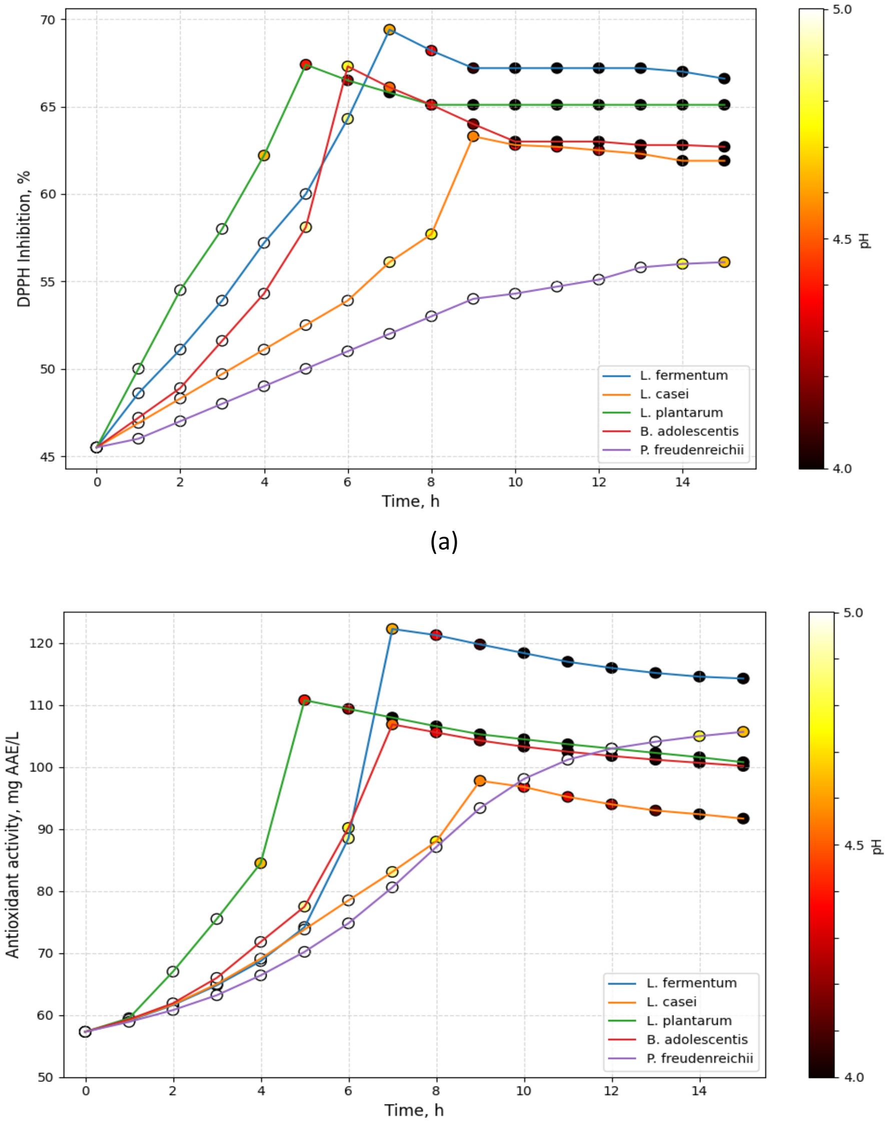
Figure 4. Heatmap of organic acids in the non-fermented spelt substrate (a) and in fermented samples at pH 5.0 ± 0.1 (b), 4.5 ± 0.1 (c), and 4.0 ± 0.1 (d). *Lf – L. fermentum, Lc – L. casei, Lp – L. plantarum, Ba – B. adolescentis, Pf – P. freudenreichii.*

The concentrations of organic acids varied with bacterial species and sample pH. In all samples, lactic, citric, acetic, malic, and propionic acids were the predominant acids, whereas oxalic, succinic, and formic acids were detected in relatively low concentrations.

During the initial fermentation stage, until the pH reached 5.0 ± 0.1, the concentrations of citric, malic, and oxalic acids decreased by approximately 20–30% compared with the non-fermented hydrolysate in samples fermented with lactic acid bacteria and *P. freudenreichii*. During the subsequent decrease in pH from 5.0 ± 0.1 to 4.5 ± 0.1, the concentrations of citric, oxalic, and malic acids increased in all samples by 17–22%, 18–22%, and 19–20%, respectively. With further acidification to pH 4.0 ± 0.1, their concentrations increased by an additional ~13%. Similar trends have been identified [31,32]. The highest concentrations of citric acid were observed in samples fermented with *B. adolescentis* (498.6, 600.7, and 678.8 mg/L) and *L. casei* (410.4, 482.3, and 545.2 mg/L), while the lowest concentrations were detected in samples fermented with *P. freudenreichii* (347.3, 405.7, and 458.4 mg/L). The highest concentrations of malic acid were detected in samples fermented with *L. casei* (85.0, 102.4, and 115.7 mg/L), whereas the lowest values were observed in samples with *B. adolescentis* (8.3, 10.0, and 11.3 mg/L).

The concentrations of acetic and propionic acids increased throughout the fermentation period. A sharp increase (10–43-fold) was observed during the initial stage of fermentation, followed by further increases of 18–22% at pH 4.5 ± 0.1 and 12–13% at pH 4.0 ± 0.1. The highest concentrations of acetic acid were detected in samples fermented with *B. adolescentis* (431.4, 520.0, and 587.6 mg/L), *L. fermentum* (259.6, 310.2, and 350.5 mg/L), and *L. plantarum* (218.3, 265.8, and 300.4 mg/L). The highest concentrations of propionic acid were observed in samples fermented with *P. freudenreichii* (268.5, 320.4, and 362.1 mg/L), followed by *L. fermentum* (78.9, 93.4, and 105.5 mg/L) and *L. plantarum* (59.3, 72.4, and 81.8 mg/L) at pH 5.0 ± 0.1, 4.5 ± 0.1, and 4.0 ± 0.1, respectively. Lactic, succinic, and formic acids were formed during fermentation. Lactic acid was dominant. The highest concentrations were detected in samples fermented with *L. plantarum* (1301.4, 1550.9, and 1725.5 mg/L) and *L. casei* (1138.2, 1350.4, and 1526.0 mg/L), while the lowest concentrations were observed in samples fermented with *B. adolescentis* (237.5, 285.6, and 322.7 mg/L). The highest succinic acid concentration was found in samples fermented with *B. adolescentis* (58.9, 72.1, and 81.5 mg/L), whereas the highest formic acid concentrations were observed in samples fermented with *L. fermentum* (48.7, 59.7, and 67.5 mg/L) and *L. plantarum* (46.5, 56.4, and 63.7 mg/L). These differences can be explained by the specific metabolic pathways characteristic of each bacterial species [30,32,33].

Changes in AOA: Fermentation of the spelt-based beverage by all tested bacterial strains resulted in a significant increase in antioxidant activity (AOA). Compared with the non-fermented substrate, DPPH radical inhibition increased from 45.5% to 53.9–69.4% (an 18–52% increase), and AOA in terms of ascorbic acid equivalents (AAE) rose from 57.3 mg AAE/L to 97.8–122.3 mg AAE/L (a 70–113% increase). The changes in AOA during fermentation are presented in Fig. 5.



(a)

(b)

Figure 5. Changes in antioxidant activity during spelt-based beverage fermentation. (a) DPPH % inhibition; (b) mg AAE/L.

AOA increased until the pH reached 4.5 ± 0.1; during further acidification to pH 4.0 ± 0.1, it decreased slightly by 2–5% and then remained relatively stable. The highest AOA was observed in samples fermented with *L. fermentum*, *L. plantarum*, and *B. adolescenti*. At pH 4.5 ± 0.1, DPPH radical inhibition was 69.4%, 67.4%, and

66.1%, and AOA reached 122.3, 110.8, and 106.9 mg AAE/L in these samples, respectively. The lowest AOA was observed in *P. freudenreichii*, which increased gradually throughout fermentation, reaching 56.1% DPPH inhibition and 105.7 mg AAE/L after 15 h. The trends in AOA closely mirrored those of TPC, consistent with previous reports indicating that phenolic compounds are the primary contributors to the antioxidant potential of fermented plant substrates [10,11,16,25,26].

CONCLUSION

The results demonstrate that fermentation of a spelt-based beverage with *B. adolescentis*, *P. freudenreichii*, *L. casei*, *L. plantarum*, and *L. fermentum* promotes the accumulation of phenolic compounds, organic acids (including short-chain fatty acids), and enhances antioxidant activity. Both TPC and AOA reached their maximum at pH 4.5 ± 0.1, although the specific bacterial strain significantly affected the bioactivity of the fermented substrate. The highest TPC was observed in samples fermented with *L. fermentum* and *L. plantarum*. *B. adolescentis* promoted elevated concentrations of acetic, citric, and succinic acids, while fermentation with *L. plantarum* and *L. fermentum* resulted in simultaneously high levels of lactic, acetic, propionic, succinic, formic, and citric acids. The highest antioxidant activity was achieved with *L. fermentum*, *L. plantarum*, and *B. adolescentis*. These findings suggest that these strains are promising starter cultures for producing a functionally bioactive spelt-based beverage. Future studies will investigate the combined effect of *L. fermentum*, *L. plantarum*, and *B. adolescentis* on the functional properties, quality, and safety of the fermented spelt beverage.

Abbreviations: AAE: Ascorbic Acid Equivalent, AOA: Antioxidant Activity, CFU: Colony-Forming Units, DPPH: 2,2-diphenyl-1-picrylhydrazyl, GAE: Gallic Acid Equivalent, HPLC: High-Performance Liquid Chromatography, TPC: Total Phenolic Content, VCC: Viable Cell Count

Author's Contributions: All authors contributed to this study.

Conflict of Interest: The authors declare no competing interests.

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