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Enhancing nutrient profile, safety, and sustainability with fermentation technology

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Enhancing nutrient profile, safety, and sustainability with fermentation technology

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Editorial: Enhancing nutrient profile, safety, and sustainability with fermentation technology

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Editorial on the Research Topic
Enhancing nutrient profile, safety, and sustainability with fermentation technology

Introduction

The global food system faces unprecedented challenges to meet the nutritional requirements of an ever changing world and growing population, coupled with the need to ensure sustainability and food security. The ancient process of fermentation and subsequent fermented foods have provided vital staples for mankind throughout history. It has evolved over the years with advanced technologies and broader substrate sources. By definition, it is a metabolic driven and transformative process which through microbial activities and enzymatic actions enhances the bioavailability, digestibility, nutritional values, safety and organoleptic properties of foods. Further to this are associated reduction and/or elimination of antinutritive constituents and toxic compounds in food.

This age-long process is experiencing a renaissance through modern scientific understanding and technological advances, offering solutions for enhancing nutrient composition, improving food safety and providing sustainable food sources. This Research Topic on "*Enhancing nutrient profile, safety, and sustainability with fermentation technology*" brings together nine innovative studies that explore different applications of fermentation across a diverse range of food products.

Nutrient profiles

Fermentation improves the nutritional profile of foods by transforming compounds into bioavailable nutrients and synthesizing bioactive compounds. The study by Zhao et al. used multiple starter culture strains for the fermentation of tomato juice. They reported changes in metabolites such as amino acids, carbohydrates, organic acids, and phospholipids, which led to greater nutritional values and antioxidant capacities. Similarly, the study by He et al. used *Pleurotus ostreatus* mycelium in solid state fermentation of soybeans and reported an increase in protein, lipid, and phenolic content and a reduction in dietary fiber and starch. The study by Maleke et al. showed that fermentation improved the acidity, fiber content, and antioxidant properties of sorghum and mopane worm flour, while also improving their water and oil holding capacities and dispersibility. These findings are consistent with other research reporting that fermentation improves functional properties in underutilized grains (1).

Reducing anti-nutritional factors is another key benefit of fermentation. The study by Nsabimana et al. combined fermentation with soaking and germination of maize and reported a significant reduction in phytate content. As a result, there was a significant improvement in iron and zinc bioavailability, reflecting the greater potential of fermentation to improve mineral bioavailability while reducing anti-nutritional factors (1). Similarly, Jia et al. developed a novel low-salt fermentation kohlrabi method, which improves its palatability and nutritional profile while minimizing risks of sodium intake.

Innovative techniques further highlight the potential of fermentation. The study by Kurchenko et al. used oligochitosans, low-molecular weight derivatives of chitosan, to stimulate lactic acid production and improve fatty acid profiles in fermented milk. This technique improves nutritional properties, which is consistent with the findings of other fermentation studies (2). The study by Murakami et al. used a novel double saccharification process to enrich amazake with isomaltose, a functional carbohydrate related to gut health. This supports the ability of fermentation to increase the availability of bioactive nutrients (1). The study by Amanipour et al. used fermentation along with dehulling and germination in gray pea flour. They reported an increase in protein and polyphenol content and antioxidant capacity. These findings are consistent with fermentation improving the antioxidant capacity and availability of bioactive compounds (3).

Safety

Fermentation contributes to food safety through the production of certain metabolites and bio-preservative compounds. The study of Kurchenko et al. investigated the influence of chitosan on the growth and productivity of *L. bulgaricus* in the presence of chitosan and its derivatives. They reported the beneficial role of chitosan, accelerating the synthesis of lactic acid and an increase in shelf life of milk. Findings from Murakami et al. showed the beneficial effects of saccharified amazake in the gut. Such improved gut health can prevent colonization of pathogenic bacteria and by extension enhanced resistance to foodborne pathogens. Likewise, the reported increase in antioxidant capacities and the presence of certain non-volatile metabolites in fermented soybean (using oyster mushroom) (He et al.) has potential food safety implications. Such antioxidant related compounds can help mitigate the growth of pathogenic microorganisms and prevent spoilage of food through inhibition of oxidation processes. These subsequently contribute to the overall safety of food.

While not explicitly mentioned in some of the studies, competitive exclusion of pathogenic microorganisms occurs during fermentation. Proliferation of fermenting microorganisms also leads to the reduction, degradation and sometimes total elimination of toxic components. Such a reduction in anti-nutrients was demonstrated in the study of Maleke et al. which might have extended to potential toxic constituents in the substrates used.

Sustainability

Fermentation is a potential solution to reduce food waste and increase sustainability. In this Research Topic, He et al. investigated antioxidant capacities and non-volatile metabolites changes after solid-state fermentation of soybean using oyster mushroom (*Pleurotus ostreatus*) mycelium. This study explored the usage of *P. ostreatus* mycelium to enhance the quality of soybean products and discussed the potential strategies to improve soybean properties for creating meat analogs. Nsabimana et al. studied the enhancement of iron and zinc bioavailability in maize by fermentation with or without soaking and germination, which can reduce micronutrient deficiencies in developing regions.

Some agricultural products are overlooked, such as gray peas and insect protein. Amanipour et al. investigated the dehulling, germination, and fermentation of the bioactive and functional properties of gray pea flour. The results could encourage industries and farmers to increase gray pea production and processing. Maleke et al. studied the effect of fermentation, malting, and ultrasonication on sorghum, mopane worm, and *Moringa oleifera*. The results indicated that ultrasonication was more effective in improving the nutritional value of the samples, followed by fermentation. The blend of bioprocessed flours with various nutritional and health properties can help address the challenges of malnutrition.

Meanwhile, innovative fermented technology can also improve human health, such as decreasing salt intake. Jia et al. investigated the non-volatile metabolites in a low-temperature and low-salt fermented Chinese kohlrabi (LSCK). Future research in optimizing the LSCK processing could decrease the salt contents in this traditional Chinese food as well as decrease water usage in traditional Chinese Hohlrabi manufacturing as desalting uses a high amount of water.

Conclusions

The studies in this Research Topic have highlighted the versatility of fermentation in enhancing nutrient profile, safety and other possibilities for better and sustainable food systems. The diverse studies presented also open new pathways for innovation in food production systems, ultimately working toward a more sustainable and nutritious food supply for future generations. As we confront global challenges of food security, malnutrition, and environmental sustainability, there is still a need for more studies to exploring novel microbial strains, investigating the scalability of these findings and others, as well as developing sustainable integration strategies to meet the needs of an ever-growing population.

Author contributions

OA: Writing – original draft, Writing – review & editing. JG: Writing – original draft, Writing – review

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Optimization of tomato (*Solanum lycopersicum* L.) juice fermentation process and analysis of its metabolites during fermentation

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Tomato (*Solanum lycopersicum* L.) is a nutritious fruit and vegetable. Fermentation can be used to enhance their nutritional value. In this study, the tomato juice was co-fermented with multistrains, optimized by uniform experimental design and response surface methodology. Superoxide dismutase activity reached 496.67 U/g and lycopene content reached 77.12μg/g when *P. pentosaceus* (53.79%), *L. casei* (13.17%), *L. plantarum* (19.87%), *L. fermentum* (13.17%). To gain insight into the dynamics of metabolites during the tomato fermentation juice process multivariate statistical analysis was performed using the UHPLC-QE-MS/MS method. The main metabolites are peptides, amino acids carbohydrates, organic acids, and phospholipids. Carbohydrates were fully retained at the end of fermentation.The content of galactitol increased from the initial 5.389 to 6.607 while the content of cytarabine decreased by 29% and uridine by 44%. Meanwhile, phospholipids (PS, PE, PC, PG, PI) were all retained by more than 70%. Terpenoids (16-deacetylgairin, (+)-Royleanone, artemisinin) were increased to varying degrees, which gives them good nutritional value and biological activity. Organic acids (malic and citric) were reduced and lactic acid content was increased, changing its original flavor and making it more palatable to the general population. The research results have demonstrated the benefits of lactic acid bacteria fermentation on tomato juice, providing a theoretical basis and reference for the fermentation metabolism process of tomato juice.

KEYWORDS

fermentation, tomato juice, superoxide dismutase, response surface methodology, UHPLC-QE-MS/MS

1 Introduction

Tomato (*Solanum lycopersicum* L.) is a colorful, sweet and sour, nutritious fruit vegetable that is produced in large quantities globally, which is grown in almost all countries, and more than 80% are consumed as processed products (1). The global tomato production exceeded 186 million tons in

2020.[1] Tomatoes contain a variety of bioactive substances, such as polyphenols, carotenoids, and vitamins, which have a positive impact on human health and well-being owing to their antioxidant, hypolipidemic, and anticancer properties (2). Recent studies have indicated that a quarter of the global tomato production is used for processing tomato products like sauces and ketchup, which are popular among consumers (3). The primary bioactive components in tomato products are carotenoids, particularly lycopene (LYC), which has been extensively utilized in the food industry as a functional ingredient in food supplements or new food products (4). Moreover, LYC has been shown to significantly contribute to cancer prevention and the management of chronic diseases in various pathological studies (5, 6). Although tomato products have been demonstrated to contain health-promoting nutrients and micronutrients, the research on tomato products mostly focuses on tomato sauces and ketchup, while there are few reports on deep-processing products derived from tomatoes (7).

Fermentation is a process that utilizes the growth and metabolic activities of microorganisms to stabilize and transform biomass. Beneficial metabolites, including extracellular polysaccharides, bioactive peptides, and lactic acid, can be generated by microorganisms in food matrices throughout the process of fermentation (8). Besides, fermentation techniques can effectively release bound bioactive compounds. Lactic acid bacteria (LAB) are widely used to ferment vegetables, dairy products, meats, and grains with bioactivities and a variety of health-promoting effects, including immunomodulatory, antiallergic, antio-besity, and antioxidant effects (9–11). The fermentation of fruit and vegetable juices by LAB not only preserves the nutrients and flavor of the juices but also generates bioactive substances through microbial metabolism. Compared to other strains, *Lactobacillus paracasei* fermentation of fermented orange juice was found to produce better flavor and sensory attributes as well as increased antioxidant activity (12). Furthermore, the bioactive compounds significantly increased in LAB-fermented Chinese wolfberry juice, particularly when using *Lactobacillus paracasei* and *Lactobacillus acidophilus* (13). Among LAB- fermented tomato juice (FTJ), *Lactobacillus plantarum* and *Lactobacillus casei* were found to be more suitable for developing FTJ with favorable flavor and health benefits (14). To address the challenges of tomato storage and diversify tomato products, this study aimed to develop a high-nutritional-value FTJ product.

In recent years, metabolomics has been applied to elucidate metabolite changes and biotransformation during food processes (15). UHPLC-QE-MS/MS(LC–MS) was widely used in many fields (e.g., medical research, environmental analysis, and food analysis) because of its high separation ability for complicated samples, relatively short and high sensitivity, and its ability to obtain rich structural information and molecular weight of each component. Food samples typically contain many small molecular weight metabolites that span a wide dynamic range of concentration and polarities (16). Therefore, LC–MS was chosen for its high sensitivity and resolution to analyze metabolites during the fermentation of tomato juice (17).

Currently, there is a paucity of information on the FTJ with LAB to enhance its bioactive function and its use of LC–MS for the analysis of non-volatile metabolites during FTJ. To the best of our knowledge, mixed LAB fermentation can produce a higher abundance of metabolites than single- strain LAB fermentation (18). To maximize FTJ's mixed bacteria fermentation mechanism, it is essential to choose appropriate dominant strains that promote it, as each strain possesses unique traits and substrate adaptations. Therefore, this study was conducted to analyze the bioactive functions of LAB- FTJ and to examine the non-volatile metabolites during fermentation.

The objectives of this study were (1) Firstly, four strains with high Superoxide dismutase (SOD) activity and high LYC content were screened by a one-way test. The process was optimized by using homogeneous setup experiments and response surface methodology (RSM) to maximize SOD activity and LYC content, to improve bioaccessibility. (2) Samples were collected under different fermentation times (0, 10, 16, 20, and 22 h). Changes in chemical composition over time were traced by LC–MS analysis. The dynamic changes of carbohydrates (galactitol, aglycone, uridine), phospholipids (PS, PE, PC, PG, PI), organic acids (malic, citric, lactic), and terpenoids ((+)-Royleanone, 16-deacetylgalloylgalloylene, and artemisinin) were analyzed throughout the fermentation process. Overall, this study provides valuable new information on the benefits of fermentation in enhancing the potential nutritional value of tomatoes and other products.

2 Materials and methods

2.1 Materials

Tomato Sauce were provided by Xinjiang University (Xinjiang, China). Methanol, toluene, from Tianjin Shengao Chemical Reagent Co., Ltd. (Tianjin, China). MRS from Hangzhou Best Biotechnology Co, Ltd. (Hangzhou, China). *Lactobacillus fermentum* (*L. fermentum*) CICC 21800, *Lactobacillus acidophilus* (*L. acidophilus*) CICC 6085, *Lactobacillus reuteri* (*L. reuteri*) CICC 6126, *Lactobacillus plantarum* (*L. plantarum*) CICC 21797, *Lactobacillus paracasei* (*L. paracasei*) CICC 22709, *Lactobacillus casei* (*L. casei*) CICC 6114, *Lactobacillus delbrueckii subsp bulgaricus* (*L. bulgaricus*) CICC 20247, *Pediococcus pentosaceus* (*P. pentosaceus*) CICC 21862, *Pediococcus acidilacriti* (*P. acidilacriti*) CICC 20720, *Lactobacillus rhamnosus* (*L. rhamnosus*) CICC 6135 were obtained from the China Center of Industrial Culture Collection (CICC) (Beijing, China). Pectinase, cellulase, and hemicellulase were purchased from Fibrochem Biochemicals Co., Ltd. (Shanghai, China). All strains were activated twice and incubated at 37°C for 24 h before use. Methanol and acetonitrile were supplied by Meguiar's. The SOD kit was purchased from Beijing Solebo Technology Co., Ltd. (Beijing, China).

2.2 Preparation and fermentation of tomato juice

First, tomato paste was mixed well with water 1:2.6. The Total Soluble Solid (TSS) was adjusted to 12.5°Brix, Then, sterilized in water at 90°C for 10 min and cooled down subsequently. Add 1.5% (w/v) pectinase, 1% (w/v) cellulase, and 1% (w/v) hemicellulase in the

1 FAO, 2020, https://www.fao.org/faostat/en/-data/QCL, accessed date 25 April 2022.

sample and enzymatically digest for 3 h at 57°C. Following, the samples were sterilized at 90°C for 30 min. After cooling, the strains were accessed into samples and placed under a Constant temperature oscillation incubator (ZD-85A, Changzhou, China, Setting 37°C) for 22 h of fermentation.

2.3 Screening of dominant strains and determination of their optimal proportions

Ten strains (*L. fermentum, L. acidophilus, L. reuteri, L. plantarum, L. paracasei, L. casei, L. bulgaricus, P. pentosaceus, P. acidilacriti, L. rhamnosus*) of fermented tomato homogenate were used and fermented in an incubator at 37°C for 22 h. After fermentation, the best four dominant strains were selected based on pH, SOD, LYC, TSS and sensory scores. The optimal ratio of these 4 strains were determined by four factors, a10-level, uniform design table. The results were analyzed and calculated through SPSS 26 and Excel (Table 1).

2.4 Optimization of the fermentation process

After determining the fermentation strain category and ratio, the SOD activity value and LYC value obtained after fermentation were used as indicators. The one-way experimental conditions were set as follows Soluble solids content (10.5, 11.5, 12.5, 13.5, 14.5°Brix), Inoculum volume (1.2.3.4.5*10^6 CFU/mL), Fermentation temperature (27.32.37.42.47°C), Fermentation time (14.18.22.26.30 h), with the above four factors as the independent variables, and SOD (Y1) and LYC (Y2) as the response values, to design a Box–Behnken test. Each factor has 3 levels (−1, 0, 1) and the design includes 5 centroids for a total of 29 sets of experiments. The factors and levels of Box–Behnken are tabulated in Table 2. Response surface results are shown in Table 3.

2.5 Measurement of physicochemical indicators

The activity of SOD was determined according to the instructions of the kit. LYC was determined using the national standard method. The soluble solid solids content was determined using a digital refractometer (TD-45, Zhejiang, China). pH was determined by a digital desktop acidimeter (PHS-3C, Shanghai, China). The sensory evaluation was performed on the fermented samples. Samples of the fermentation broth produced under different conditions were numbered, and then 10 people trained in the sensory evaluation were asked to rate the fermentation broth product's sensory, flavor, acceptability, and hypothetical purchase intent for a total score of 90. The details were shown in Table 4.

2.6 Metabolites extraction and LC–MS analysis

Each sample (200 μL) was mixed with 800 μL of extraction solution (methanol: acetonitrile = 1:1, containing an isotope-labeled internal standard mixture) in an EP tube. The samples were then vortexed for 30 s, extracted by low-temperature ultrasound for 30 min (5°C, 40 KHz), and allowed to stand for 30 min at −20°C. The samples were then centrifuged again for 15 min (13,000 g, 4°C), and the supernatant was dried with nitrogen, followed by the addition of 120 μL of the compound solution (acetonitrile: water = 1:1). The sample was vortexed again for 30s, extracted by low-temperature ultrasonic extraction for 5 min (5°C, 40 KHz), centrifuged for 10 min (13,000 g, 4°C), and the supernatant was put into vials to be analyzed on the machine. In addition, 20 μL of supernatant from each sample was mixed as quality control (QC).

Metabolite detection was performed on an ACQUITY UPLC HSST3 column (100 mm × 2.1 mm i.d., 1.8 μm, Waters, Milford, USA) with two mobile phases: 95% ultrapure water +5% acetonitrile (containing 0.1% formic acid) and 5% ultrapure water +47.5% acetonitrile +47.5% isopropanol +5% ultrapure water (containing 0.1% formic acid). The two mobile phases were 0.1% formic acid, the temperature of the autosampler was 40°C, and the injection volume was 3 μL. The ion source was electrospray ionization (ESI), and the ionization modes: positive (ESI+) and negative (ESI-) scanning modes were used to collect mass spectrometry signals. The scanning range was 70 ~ 1,050 m/z; the intrathecal gas flow rate was 50 Arb, the

TABLE 1 Uniform design table for the experiment.

No.	*Pediococcus pentosaceus* (%)	*Lactobacillus casei* (%)	*Lactobacillus plantarum* (%)	*Lactobacillus fermentum* (%)
1	40	13	19	21
2	41	15	24	19
3	42	17	18	17
4	43	19	23	15
5	44	21	17	13
6	45	12	22	22
7	46	14	16	20
8	47	16	21	18
9	48	18	15	16
10	49	20	20	14

auxiliary gas flow rate was 13 Arb, and the capillary temperature was 325°C. The resolution of the full mass spectrum was 60,000, and the resolution of the MS2 was 7,500. The collision energies were 20, 40, and 60 eV.

2.7 Data processing and statistical analysis

Data were expressed as mean plus or minus standard deviation and analyzed using SPSS26 software [In SPSS 26, we use the one-way

TABLE 2 Factors and levels of the experiment.

Factors	Levels		
	–1	0	1
Fermentation temperature (°C)	32	37	47
Fermentation time (h)	18	22	26
Inoculum volume (*10^6 CFU/mL)	2.0	3.0	4.0
Soluble solid content (°Brix)	11.5	12.5	13.5

TABLE 3 The experiments and results of RSM for the FTJ fermentation process.

No.	Factors				Response	
	Temperature (°C)	Time (h)	Inoculation amount (*10^6CFU/mL)	Soluble solid content (°Brix)	SOD activity (U/mL)	LYC (μg/g)
1	37	18	2	12.5	446.09	72.01
2	37	22	3	12.5	507.71	77.69
3	37	26	3	11.5	453.06	73.63
4	42	22	3	13.5	434.46	74.71
5	42	22	3	11.5	449.24	74.17
6	32	22	2	12.5	463.62	71.19
7	32	22	2	12.5	453.18	71.46
8	37	26	4	12.5	441.22	73.63
9	42	18	3	12.5	442.63	70.92
10	37	22	2	12.5	431.77	74.44
11	42	26	3	12.5	439.04	72.01
12	37	26	3	13.5	459.96	75.80
13	37	22	3	12.5	508.07	77.97
14	37	18	3	11.5	467.97	74.99
15	42	18	3	12.5	455.31	73.91
16	32	22	4	12.5	432.41	73.91
17	37	22	3	12.5	501.41	77.43
18	42	22	4	12.5	440.63	73.10
19	38	26	2	12.5	460.23	75.80
20	32	26	3	12.5	461.62	71.74
21	37	18	3	13.5	432.72	74.18
22	37	18	4	12.5	451.92	76.09
23	37	22	3	12.5	509.67	77.17
24	37	22	2	11.5	457.04	73.65
25	37	22	3	12.5	499.37	76.63
26	37	22	4	13.5	432.67	77.70
27	42	22	2	12.5	447.08	74.45
28	37	22	4	11.5	437.18	74.72
29	32	22	3	11.5	438.09	72.83

TABLE 4 Sensory attributes of the FTJ products.

Category	Attributes	Score
Appearance	Red color	0–10
	Clarity	0–10
Flavor	Tomato flavor	0–10
	Sour flavor	0–10
Taste	Acid-sugar ratio	0–10
	Tomato taste	0–10
	Astringency	0–10
Overall	Acceptability	0–10
	Purchase intention	0–10

ANOVA test to compare means. Statistical significance ($p < 0.05$; Duncan test)]. Plotting was done using Origin 2021 software. Multivariate analysis was performed using SIMCA 16.0 software. Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) were performed to reduce the dimensionality of the data by visualizing sample distribution and grouping. Subsequently, supervised Orthogonal Projection-Least Squares Discriminant and Applied Analysis (OPLS-DA) of the underlying structure was performed, and additionally, to assess the importance of the variables in the projection, the importance of the projected variables of the initial principal components (VIP) was determined in Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA). This technique allows for summarizing and determining the contribution of each variable. Metabolites with VIP > 1 and $p < 0.05$ were considered as significantly changed metabolites.

3 Results

3.1 Strain screening and ratio determination

Each strain has its characteristics during the fermentation process, so it is important to consider the characteristics of each strain when selecting the appropriate strain for tomato juice. The effect of multi-strain association on fermentation is more beneficial than single-train fermentation due to the reciprocal symbiotic interaction between microorganisms and products derived from mixed fermentation containing more metabolites (19). In this experiment, 10 strains were used for fermentation, SOD, LYC, pH, TSS, and sensory during fermentation were determined, and finally four strains were selected for mixed fermentation. The results were shown in Table 5.

SOD is an important component of the antioxidant enzyme system in biological systems and has a strong antioxidant ability. SOD is not only naturally present in fruit and vegetable juices, it is also produced during the metabolism of LAB (20). After the fermentation of tomato juice, the SOD activity increased in all groups. Compared to the original solution producing, the *L. casei, L. plantarum, L. fermentum, L. paracasei* increased the SOD by 41.44, 35.63, 49.02, and 36.85 U/g, respectively. Explanations for these differences may include strain-specific functions, differences in growth rates and environments, and differences in carbon source utilization.

Xinjiang tomato fruits contain higher levels of LYC than those from other regions (21). LYC has been shown to have beneficial effects on human health. The LYC content produced by different strains varied. *P. pentosaceus, L. fermentum, P. acidilactici,. L. plantarum* was increased by 9.7, 8.2, 6.0, and 5.8%, respectively, compared to the unfermented samples. It may be due to fermentation hydrolysis and the destruction of the cell walls of tomato cells by LAB during the fermentation process. The destruction of the cells leads to a decrease in the pectin content and reduces the size of the tissue fragments, which reduces gravitational separation and promotes the release of LYC, thereby increasing the content (22).

The proportion of flavoring substances influences affects the sensation. The decrease in pH during fermentation may be due to the production of organic acids, and the absorption of a portion of glycogen by microorganisms, which leads to a decrease in TSS and destroys the flavor of the fermented juice. The highest sensory evaluation scores were given by *L. fermentum, L. plantarum, P. pentosaceus*, and *L. paracasei*, with scores of 90.33, 89.00, 88.33, and 87.66, respectively. For these results, the differences are due to the unique properties of each strain resulting in fermented samples with different acidic flavors and different organic acid compositions that give each sample a unique flavor. Besides, the volatile compound composition of the FTJ varies from strain to strain, and the senses will be different (23).

Combined fermentation of multiple strains can make up for the defects of other single strains in fermentation. Based on the three factors of high SOD, LYC, the best four strains, *P. pentosaceus, L. casei, L. plantarum*, and *L. fermentum* were selected for mixed fermentation. The optimum percentage of each strain was determined using a homogeneous design and the results are shown in Table 6, and the equation follows:

$$Y = 267.485 + 1284.208X_1 - 288.073X_2^2 - 2436.117X_3^2 - 8973.864X_1X_4 - 6613.659X_2X_3 + 5530.703X_2X_4 + 12747.912X_3X_4$$

$R^2 = 0.999$ and $p < 0.01$, indicating that the equation is well-fitted and accurately predicts optimal conditions. In conclusion, the optimal inoculation rates of FTJ were predicted to be *P. pentosaceus* (53.79%), *L. casei* (13.17%), *L. plantarum* (19.87%), *L. fermentum* (13.17%), with a corresponding SOD activity of 525.76 U/g. Based on the optimal inoculation rate described above, the total strain inoculum of the four strains was maintained at 2×10^6 CFU/mL and the TSS was maintained at 13.5°Brix. Fermentation for 22 h at 37°C. In the validation test, SOD activity reached 486.35 U/g.

3.2 Response surface experimental results and analysis of variance

Different fermentation environments have different effects on the results and to achieve the best product, it is necessary to optimize the fermentation conditions. According to the preliminary experiments showed no significant changes in pH and TSS content. Therefore, SOD and LYC were used as dual response values for the optimization of fermentation conditions.

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TABLE 5 Physicochemical properties of FTJ fermented by ten strains.

No.	Strains	SOD activity (U/g)	LYC (μg/g)	pH	TSS	Sensory Evaluation (Score)
1	Unfermented	387.00 ± 2.00^{e}	68.75 ± 1.09^{de}	3.98 ± 0.03^{cd}	13.80 ± 0.10^{cde}	83.33 ± 1.52^{cd}
2	*P. pentosaceus*	420.47 ± 3.01^{c}	75.43 ± 1.18^{a}	4.02 ± 0.00^{ab}	14.20 ± 0.10^{ab}	88.33 ± 1.52^{ab}
3	*L. casei*	428.44 ± 1.66^{b}	72.24 ± 0.71^{bc}	4.02 ± 0.00^{ab}	14.10 ± 0.26^{ab}	85.00 ± 1.73^{bcd}
4	*L. rhamnosus*	397.71 ± 3.85^{d}	71.02 ± 1.45^{cd}	3.93 ± 0.00^{e}	13.73 ± 0.15^{e}	85.66 ± 3.05^{bcd}
5	*L. plantarum*	422.63 ± 1.64^{bc}	72.74 ± 1.49^{abc}	4.00 ± 0.01^{bcd}	14.16 ± 0.05^{ab}	89.00 ± 2.00^{ab}
6	*L. fermentum*	436.02 ± 3.41^{a}	74.41 ± 1.06^{ab}	4.01 ± 0.01^{bc}	13.76 ± 0.05^{de}	90.33 ± 2.51^{a}
7	*L. acidophilus*	419.35 ± 0.69^{c}	67.79 ± 1.29^{e}	4.02 ± 0.01^{ab}	14.06 ± 0.11^{abc}	83.66 ± 1.52^{cd}
8	*L. reuteri*	422.48 ± 3.35^{c}	72.07 ± 1.59^{bc}	3.97 ± 0.01^{d}	14.26 ± 0.20^{a}	85.33 ± 2.08^{bcd}
9	*P. acidilactici*	400.33 ± 4.39^{d}	72.89 ± 1.79^{abc}	4.02 ± 0.00^{ab}	14.03 ± 0.15^{abcd}	82.66 ± 2.08^{e}
10	*L. bulgaricus*	395.48 ± 3.06^{d}	71.56 ± 2.21^{bcd}	4.04 ± 0.00^{a}	13.96 ± 0.15^{bcde}	86.33 ± 2.08^{abcd}
11	*L. paracasei*	423.85 ± 2.40^{bc}	69.12 ± 1.47^{de}	4.02 ± 0.02^{ab}	14.03 ± 0.05^{abcd}	87.66 ± 3.21^{abc}

Values are from the mean ± standard deviation (SD), and different lowercase letters in the upper right corner of the values in the same column represent significant differences between the values ($p < 0.05$).

TABLE 6 Results of uniform design experiments.

No.	*P. pentosaceus* (%)	*L. casei* (%)	*L. plantarum* (%)	*L. fermentum* (%)	SOD activity (U/g)
1	40	13	19	21	430.10
2	41	15	24	19	449.83
3	42	17	18	17	426.90
4	43	19	23	15	409.10
5	44	21	17	13	432.90
6	45	12	22	22	423.10
7	46	14	16	20	380.13
8	47	16	21	18	415.46
9	48	18	15	16	416.33
10	49	20	20	14	420.10

The metabolic activity of LAB is affected by fermentation temperature, thereby affecting the fermentation quality (24). As shown in Figure 1A, the SOD activity is 488.78 U/g at 37°C, then decreased with increasing temperature. LYC also reached a maximum value of 73.13 μg/g at 37°C, after which it gradually decreased. The appropriate growth temperature for LAB is generally around 37°C, so the decrease in both SOD activity and LYC content may be due to over-high temperature. The intolerance of bacteria to heat can lead to a decrease in enzyme metabolism and activity (25). Hence, the optimal fermentation temperature was set at 37°C.

The SOD activity and LYC content were affected by the fermentation time as shown in Figure 1B. When the fermentation time is less than 14 h, the growth and reproduction of LAB were insufficient, metabolites were not accumulated enough, and the SOD activity and LYC content were low. If the time is too long, it will lead to a decrease in SOD activity and LYC content because insufficient nutrients will lead to an increase in the number of dead bacteria and an excessive accumulation of metabolic wastes. SOD activity increased rapidly between 14 h and 22 h and was 493.06 U/g at 22 h, afterwards decreased. The LYC reached a maximum value of 72.20 μg/g at 26 h and then decreased with increasing fermentation time. Therefore, the optimal fermentation time is 22–26 h.

As shown in Figure 1C, the SOD activity was (486.95 U/g) when the TSS content was adjusted to 13.5°Brix, and the LYC reached 71.14 μg/g at a TSS content of 11.5°Brix. From these results, we chose the range of 11.5°Brix-13.5°Brix.

From Figure 1D, it can be observed that When the strain number concentration was 10^6 CFU/mL, the bacterial population was insufficient and the bacterial metabolic rate was slow. It induced a decrease of SOD activity and LYC. However, the values of the indicators increased with the increase of the inoculum. The LYC reached 72.43 μg/g when the strain number concentration was 2×10^6 CFU/mL. the maximum SOD activity was 486.92 U/g. When the strain number con-centration was 3×10^6 CFU/mL. If the inoculum exceeds 3×10^6 CFU/mL, it may result in excessive fermentation, which can result in destabilization of terpenoids and loss of biological enzyme activity. As a result, the optimal inoculum amount ranges from 2×10^6–3×10^6 CFU/mL.

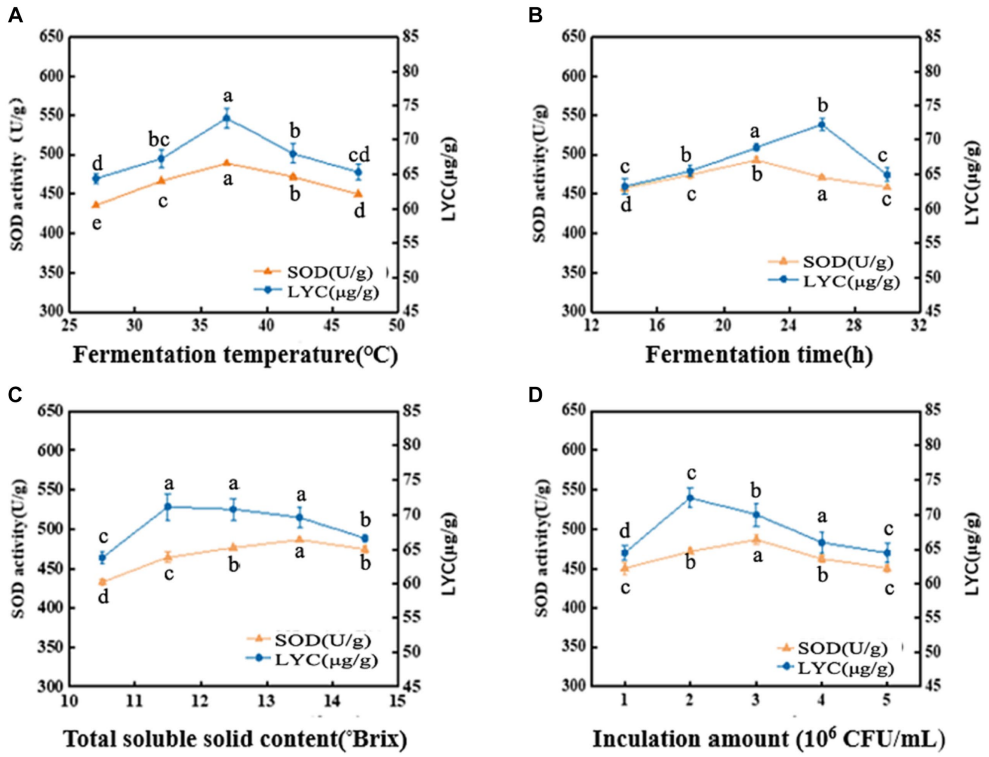

FIGURE 1
Effects of fermentation temperature **(A)**, fermentation time **(B)**, soluble solid content (°Brix) **(C)**, and inoculation amount **(D)** on SOD activity and LYC content. Different lowercase letters represent significant differences between values ($p < 0.05$).

Experiments showed that four factors had effects on both SOD activity and LYC. The design and results of the Box–Behnken test were shown in Table 3. The regression equations are modeled below:

$$Y1 = 503.10 - 0.1538A + 10.48B - 4.47C - 9.97D - 8.49AB + 6.19\,AC - 0.0775\,AD - 6.53\,BC + 7.90BD + 5.19CD - 29.59A^2 - 12.11B^2 - 31.52C^2 - 32.09D^2$$

$$Y2 = 77.25 + 1.03A\ 0.7420B + 1.02C + 0.4004D - 0.5100AB - 1.02\,AC - 0.2075\,AD - 1.17\,BC + 0.5587BD - 0.5475CD - 3.18A^2 - 0.17B^2 - 1.15C^2 - 0.9473D^2$$

Anova and significance test for SOD activity and LYC. R^2, adjusted R^2, final results for flow rate flow %, and proper accuracy are shown in Table 7.

The test of significance of the coefficients of the model regression equation for Y1 (Figure 2A) shows that the linear coefficients (B, D, AB, AD, BD, BC, A^2, B^2, C^2, and D^2) are highly significant and the rest of the terms are not significant. The test of significance of coefficients of regression equation of the Y2 model (Figure 2B) shows that linear coefficients (A, B, C, AC, BD, A^2, B^2, C^2, and D^2) are highly significant and the rest of the terms are not significant ($p < 0.01$).

As can be seen from Table 7, the correlation coefficient R^2 value for SOD activity was 0.9877 and for LYC it was 0.9605, indicating that the model can be used for the analysis and prediction of the fermentation process. By predicting the regression model, the best treatment for FTJ was obtained as follows: fermentation temperature of 37.09°C, LAB inoculum of 2.95×10^6 CFU/mL, fermentation time of 22.20 h, and TSS of 12.45°Brix. In these conditions, the predicted value of FTJ SOD activity was 505.79 U/g, and the predicted value of LYC was 77.33 μg/g.

After comprehensive consideration, the optimal conditions were modified to a fermentation time of 22 h, fermentation temperature of 37°C, LAB inoculum of 3×10^6 CFU/mL, and sugar addition of 12.5°Brix. Under these conditions, we conducted experiments and verified that the SOD activity of the tomato enzyme was 496. 67 U/g and the LYC content was 77.12 μg/g.

3.3 Multivariate statistical analysis

LC–MS technique was used to characterize the metabolites in the fermentation broth to gain insight into the metabolites at different time stages during the fermentation process. A total of 1867 ionic metabolite signatures were generated at five stages, A, B, C, D, and E, during the fermentation process, including 1,020 positive ion patterns and 847 negative ion patterns. The metabolites were further analyzed by applying PCA and OPLS-DA models to the processed metabolite lists, which can effectively reduce the data dimensionality and improve the interpretability and validity of the data. PCA responds to the overall temporal and within-sample variability, reveals the distribution trends among various samples, and identifies possible discrete dispersal points,

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TABLE 7 Analysis of variance of SOD activity and LYC in FTJ.

Source	SOD activity (U/mL)		TFC (mg/mL)	
	F-value	*P*-value	*F*-value	*P*-value
Model	80.48	<0.0001**	24.34	<0.0001**
A-Temperature	0.0157	0.9020	31.69	<0.0001**
B-Fermentation time	105.46	<0.0001**	23.81	0.0002**
C-Inoculum amount	13.31	0.0026**	31.47	<0.0001**
D-Amount of added sugar	66.09	<0.0001**	4.80	0.0458*
AB	33.71	<0.0001**	5.48	0.0345*
AC	10.09	0.0067**	12.28	0.0035**
AD	0.0016	0.9688	0.5107	0.4866
BC	19.97	0.0005**	28.96	<0.0001**
BD	29.24	<0.0001**	6.58	0.0224*
CD	7.09	0.0185*	3.56	0.0803*
A^2	373.83	<0.0001**	194.80	<0.0001**
B^2	197.96	<0.0001**	68.97	<0.0001**
C^2	424.22	<0.0001**	25.27	0.0002**
D^2	439.56	<0.0001**	17.26	0.0010**
Lack of Fit	0.6268	0.7502^{ns}	1.39	0.4013^{ns}
R^2	0.9877		0.9605	
Adj. R^2	0.9755		0.9211	
Pred. R^2	0.9494		0.8097	
Adeq precision	26.6468		16.3740	
C.V.%	0.8736		0.7804	

*$p < 0.05$, **$p < 0.01$.

which can intuitively reflect the similarities or differences among the samples. As shown in Figure 3, fermentation samples at 0 h–10 h, 0 h–16 h, 0 h–20 h, and 0 h–22 h were separated by PCA principal component differentiation. All samples and QCs were within the 95% confidence interval, indicating good stability and reproducibility of the experiment.

In this work, we also analyzed the metabolite variation using the OPLS-DA model. OPLS-DA is an orthogonal correction that filters out uncorrelated orthogonal signals, ensuring a valid and more reliable model. In the model, there were four groups, and their OPLS-DA score plots (Figure 4) showed excellent model parameters (0 h–10 h: $R^2Y = 1$, $Q2 = 0.963$; 0 h–16 h: $R^2Y = 1$, $Q^2 = 0.983$; 0 h–20 h: $R^2Y = 1$, $Q^2 = 0.990$; 0 h–22 h, $R^2Y = 1$, $Q^2 = 0.993$). In cross-validation and response alignment, tests showed that no overfitting occurred in the OPLS-DA model (Supplementary Figure S1). Therefore, the OPLS-DA model is valid with high predictive power and can be used to explore metabolic differences at different stages during the fermentation of FTJ.

3.4 Screening for differential metabolites

3.4.1 Determination of differential metabolites

The projection of the first principal component (VIP) from the OPLS-DA analysis was utilized to assess the strength and interpretability of the effect of expression patterns between groups. Typically, differential metabolites were identified as VIP > 1. In this study, the metabolites with VIP > 1 and $p < 0.05$ were selected to screen four groups (0–10 h, 0–16 h, 0–20 h, and 0–22 h) in a positive–negative ion mode. Overall, we identified 168 differential metabolites throughout the fermentation process, 75 differential metabolites during 0–10 h, 111 differential metabolites during 0–16 h, 136 differential metabolites during 0–20 h, and 166 differential metabolites during 0–22 h (Supplementary Table S1). Among these differential metabolites, organic acids, amino acids, and their derivatives, etc. accumulated mainly due to lactobacilli metabolism, whereas there were lipids and lipid-like molecules mainly consumed due to fermentation, suggesting that carbohydrates, certain amino acids, and lipids were the main precursors of the metabolites.

In addition, we categorized and ranked the major differential metabolites (Figure 5) to gain more insight into the changes in differential metabolites during FTJ. The results showed that the major different metabolites during FTJ were peptides, amino acids, and carbohydrates. These metabolites have different metabolic profiles in different fermentation stages. It is noteworthy that the metabolic properties of the primary metabolites showed relative differences at different stages of the fermentation process. The species and relative abundance of the difference metabolites were the primary ways in which they were expressed. We also constructed a hierarchical cluster

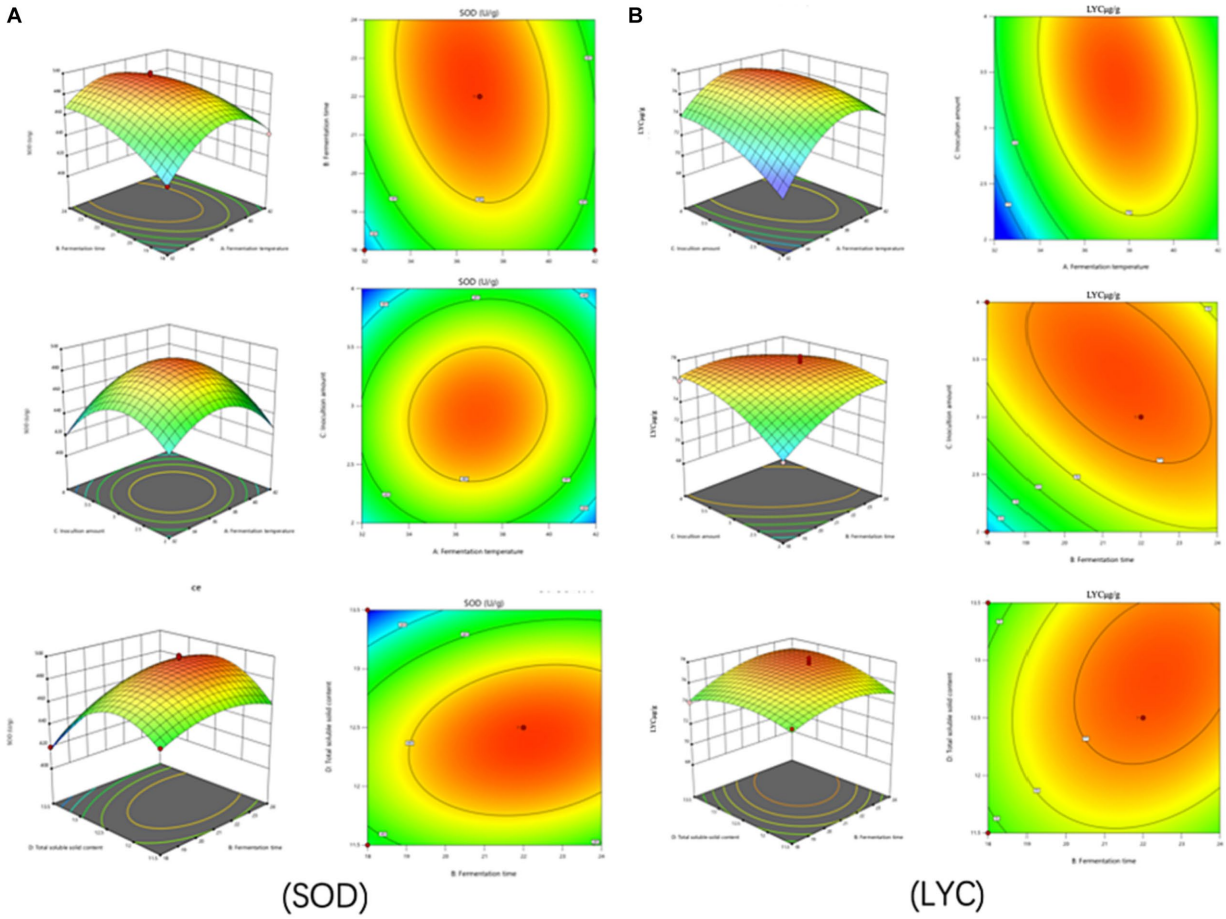

FIGURE 2
Effects of interaction of various factors on **(A)** (SOD) activity and **(B)** (LYC) concentration of FTJ.

analysis (HCA) (Figure 6), the findings of which were consistent with the differential metabolites.

3.4.2 Dynamics of carbohydrates, phospholipids, organic acids, and terpenoids during fermentation

Throughout the fermentation process, the total amount of carbohydrate matter decreased from an initial 1118.6 to 1109.6. Galactitol is commonly used as a low-calorie sweetener and can be a potential component of biobased chemicals. During fermentation, the content of galactitol increased from an initial 5.389 to 6.607 (Figure 7A), which may be related to the hydrolysis of sucrose, and other complex polysaccharides in tomato enzymes (26). The content of cytarabine decreased by 29% (Figure 7B), which may be due to enzymatic deglycosylation. Uridine decreased by 44% (Figure 7C) due to the use of lactobacillus fermentation which requires carbohydrates as an energy source for growth (19).

Malic acid is highest in unfermented tomato juice and decreases with longer fermentation time (Figure 7D). LAB can metabolize malic acid using malic acid lactase, which decarboxylates it to lactic acid using NAD^+ and $Mn2^+$-dependent malic acid lactase (27–29). However, *L. casei* can also utilize malic enzymes to break down malate into pyruvate, allowing them to use malate as a carbon source for growth (30). Lactic acid is the primary organic acid produced during LAB fermentation. Its content slightly increases with the duration of fermentation (Figure 7E). Lactic acid is produced by LAB through sugar metabolism, but this is not the only way LAB produces this metabolite. In fact, polyols (e.g., glycerol) or acids (e.g., malic acid) may also be metabolized to produce lactic acid (31). Lactic acid and typically low pH can damage cell walls and membranes, altering membrane potential and active transport (32), leading to energy depletion and cell death. Therefore, the preservation of fermented juices is favored due to the high lactic acid content during fermentation. Citric acid is known to be a key intermediate in the tricarboxylic acid (TCA) cycle (33). Therefore, citric acid increased at the beginning of fermentation but decreased thereafter (Figure 7F). Citric acid can be transformed by citrate lyase in LAB to oxaloacetate and acetic acid, whereas oxaloacetate can be converted to pyruvate, which can then be converted by LAB to the flavoring molecule 3-hydroxybutanone (34).

Terpenoids are a diverse group of plant secondary metabolites consisting of several isoprene units (35). These bioactive compounds confer a wide range of biological activities such as anticancer, antiallergic, antimicrobial, and antioxidant activities (36, 37). When the fermentation process was completed, 99% of the total terpenoid

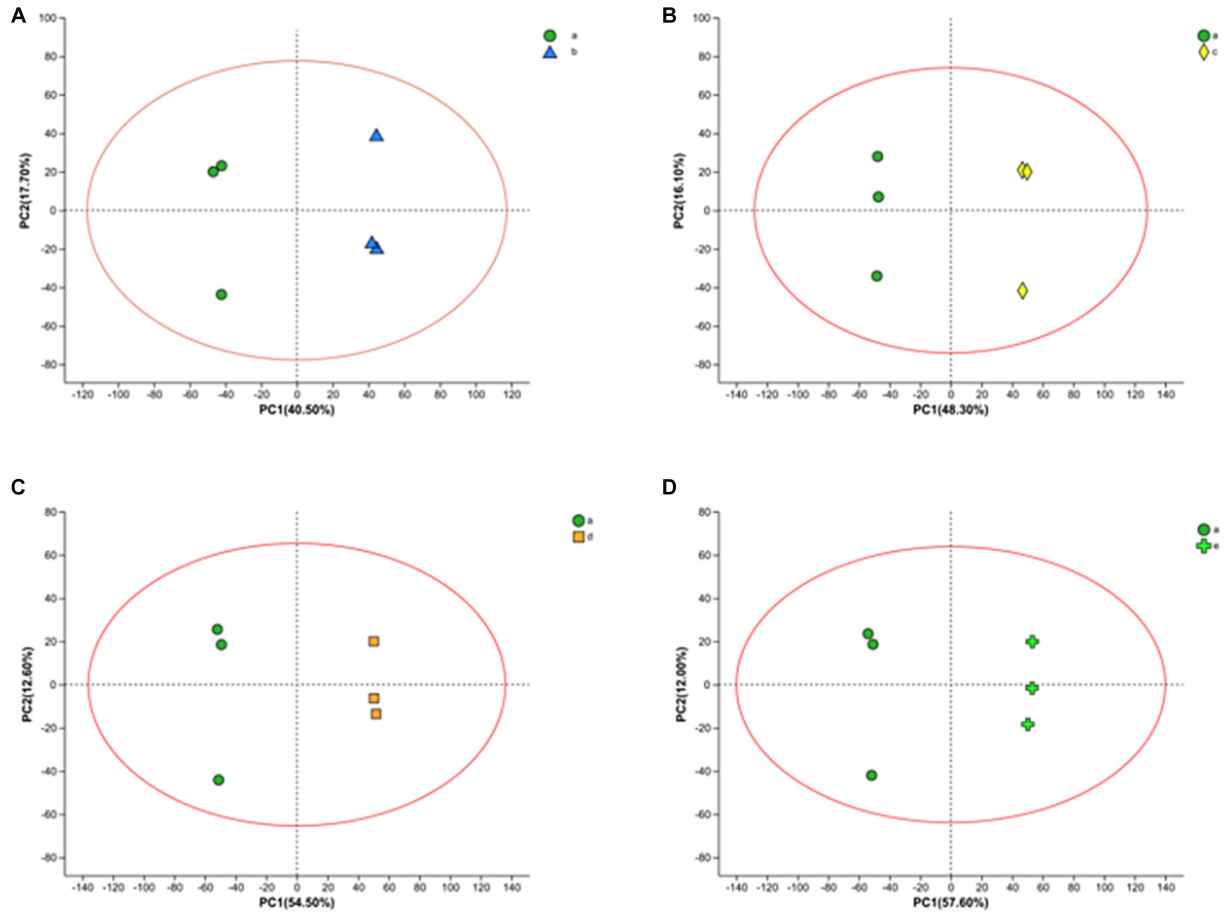

FIGURE 3
PCA score plots of FTJ during different multi-strain fermentation stages [**(A)** 0–10 h; **(B)** 0–16 h; **(C)** 0–20 h; **(D)** 0–22 h].

content was retained, with 1.21-fold up-regulation of 16-deacetylgairin and 1.22- go down-regulation of (+)-Royleanone (Figures 7G–I). Artemisinin is a sesquiterpene endoperoxide with potent antimalarial properties (38). Artemisinin was also slightly elevated during fermentation. It indicated that terpenoids were extracted from the berries during the fermentation process (39).

Phospholipids are major structural components of cell membranes and are essential for various cellular processes (40). In previous studies, it was shown that phosphatidylserine (PS) and phosphatidylethanolamine (PE) are high quality phospholipids. Studies have shown that the combined use of PS and PE lipids can be effective in the treatment of some cancers (41). The quantitative results of PS, PE, PC, PG, and PI were shown in Table 8. The total amount of all of them decreased to different degrees after fermentation. PC content decreased from 41.138 to 35.755 and PE content decreased from 56.543 to 46.964. Although the intensity of PS was lower compared to the others, it also decreased after fermentation with PS content decreasing from 11.119 to 9.186 (Table 8). The results showed that most of these species contain unsaturated fatty acyl fractions, including oleic (18:1), linoleic (18:2), and linolenic (18:3) acid chains. Specifically, esterified polyunsaturated 18:2 and 18:3 chains predominated. This result suggests that phospholipids containing 18:2 and 18:3 chain acyl groups underwent degradation in microorganisms during fermentation.

Lipidomics analysis was conducted according to previous studies (42), and we predicted that the phospholipids in this study may follow a similar degradation pathway. In the presence of hydrolytic enzymes from the microbial community, phospholipids undergo a lipolytic reaction that releases free fatty acids, namely phospholipids PE and PC, and simultaneously generates solubilized phospholipids. These changes could be the primary cause of the decline in phospholipid content. Various fatty acids elongate fatty acids, leading to the production of very long-chain fatty acids with a wide range of chain lengths (42). Fatty acids can be converted to hydroxy fatty acids through a series of microbial fatty acid hydroxylase reactions. Hydroxy fatty acids can be further transformed into hydroxy fatty acid branched-chain fatty acid esters by acylation reactions. Moreover, hydroxy fatty acids may be integrated into sphingolipids such as ceramides (43), thus contributing to sphingolipid accumulation.

4 Conclusion

In this study, the suitability of 10 probiotic strains for tomato juice fermentation was investigated and 4 of the most suitable strains were

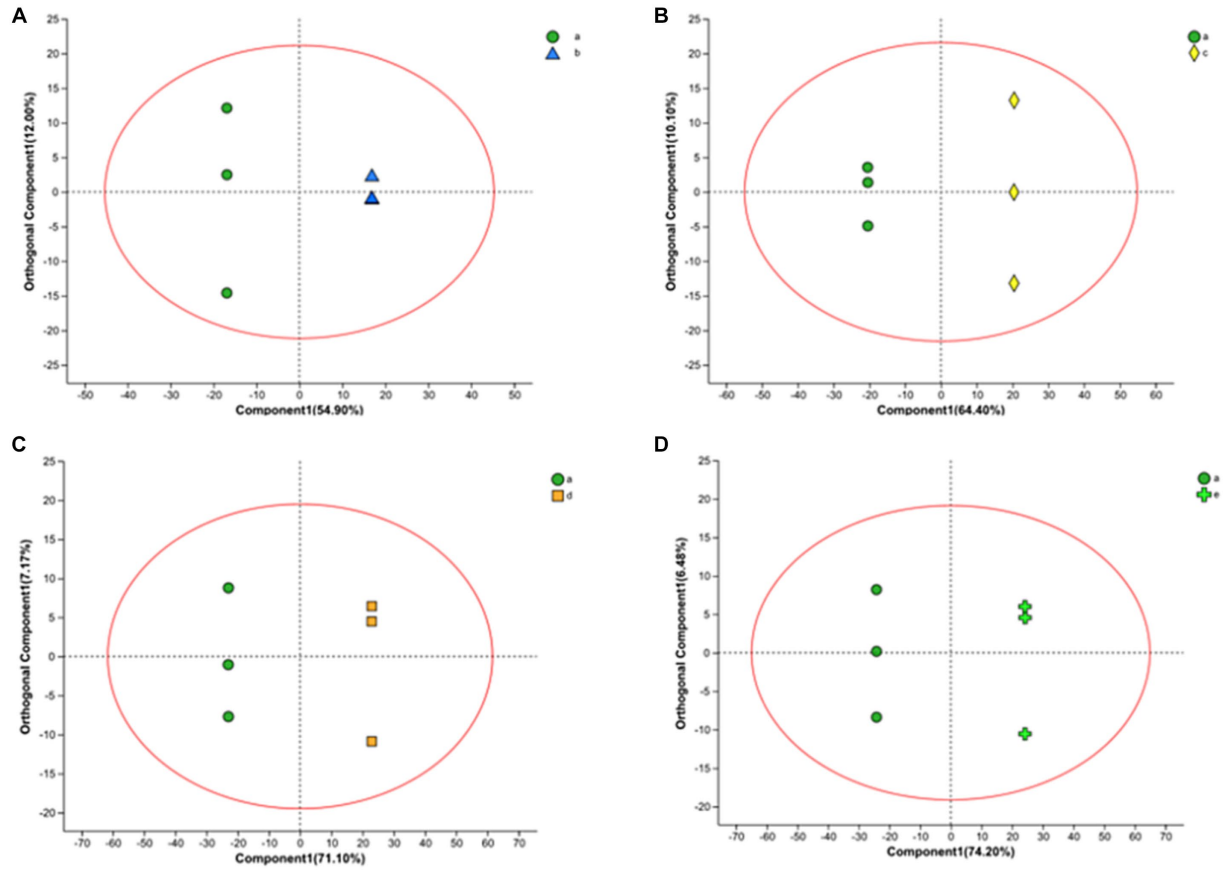

FIGURE 4
OPLS-DA in positive ion mode yields scatter plot [**(A)** 0–10 h; **(B)** 0–16 h; **(C)** 0–20 h; **(D)** 0–22 h].

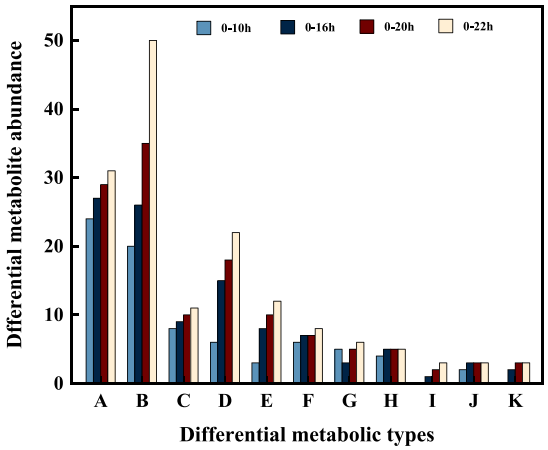

FIGURE 5
Changes of differential metabolites in FTJ during different multi-strain fermentation stages [A: Peptides; B: amino acid; C: heterocyclic compounds; D: phospholipid; E: carbohydrate; F: organic acid; G: steroid; H: amine; I: terpene; J: sulfur-containing compounds; K: fatty acid].

selected. The FTJ process was optimized using a homogeneous design and RSM. The optimal fermentation conditions for FTJ fermentation were determined as follows: fermentation time of 22 h, TSS content of 13°Brix, fermentation temperature of 37°C, and inoculum concentration of 3×10^6 CFU/mL.

We analyzed key differential metabolites of fermented tomato juice using UHPLC-QE-MS/MS and identified 168 different metabolites: including carbohydrates, phospholipids, organic acids, and terpenes. Changes in carbohydrates such as galactitol, aglycone, and uridine were related to the hydrolysis of sucrose and polysaccharides in the fermented tomato juice and their need for LAB-fermentation as an energy source for growth. The changes in phospholipids (PS, PE, PC, PG, PI) are since most of these species contain unsaturated fatty acyl portions and they can undergo degradation during microbial fermentation. Organic acids (malic, citric, lactic) can be altered because each has a different metabolic pathway. During fermentation, 16-deacetylgairin, (+)-Royleanone, and artemisinin have different degrees of variation with fermentation time due to the terpenoids that can be extracted from the stock solution. Due to the limitations of the specific strains and experimental conditions used in this study, there may be some deficiencies in general applicability, and further studies involving a wider range of strains and fermentation conditions are needed to improve the applicability of the results. All in all, this study provides further insight into the fermentation process of tomato juice and the dynamic changes of metabolites during fermentation and lays the foundation for the study of fruit and vegetable fermentation.

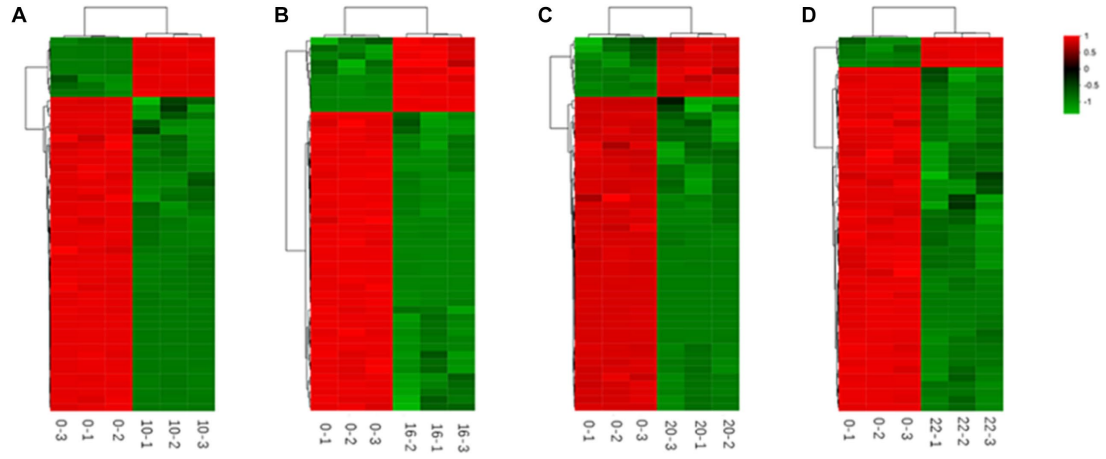

FIGURE 6
Heat maps of metabolites differing by fermentation times [**(A)** 0–10 h; **(B)** 0–16 h; **(C)** 0–20 h; **(D)** 0–22 h].

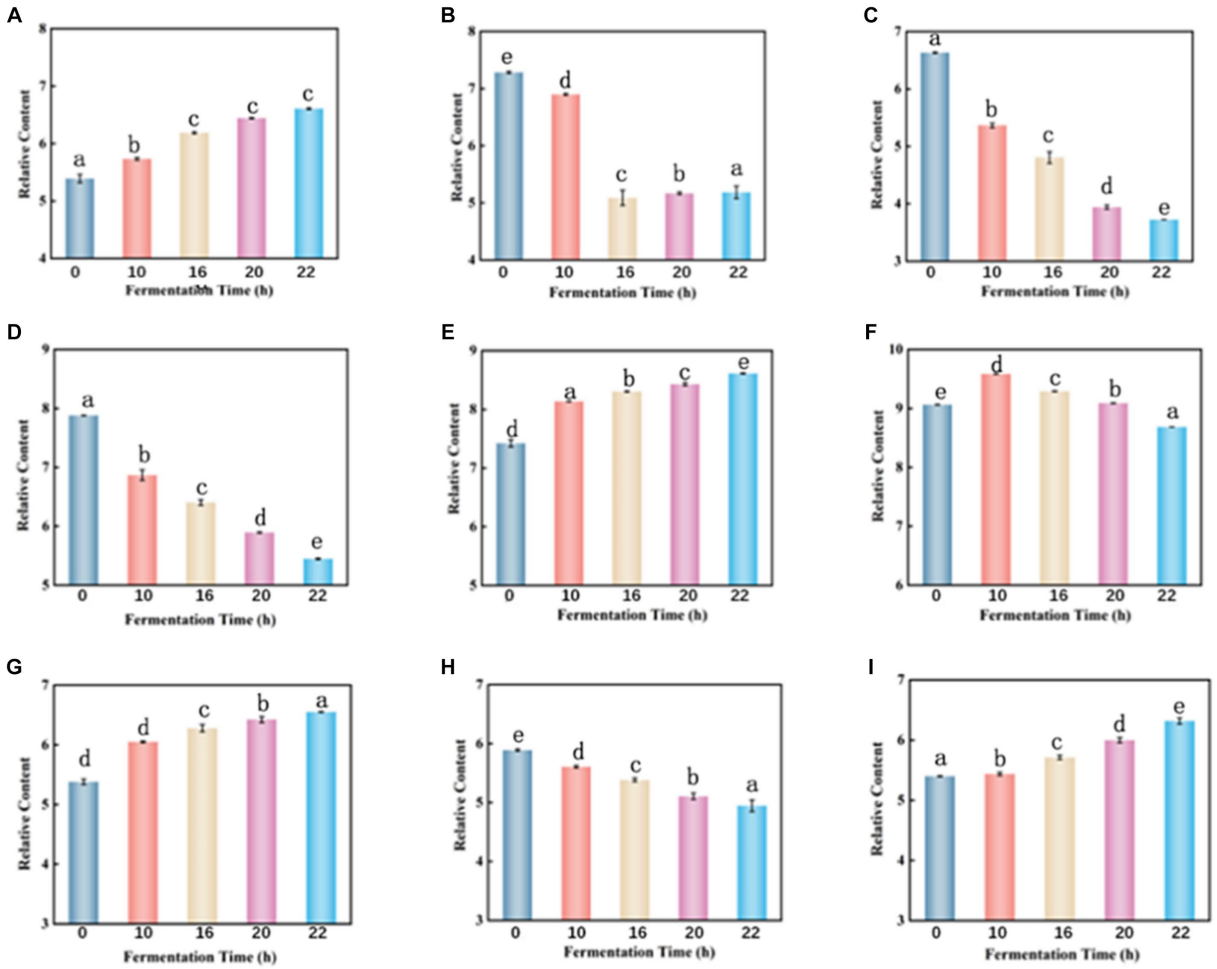

FIGURE 7
Dynamic changes of key differential metabolites in FTJ during different multi-strain fermentation stages [**(A)** Galactitol; **(B)** cytarabine; **(C)** uridine; **(D)** malic acid; **(E)** lactic acid; **(F)** citric acid; **(G)** 16-deacetylgeyerline; **(H)** (+)-Royleanone; **(I)** artecanin]. Different lowercase letters represent significant differences between values ($p < 0.05$).

TABLE 8 Dynamic changes of key phosphatidylinositol in FTJ during different multi-strain fermentation stages.

Name	Marker compound	Ion model	0	10	16	20	22
PS	LysoPS(18:2(9Z,12Z)/0:0)	Pos	6.4836	5.8108	5.1117	4.7881	4.2589
	LysoPS(18:1(9Z)/0:0)	Neg	5.5759	5.6997	5.7075	5.7909	5.7958
PE	LysoPE(18:2(9Z,12Z)/0:0)	Pos	7.5012	6.9392	6.4898	6.3124	6.2461
	LysoPE(16:0/0:0)	Pos	6.0563	4.9907	3.6795	3.6507	3.6233
	PE(14:0/14:1(9Z))	Pos	5.7653	5.7884	5.8141	5.8289	5.8220
	LysoPE(0:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	Neg	5.5831	5.5824	5.5681	5.5908	5.5807
	PE(16:0/0:0)	Neg	5.7182	5.1365	4.8070	4.7156	4.7254
	LysoPE(0:0/18:3(9Z,12Z,15Z))	Neg	6.4544	5.8502	5.3014	4.8768	4.6092
	LysoPE(18:3(9Z,12Z,15Z)/0:0)	Neg	6.1163	5.1672	4.4981	4.2480	3.9381
	LysoPE(20:5(5Z,8Z,11Z,14Z,17Z)/0:0)	Neg	5.8900	5.9239	5.9135	5.9310	5.9208
	PE(18:2/0:0)	Neg	7.4580	6.9304	6.6129	6.4954	6.4979
PC	LysoPC(18:2(9Z,12Z)/0:0)	Pos	7.9526	7.0109	6.4531	6.2886	6.0624
	PC(18:1(12Z)2OH(9,10)/2:0)	Pos	2.9471	5.6862	5.7116	5.8288	6.0596
	LysoPC(18:1(11Z)/0:0)	Pos	6.6764	6.0620	5.6456	5.3707	5.1022
	LysoPC(0:0/18:2(9Z,12Z))	Pos	5.7736	4.7333	3.6030	3.6329	3.6078
	LysoPC(18:3(6Z,9Z,12Z)/0:0)	Pos	6.7973	5.5602	4.8267	4.7051	4.3512
	LysoPC(14:1(9Z)/0:0)	Neg	5.4462	5.3959	5.3507	5.3351	5.3038
	PC(17:2(9Z,12Z)/0:0)	Neg	5.5451	5.7233	5.3914	5.3273	5.2678
PG	PG(16:0/0:0)	Neg	6.3946	5.2538	6.0129	5.8495	5.6615
	LysoPG(16:0/0:0)	Neg	5.6216	5.5076	5.2768	5.0261	4.8116
	LysoPG(18:2(9Z,12Z)/0:0)	Neg	5.7499	5.3527	4.9343	4.6437	4.2765
PI	PI(20:5(5Z,8Z,11Z,14Z,17Z)/0:0)	Pos	6.3779	6.1558	5.8257	5.4085	5.0213
	LysoPI(18:2(9Z,12Z)/0:0)	Neg	7.7322	7.5037	7.2196	6.9157	6.6915
	LysoPI(16:0/0:0)	Neg	7.0630	6.4191	5.8240	5.4846	5.2309

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

LZ: Writing – original draft. RM: Writing – review & editing. JH: Writing – review & editing. LW: Writing – review & editing. YM: Writing – review & editing. BL: Writing – review & editing. HZ: Writing – review & editing. KC: Writing – review & editing. AA: Writing – review & editing.

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Conflict of interest

KC was employed by Xinjiang Huize Food Limited Liability Company.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Corrigendum: Optimization of tomato (*Solanum lycopersicum* L.) juice fermentation process and analysis of its metabolites during fermentation

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In the published article, Supplementary Table 1 and Supplementary Figure 1 were mistakenly not included in the publication. The missing material has been published in the original article.

SUPPLEMENTARY FIGURE 1
Plot of cross-validation and alternate testing of OPLS-DA model (ESI+).

SUPPLEMENTARY TABLE 1
Detailed list of metabolites differing between groups.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Non-targeted metabolomic analysis of non-volatile metabolites in a novel Chinese industrially fermented low-salt kohlrabi

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Low-temperature and low-salt fermented Chinese kohlrabi (LSCK) represents a novel approach to producing low-salt kohlrabi without the need for desalination during processing, as compared to traditional techniques. However, the profile of its non-volatile metabolites remains unclear. In order to investigate the non-volatile metabolites and their changes in LSCK during fermentation, the LSCKs fermented for 0 day (0D), 45 days (45D) and 90 days (90D) were analyzed using LC-MS/MS non-targeted metabolomics coupled with multivariate statistical analysis. The results showed that 60, 74, and 68 differential metabolites were identified in the three groups A1 (0D and 45D), A2 (0D and 90D), and A3 (45D and 90D) (VIP >1, $p < 0.05$, Log2FC >1), respectively. The differential metabolites were mainly amino acids, peptides, and analogues, fatty acyls, organic acids and derivatives, and carbohydrates and carbohydrate conjugates. Seventeen common differential metabolites were identified in A1, A2, and A3 groups. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis suggested that the alanine, aspartate and glutamate metabolism, butanoate metabolism, α-linolenic acid metabolism, arginine biosynthesis, and phenylalanine metabolism were significantly correlated with the differential metabolites. The present study elucidates for the first time the changes in non-volatile differential metabolites and their associated metabolic pathways in the novel Chinese low-salt kohlrabi, providing a theoretical basis for improving the industrial fermentation process of this innovative product.

KEYWORDS

kohlrabi, low-temperature and low-salt fermentation, LC-MS/MS, differential metabolites, metabolic pathways

1 Introduction

Kohlrabi (*Brassica juncea* var. *megarrhiza* Tsen et Lee), a member of the cruciferous *Brassica* annual herb family, is widely cultivated in China. Known for its high nutritional value, kohlrabi is rich in vitamins, proteins, and carbohydrates. It also serves as a quality source of biologically active components, such as thioglucosides and indole derivative

(1). However, raw kohlrabi has high levels of isothiocyanate, which gives a strong mustardy and bitter flavor and makes it unsuitable for direct consumption. After fermentation, kohlrabi develops a moderately sweet and salty taste with a robust soy sauce flavor (2). Fermented kohlrabi is a renowned Chinese specialty and is one of the four most famous pickles in Sichuan Province, alongside mustard tubers, Dongcai, and mustard vein (3).

Traditional fermentation methods for kohlrabi often use a high salt concentration (15–20%, w/w). Long-term consumption of high-salt foods can elevate sodium ion levels in the human body, resulting in sodium-potassium imbalance and thus leading to diseases such as hypertension and atherosclerosis (4). Consequently, desalination is required to produce low-salt kohlrabi. However, this process not only causes a significant loss of nutrients such as proteins but also increases production costs for treating sodium-containing wastewater. Therefore, low-salt fermentation techniques are gaining global attention. Salt plays a crucial role in forming food taste and flavor (5). Liang et al. (6) reported that a 6% salt concentration in Chinese sauerkraut fermentation promoted higher lactic acid bacteria abundance and better texture. Fermentation temperature also affects the physicochemical properties of fermented products by influencing microbial metabolites. For instance, Aung and Eun (7) found that laver fermented at 25°C produced more flavonoids and enhanced α-amylase inhibitory activity than that fermented at 30°C.

Compared to traditional kohlrabi, the production of LSCK uses less salt and does not require desalination. LSCK has higher protein and reduced sugar levels and possesses elevated concentrations of flavor substances such as alcohols, ketones, pyrazines, ethers, and nitriles, as documented in our previous study (8). Moreover, the volatile differential metabolites of LSCK were analyzed in our previous study (9). Given kohlrabi's richness in nutritional and functional metabolites, a thorough investigation into both volatile and non-volatile metabolites of LSCK is necessary. However, there is a paucity of literature on the non-volatile metabolites and their changes in this novel low-salt, industrially fermented kohlrabi. Furthermore, the mechanisms underlying the formation of these differential non-volatile metabolites remain unclear. In this study, the non-volatile metabolites of LSCKs with different fermentation periods (0, 45, and 90 days) were analyzed using non-targeted LC-MS/MS metabolomics, and differential metabolites were screened by multivariate statistical analyses. The present study provides a theoretical basis for improving LSCK processing and utilizing its nutritional and functional metabolites.

2 Materials and methods

2.1 Preparation of the novel low-salt industrially fermented kohlrabi LSCK

The industrially fermented LSCK was prepared as described in our previous studies (9). The kohlrabies were naturally air-dried outdoors with an average temperature of 7–12°C for 20–40 days after harvesting. After air-drying, the kohlrabies were washed in water at 60 ± 2°C, then dried at 35–40°C for 15 min in a drier. The pretreated kohlrabies were then mixed with 2.5% (w/w) salt and pickled at 4 ± 1°C for 2 days. Then, 1.5% (w/w) salt was added to the once pickled kohlrabies and followed by fermentation at 4 ± 1°C for 0, 45, and 90 days, termed 0D, 45D, and 90D, respectively.

2.2 Metabolite extraction from LSCK

Metabolite extraction was performed as described in our previous study (10). Fifty micrograms of LSCK were transferred into a clean 2 mL-microtube with a 6 mm grinding bead. Then, 400 μL of extraction solution [methanol:water = 4:1, (v:v)] combined with 0.02 mg/mL of internal standard (L-2-chlorophenylalanine) was used for LSCK metabolite extraction. LSCK was completely ground by using the frozen tissue grinder (Wonbio-96C, Shanghai Wanbo Biotechnology, Shanghai, China) for 6 min at −10°C and 50 Hz. Subsequently, the LSCK metabolite was extracted by low-temperature ultrasonic extraction (KW-100TDV, Kunshan Shumei, Kunshan, China) for 30 min at 5°C and 40 kHz. The extracted LSCK samples were stored at −20°C for 30 min and centrifuged (H1850R, Cence, Changsha, China) at 13,000 g for 15 min at 4°C. The supernatant was used for LC-MS/MS analysis.

2.3 Mass spectrometry conditions

The LC-MS/MS analysis for LSCK extract was performed on a Thermo UHPLC-Q Exactive HF-X system equipped with an ACQUITY HSS T3 column (100 mm × 2.1 mm i.d., 1.8 μm; Waters, United States) at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The mobile phases were comprised of solvent A and solvent B. Solvent A was 0.1% formic acid in water: acetonitrile solution (95:5, v/v), while solvent B was 0.1% formic acid in acetonitrile: isopropanol: water solution (47.5:47.5:5, v/v/v). The separation and MS conditions were detailly described in our previous study (10). The optimal conditions were source temperature 425°C; sheath gas flow rate 50 arb and aux gas flow rate 13 arb; ion-spray voltage floating (ISVF) −3,500 V in negative mode and 3,500 V in positive mode, respectively. Normalized collision energy, 20–40–60 V rolling for MS/MS. Full MS resolution was 60,000, and MS/MS resolution was 7,500. Data acquisition was performed with the Data Dependent Acquisition (DDA) mode. The detection was carried out over a mass range of 70–1,050 m/z.

2.4 Statistical analysis

The LC/MS raw data was pretreated by Progenesis QI (Waters Corporation, Milford, United States) software. The non-volatile metabolites were identified by searching the primary databases HMDB,[1] Metlin,[2] and Majorbio Database. The R package "ropls" (Version 1.6.2) was employed to perform principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA), and 7-cycle interactive validation evaluating the stability of the model. The non-volatile metabolites with VIP >1, $p < 0.05$ were considered as

1 http://www.hmdb.ca/
2 https://metlin.scripps.edu/

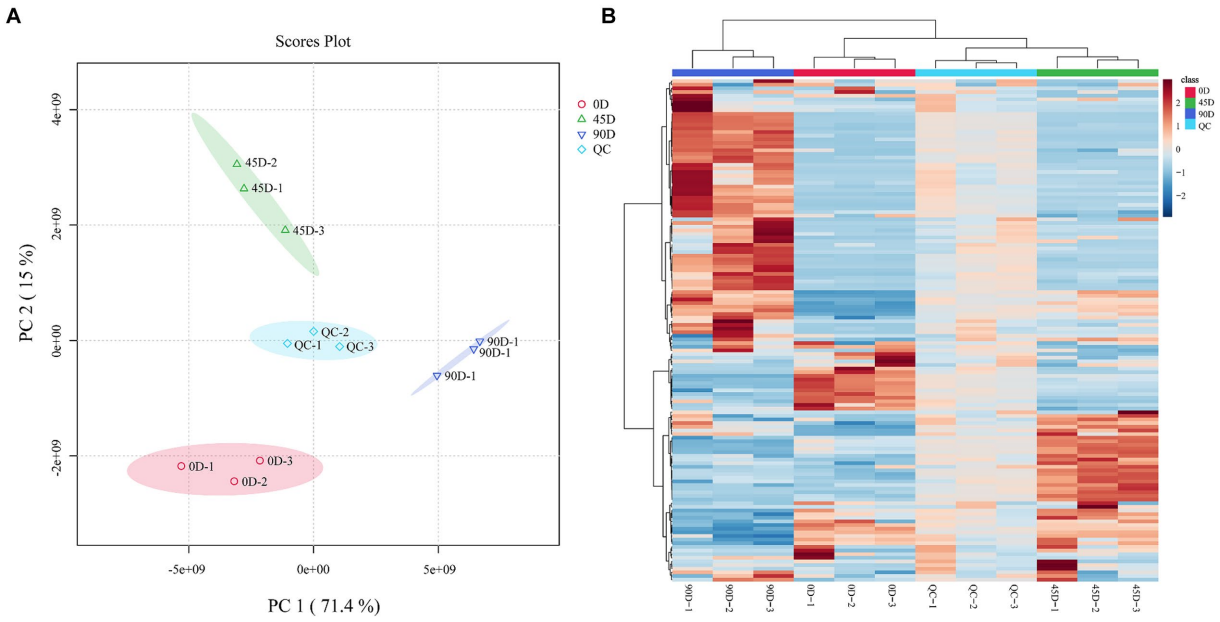

FIGURE 1
Principal component analysis (PCA) plot of the metabolites for 0D, 45D, 90D, and QC samples **(A)**, hierarchical clustering analysis (HCA) of nontargeted metabolomics for 0D, 45D, 90D, and QC samples **(B)**. 0D, 45D, and 90D represent the LSCK fermented for 0 days, 45 days, and 90 days, respectively, QC stands for quality control.

significantly differential non-volatile metabolites based on the Variable Importance in the Projection (VIP) obtained by the OPLS-DA model and the *p*-value obtained by Student's *t*-test. Differential non-volatile metabolites among the A1, A2, and A3 groups were mapped into their biochemical pathways through metabolic enrichment and pathway analysis based on KEGG database.[3] Python packages "scipy.stats"[4] was used to perform enrichment analysis to obtain the most relevant biological pathways for experimental treatments.

3 Results and discussion

3.1 PCA analysis for LSCK non-volatile metabolites

In order to reveal the effect of the fermentation period on LSCK non-volatile metabolites, the metabolite profiles of the three different kohlrabies were evaluated by LC-MS/MS coupled with multivariate statistical methods. As shown in Figure 1A, the quality control (QC) group closely distributed and clustered in the center, indicating high reproducibility and reliability of the data. The 0D, 45D, and 90D samples were distributed in distinct regions, suggesting significant differences in metabolites among the different LSCKs. Based on the PCA results, the three treatment groups were established A1 (0D and 45D), A2 (0D and 90D), and A3 (45D and 90D). PC1 contributed 71.4%, while PC2 contributed 15%, for a total contribution of 86.4%, indicating that the PCA model had good interpretability. In order to better visualize the differences between the LSCK samples, hierarchical clustering analysis (HCA) was performed in the form of heat maps, as shown in Figure 1B. It was obvious that the non-volatile metabolite profiles were significantly different among LSCKs.

3.2 OPLS-DA analysis for LSCK non-volatile metabolites

OPLS-DA analysis can better remove confounding factors unrelated to categorical information and further enhance the model's analytical ability. Figures 2A,C,E show the plots of OPLS-DA scores for the three treatment groups, A1 (0D and 45D), A2 (0D and 90D), and A3 (45D and 90D), respectively. All three treatment groups were significantly separated from each other, suggesting that different fermentation periods caused the significant differences in non-volatile metabolite compositions and contents among the LSCKs. The parameters of the OPLS-DA model are listed in Table 1. The R^2X scores in the model were all larger than 0.5. Moreover, the scores of R^2Y and Q^2 were both larger than 0.9, indicating the model could effectively explain and predict the differences between the metabolites in each group.

To further demonstrate the validity of the results, 200-loop iteration permutation tests were performed, as shown in Figures 2B,D,F. Q^2 represents the predictive ability of the OPLS-DA model. All Q^2 points located on the left side of the plot were lower than the original Q^2 points on the right side. Furthermore, the regression lines of Q^2 were all intersected with the negative half-axis of the *Y*-axis, indicating that the model had good reliability and stability. On the other hand, no over-fitting was observed.

3 http://www.genome.jp/kegg/
4 https://docs.scipy.org/doc/scipy/

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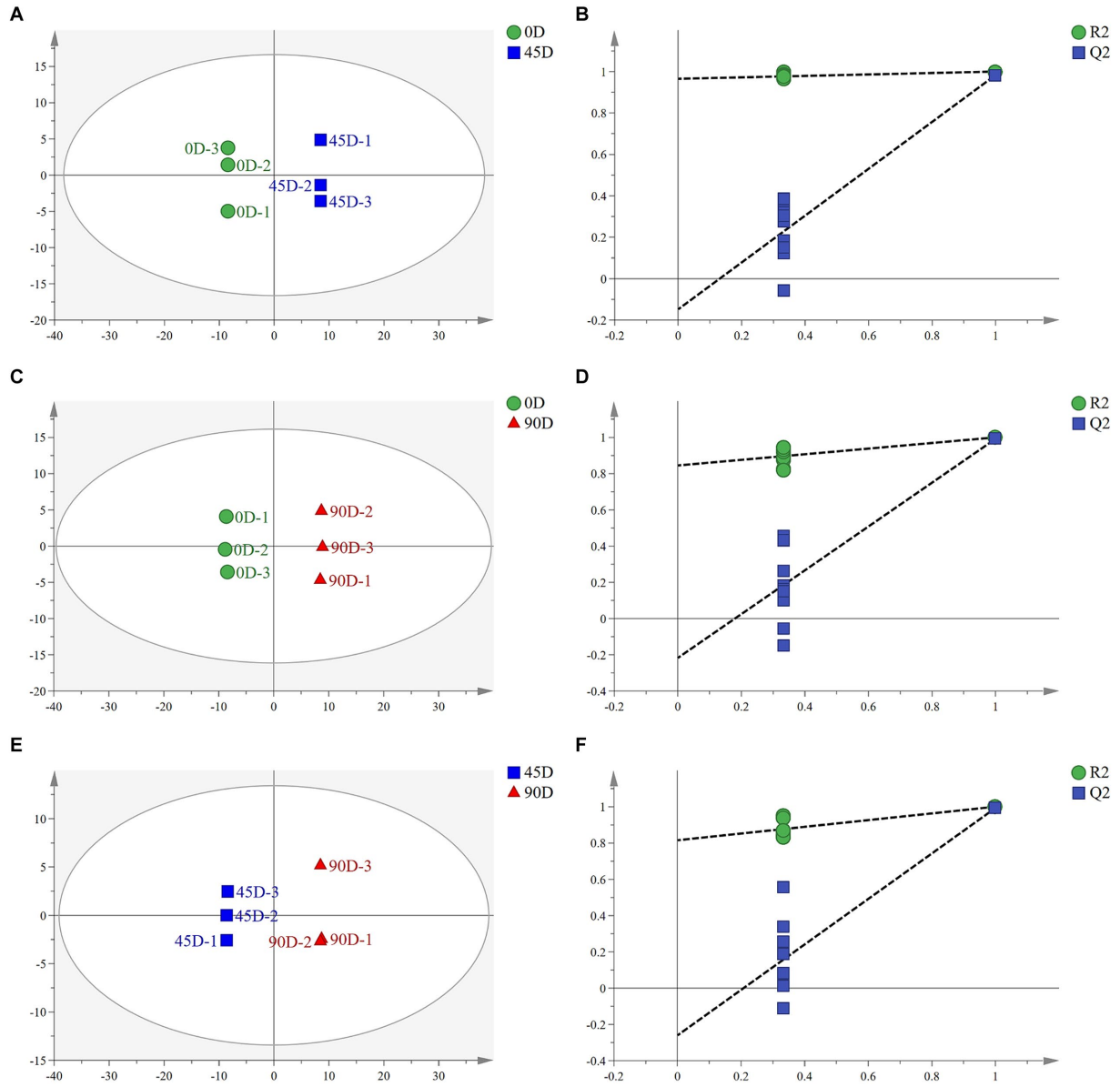

FIGURE 2
Scatter plots and permutation test of OPLS-DA models for LSCK, **(A,C,E)** indicates the OPLS-DA analysis for A1, A2, and A3, while **(B,D,F)** represents permutation test for A1, A2, and A3.

TABLE 1 Parameters of the OPLS-DA models of LSCK.

Group	R^2X	R^2Y	Q^2
A1 (0D–45D)	0.729	1.000	0.983
A2 (0D–90D)	0.763	1.000	0.991
A3 (45D–90D)	0.714	1.000	0.993

3.3 Classification of non-volatile metabolites in LSCKs

A total of 138 metabolites were detected and annotated from LSCKs based on LC-MS/MS untargeted metabolomics (Supplementary Table S1). Among which, a total of 124 metabolites were classified in 13 groups including more than 1 metabolites, as shown in Figure 3. The 13 groups were amino acids, peptides, and analogues (29, 23.39%), fatty acyls (19, 15.32%), organic acids and derivatives (14, 11.29%), carbohydrates and carbohydrate conjugates (15, 12.10%), benzene and substituted derivatives (10, 8.06%), organooxygen compounds (7, 5.65%), imidazopyrimidines (6, 4.84%), phenols (4, 3.23%), pyridines and derivatives (3, 2.42%), diazines (3, 2.42%), pyrimidine nucleosides (2, 1.61%), steroids and steroid derivatives (2, 1.61%), and others (10, 8.06%). According to the compositions and contents of metabolites, non-volatile metabolites in the first five groups were considered the major metabolites in LSCKs. Amino acids, peptides, and analogues accounted for the highest proportions in LSCKs. Amino acids, strongly associated with food taste and odor, are the main contributors to fermented foods flavors

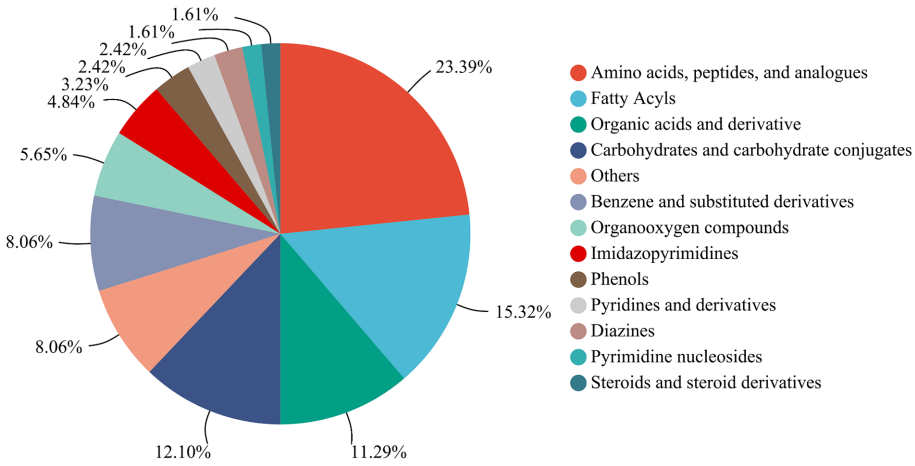

FIGURE 3
Pie chart of the number of different types of all non-volatile metabolites.

(11). A variety of amino acids, including L-glutamic acid, L-aspartic acid, L-glutamine, L-serine, L-phenylalanine, L-cysteine, L-leucine, L-valine, and others, were detected in the LSCKs. Free amino acids play different roles in the composition of fermented foods flavors. Alanine and arginine mainly provide sweetness accompanied by a monosodium-like taste, while glutamic acid and aspartic acid are the main contributors to the freshness of fermented foods, and tryptophan and phenylalanine affect astringent and bitter tastes of foods (12). During the fermentation of LSCK, microorganisms could utilize nutrients to synthesize various types of free amino acids, providing the fermented LSCK characteristic flavor.

3.4 Classification and identification of differential non-volatile metabolites in LSCKs

In order to further investigate the differences in non-volatile metabolites of LSCKs, 60, 74, and 68 differential metabolites were, respectively, screened from the three treatment groups of A1 (0D–45D), A2 (0D–90D), and A3 (45D–90D) ($p < 0.05$, VIP >1, and Log2FC >1), as shown in Figure 4. Five most significant differential metabolites were marked in Figures 4A–C. In A1 (0D–45D) group, the differential metabolites (−)-epigallocatechin, (10S)-juvenile hormone III diol, γ-aminobutyric acid, and 4-acetamido-2-aminobutanoic acid were up-regulated, while 3-(2-hydroxyphenyl) propanoic acid was down-regulated. In A2 (0D–90D) group, L-kynurenine, dodecanedioic acid, dehydroepiandrosterone, and acetoacetic acid were up-regulated, whereas diphenylamine was down-regulated. However, in A3 (45D–90D) group, the two differential metabolites vanillylmandelic acid and L-kynurenine were up-regulated, and three differential metabolites diphenylamine, N-acetyl-D-glucosamine, and γ-aminobutyric acid were down-regulated. Throughout the fermentation process, the number of up-regulated metabolites exceeded that of downregulated ones, indicating an overall increase in metabolite levels. This increase suggests enhanced synthesis of flavor precursor substances and flavor compounds (13).

Amino acids, peptides and analogs, fatty acyls, organic acids and derivatives, and carbohydrates and carbohydrate conjugates were the common major differential metabolites in the three LSCKs, as shown in Figure 4D and Supplementary Table S2. During LSCK fermentation, microorganisms metabolize nutrients such as sugars, fats, and proteins through enzymatic reactions, producing a variety of metabolites such as organic acids, amino acids, and fatty acids (14). Figures 5A–C show the changes in differential metabolites in groups A1 (0D–45D), A2 (0D–90D), and A3 (45D–90D), respectively.

3.4.1 Amino acids, peptides, and analogues in LSCKs

Amino acids, peptides, and analogues accounted for the largest proportion of all differential metabolites. Compared to 0D, 13 and 14 amino acids, peptides, and analogues showed significant differences at 45D (9 up-regulated and 4 down-regulated) and 90D (7 up-regulated and 7 down-regulated), respectively. Furthermore, 90D showed different trends in amino acid differential metabolites compared with 45D (6 up-regulated and 7 down-regulated). Amino acid metabolism is a key factor influencing fermented foods flavors (15). Alanine and L-aspartic acid, which provide sweetness and freshness, were produced in the highest amount at 90D (90D > 45D > 0D), and these free amino acids positively contributed to the sensory properties of kohlrabi (16). Similarly, the two essential amino acids L-phenylalanine and L-isoleucine increased progressively with fermentation time. Microorganisms biosynthesize biocatalysts such as cellulases and proteases during fermentation, which degrade kohlrabi cell walls, decompose proteins, and release free amino acids (12). Moreover, peptides and proteins can also be metabolized by microorganisms, producing various amino acids (17). On the other hand, some amino acids, such as L-glutamic acid, L-asparagine, and aminoadipic acid, decreased during LSCK fermentation, likely due to their utilization by microorganisms. For example, lactic acid bacteria (LAB) can metabolize amino acids to produce flavor substances such as phenyl lactic acid, phenyl acetate, and phenylethanol (18). Interestingly, L-valine was the only essential amino acid decreased during the later stage of fermentation, with the lowest levels observed at 90D. L-valine serves as a nitrogen source for yeast (19), which is

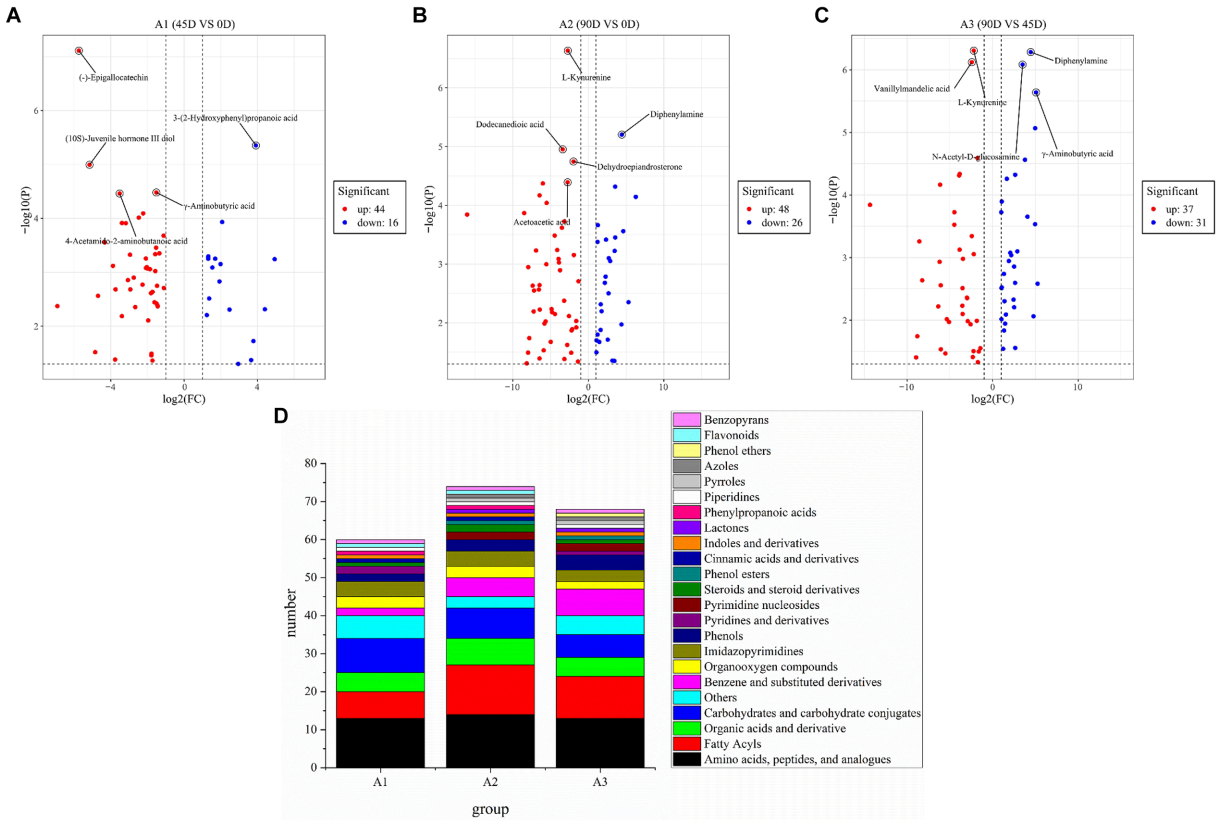

FIGURE 4
Volcano plot of the differential non-volatile metabolites of 0D–45D **(A)**, 0D–90D **(B)**, 45D–90D **(C)** and classification information for differential metabolites of LSCK **(D)**.

probably utilized by yeast during fermentation. As a bitter amino acid, the decrease of valine is beneficial to LSCK flavor (20). The decrease of branched chain amino acid like L-valine and L-leucine in the later fermentation stage was probably due to their metabolism by LAB and yeast, producing branched aldehydes and alcohols, which contributed greatly to the unique flavor of fermented foods (21). Therefore, the degradation and release of free amino acids occur simultaneously during microbial fermentation in LSCK, which was consistent with a previous study (22). Amino acids not only greatly enhance flavor but are also key bioactive compounds that dominate various life activities in plants. They are involved in the synthesis of amines, proteins, alkaloids, enzymes, vitamins, terpenoids, purines, and pyrimidines. Additionally, amino acids are essential for plant stress defense and reducing abiotic stresses (23).

3.4.2 Fatty acyls

Most of the fatty acyls increased during LSCKs fermentation. Compared to 0D, 7 fatty acyls in 45D were up-regulated. While, 10 fatty acyls were up-regulated and 3 fatty acyls were down-regulated in 90D, compared to 0D. Eleven differential fatty acyls were detected in 90D and 45D, among which 8 fatty acyls decreased in 45D. Most of the up-regulated fatty acyls were unsaturated fatty acid during fermentation. Unsaturated fatty acids are a class of essential fatty acids with various biological functions, such as promoting human growth and development, maintaining cellular homeostasis, and lowering blood pressure and lipids (24). (9Z,12Z,15Z)-Octadecatrienoic acid, also known as α-linolenic acid, has multiple functions, including improving cardiovascular health, enhancing immunity, and providing anti-inflammatory effects (25). During kohlrabi fermentation, *Lactobacillus plantarum* and *Bifidobacterium* metabolize fats to produce α-linolenic acid and convert it into longer-chain ω-3 fatty acids like eicosapentaenoic acid and docosahexaenoic acid (26). The content of α-linolenic acid in 45D and 90D was 10.47 and 17.48 times higher than that of 0D, respectively. The increase in medium-chain fatty acids like pelargonic acid, dodecanoic acid, heptanoic acid, and isovaleric acid is correlated with some biosynthesis pathways. Microorganisms such as *Lactobacilli*, *Lactococcus* spp., and *Streptococcus* spp., with lipolytic enzymes, also participate in the formation of medium-chain fatty acids (27). The content of some fatty acids, such as pelargonic acid and dodecanoic acid, decreased significantly in the later stage of fermentation (45–90 days), likely due to microbial metabolic activity and membrane lipid degradation (28). Pelargonic acid and dethiobiotin were differential fatty acyl metabolites present in all three groups (A0, A1 and A2). Pelargonic acid, a saturated fatty acid with a putrid and irritating odor, decreased significantly in the later stage of fermentation (45–90 days), resulting in the lowest levels in 90D and promoting positive flavor formation in LSCK. Dethiobiotin is a medium-chain fatty acid widely used in biomedical applications as a precursor for synthesizing biotin (29). The described changes in differential fatty acyl metabolites positively influenced the flavor improvement and nutrient enrichment of LSCKs.

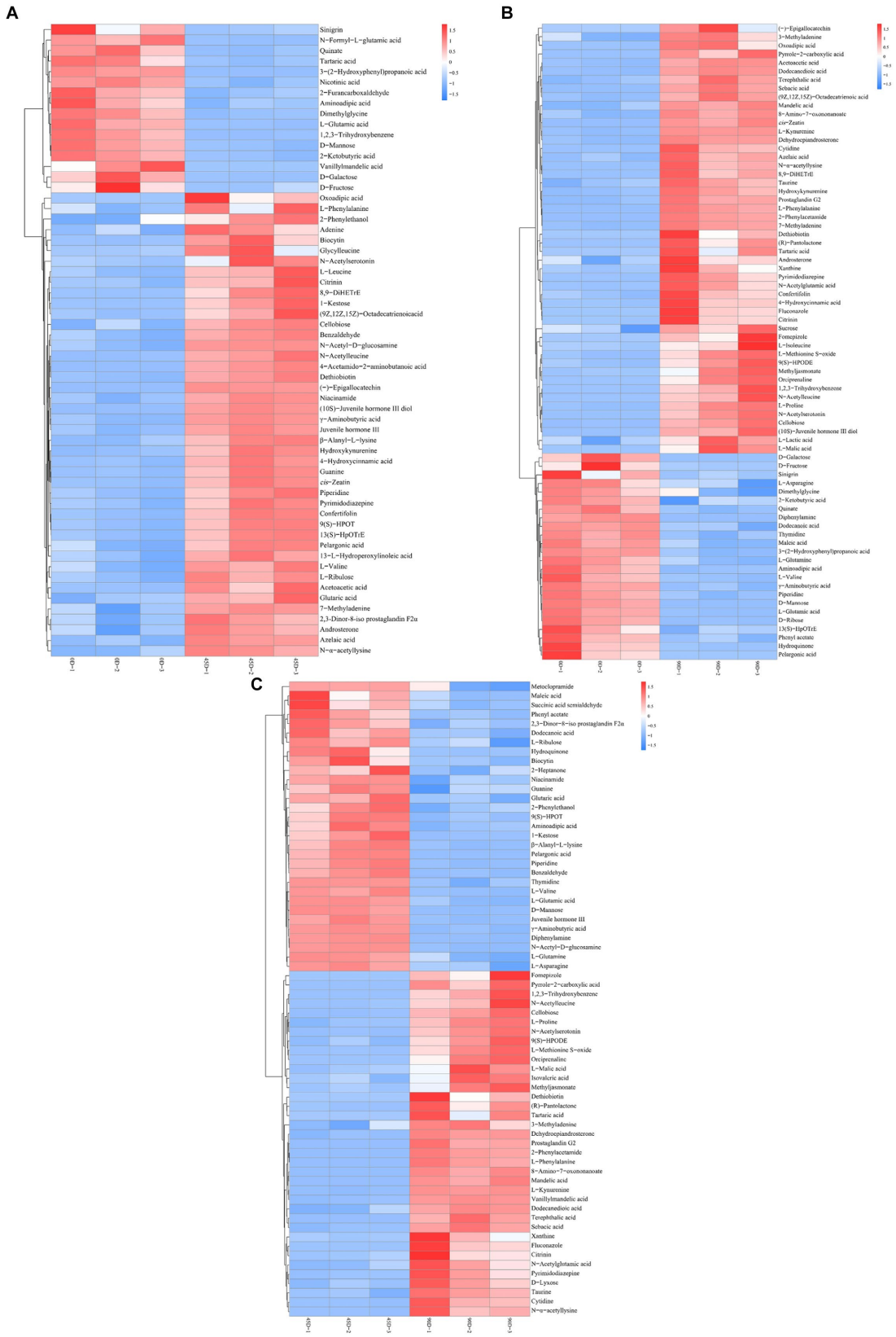

FIGURE 5
Hierarchical cluster analysis of main differential metabolites in LSCK, **(A–C)** indicated the differential metabolites of groups A1, A2, and A3 respectively.

3.4.3 Organic acids and derivatives

The compositions and contents of organic acids play a critical role in fermented vegetables flavors. Most of the organic acids significantly increased in the earlier fermentation stage (0–45 days) and decreased in the later stage (45–90 days). It has been documented that low-salt fermented foods with relatively higher water activity may promote the growth of LAB (30), which could affect the production of organic acids and thus affect the taste and acceptability of LSCK. During fermentation, microorganisms utilize nutrients such as glucose, sucrose, and fructose to produce organic acids through metabolic pathways, including glycolysis, the pentose phosphate pathway, and the citric acid cycle (TCA cycle). This metabolic activity changes the pH of the fermentation environment, contributing to the characteristic flavor of fermented kohlrabi and reducing contamination by spoilage microorganisms (31). The decline in organic acids during the late fermentation stage (45–90 days) can be attributed to various factors. Some studies have suggested that microorganisms prioritize organic acids as a carbon source in the later stages of vegetable fermentation due to the depletion of nutrients such as carbohydrates and proteins, and the accumulation of total acids during fermentation leads to a decrease in pH, which affects microbial reproduction and metabolism (32). Lactic acid was a differential metabolite in group A2 (0D–90D), which continuously increased during fermentation. Lactic acid is the most common core organic acid in fermented vegetables, usually produced by *Lactobacillus*, and contributes greatly to many fermented foods flavors. The increase in lactic acid during fermentation is mainly dependent on the Embden-Meyerhof pathway (EMP) as well as the degradation of other organic acids (e.g., malic and citric acid) (33). Pyruvate, an essential intermediate product in basic metabolic pathways such as glycolysis, malate-lactate fermentation, and the TCA cycle, increased in the early stage (0–45 days) and decreased in the later stage (45–90 days). Environmental limitations (pH and oxygen content) reduce the rate of pyruvate production during the later stage of fermentation. Under anaerobic conditions, pyruvate produced from glycolysis is ultimately converted to lactic acid through lactic acid fermentation (34). As a key substance in the central carbon metabolic pathway, pyruvate is also consumed in metabolic pathways such as the TCA cycle and branched-chain amino acid synthesis (35). Malic acid was the differential metabolite between group A2 (0D–90D) and group A3 (45D–90D) and gradually increased during fermentation. With its strong acidity, malic acid directly enhances the sourness of fermented foods (36). Organic acids are also essential in regulating plant vital activities. For instance, salicylic acid plays a primary role in plant defense and immune responses (37), while benzene-ring-containing carboxylic acids and their derivatives can induce and produce resistance in a wide variety of plants (38). The production of organic acids during kohlrabi fermentation involves many complex metabolic networks and enzyme-catalyzed reactions, necessitating further investigation into the flavor composition and related metabolic pathways of fermented kohlrabi.

3.4.4 Carbohydrates and carbohydrate conjugates

Most of the carbohydrates and carbohydrate conjugates declined with the extension of fermentation period. For example, differential metabolites such as D-glucose, D-fructose, D-galactose, and D-ribose decreased continuously, reaching their lowest in 90D. Carbohydrate metabolism is fundamental to microbial growth and the formation of flavor compounds. Guérin et al. (39) reported that the characteristic sweetness of fermented products was largely dependent on the release of monosaccharides, especially glucose, fructose, and galactose. It is well known that *Lactobacillus lactis* utilizes straight-chain starch and sucrose to release D-glucose, which is finally metabolized to produce pyruvic acid and acetyl-coenzyme A via the EMP pathway. Acetyl-coenzyme A is a substrate for the further production of flavors (e.g., organic acids and ethanol) (40). The sucrose content in LSCK did not change remarkably during fermentation since the molecular structure of sucrose was more complex compared to other monosaccharides, requiring multiple metabolic steps to be fully utilized by microorganisms. Microorganisms primarily metabolize glucose and fructose in the early fermentation stages, while sucrose utilization remains relatively low, consistent with a previous study (41). Similar to sucrose, cellobiose, a disaccharide composed of two glucose molecules produced by the enzymatic hydrolysis of lignocellulose or cellulose (42), significantly increased among the three treatment groups (A1, A2, and A3) during fermentation. This increase may be related to the abundant dietary fiber in kohlrabi (43). D-mannose, another common differential metabolite among the three groups (A1, A2, and A3), declined significantly during fermentation, likely due to its metabolism by yeasts in LSCK (44).

3.5 Common differential metabolites in LSCKs

Venn diagrams were constructed for statistically analyzing the differential metabolites in the three groups (A1, A2, and A3), as shown in Figures 6A,C. The three treatment groups totally shared 17 common metabolites (Figure 6B), including 7 amino acids, peptides, and analogues (L-phenylalanine, aminoadipic acid, L-valine, L-glutamic acid, N-alpha-acetyllysine, N-acetylleucine, and γ-aminobutyric acid), 3 carbohydrates and carbohydrate conjugates (tartaric acid, cellobiose, and D-mannose), 2 fatty acyls (pelargonic acid and dethiobiotin), 1 benzopyrans (citrinin), 1 indoles and derivative (N-acetylserotonin), 1 phenol (1,2,3-trihydroxybenzene), 1 piperidine (piperidine), and 1 other (pyrimidodiazepine). It was obvious that these 17 differential metabolites varied significantly, which could be used as potential biomarkers for distinguishing the three different LSCKs. The 0D produced the highest levels of D-mannose, aminoadipic acid, and L-glutamic acid, which decreased significantly as fermentation progressed. L-Glutamic acid, an amino acid that provides fresh flavor, can be converted to other amino acids through various biochemical pathways during fermentation. For example, glutamic acid can react with α-ketoglutarate to form alanine via glutamic oxaloacetic transaminase (GOT) in the alanine, aspartate, and glutamate metabolism pathway. Additionally, a portion of glutamate can also be converted to aspartic acid, catalyzed by GOT, leading to a significant increase in aspartic acid content during LSCK fermentation (45). The 45D had higher differential metabolites of L-valine, γ-aminobutyric acid, piperidine, and pelargonic acid. L-valine and γ-aminobutyric acid, categorized as amino acids, peptides, and analogues, initially increased and then decreased during LSCK fermentation. This increase during the early stages might be due to protein degradation in LSCK, while the subsequent decrease could result from protein synthesis and participation in other metabolic processes, consistent with a previous studies (46). In 90D, the most

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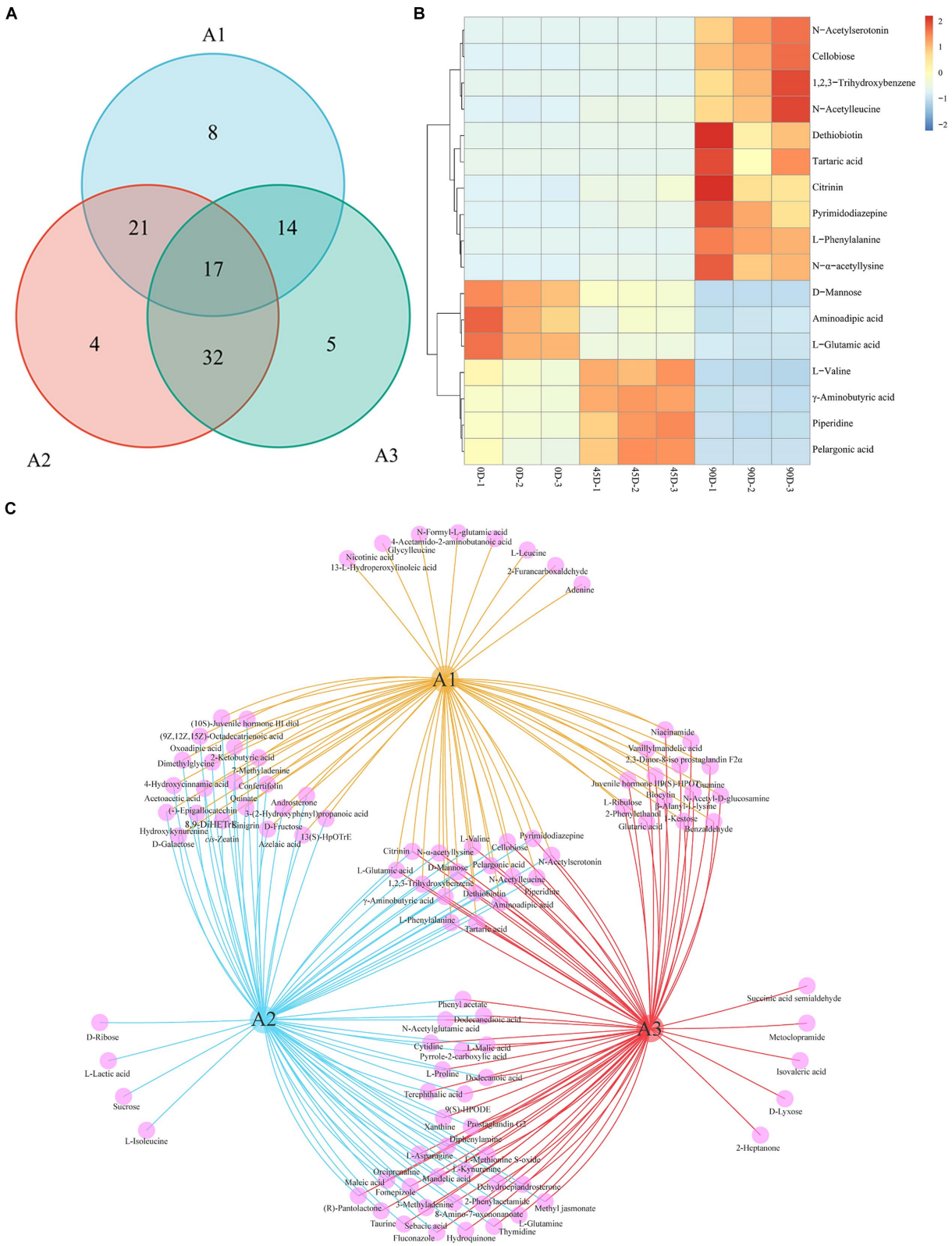

FIGURE 6
Venn diagram and common differential metabolites analysis for the three treatment groups of LSCK. **(A)** The number of metabolic sets of Venn diagram. **(B)** Hierarchical clustering heatmap of common differential metabolites. **(C)** Venn diagram visualization results.

abundant differential metabolites were N-acetylserotonin, cellobiose, 1,2,3-trihydroxybenzene, N-acetylleucine, dethiobiotin, tartaric acid, citrinin, pyrimidodiazepine, L-phenylalanine, and N-α-acetyllysine. Tartaric acid with an excellent antioxidant property gradually increased during fermentation, potentially enhancing the antioxidant activity of LSCK (47).

3.6 KEGG enrichment and metabolic pathway analysis

To investigate the disturbed metabolic pathways, pathway enrichment analysis on all the differential non-volatile metabolites was performed using the KEGG database [$-\log10(p)>1.3$, impact >0.1]. As shown in Figure 7A, the most enriched pathways were alanine, aspartate and glutamate metabolism (impact = 0.72), butanoate metabolism (impact = 0.36), α-linolenic acid metabolism (impact = 0.35), arginine biosynthesis (impact = 0.19), and phenylalanine metabolism (impact = 0.47). However, the KEGG enrichment pathways were different among the A1 (0D–45D), A2 (0D–90D), and A3 (45D–90D) groups. As for A1 (0D–45D), the top five pathways were valine, leucine and isoleucine biosynthesis, butanoate metabolism, amino sugar and nucleotide sugar metabolism, aminoacyl-tRNA biosynthesis, and galactose metabolism (Figure 7B). For the A2 (0D–90D) group, the top five pathways were aminoacyl-tRNA biosynthesis, valine, leucine, and isoleucine biosynthesis, galactose metabolism, alanine, aspartate and glutamate metabolism, and arginine biosynthesis (Figure 7C). While for the A3 (45D–90D) group, the primary pathways were alanine, aspartate and glutamate metabolism, aminoacyl-tRNA biosynthesis, arginine biosynthesis, butanoate metabolism, and D-glutamine and D-glutamate metabolism (Figure 7D). These results suggested that the main metabolic pathways in LSCKs changed with the fermentation period, resulting in differential metabolites in LSCKs. Consequently, the metabolic processes need to be further investigated to more accurately monitor the flavor changes of LSCKs during fermentation.

To better analyze the transformation of metabolites during LSCK fermentation, the five important metabolic pathways were integrated into a metabolic network, as seen in Figure 8. In the alanine, aspartate and glutamate metabolism pathway, the L-asparagine reacted with asparagine-oxo-acid transaminase to produce 2-oxosuccinamate,

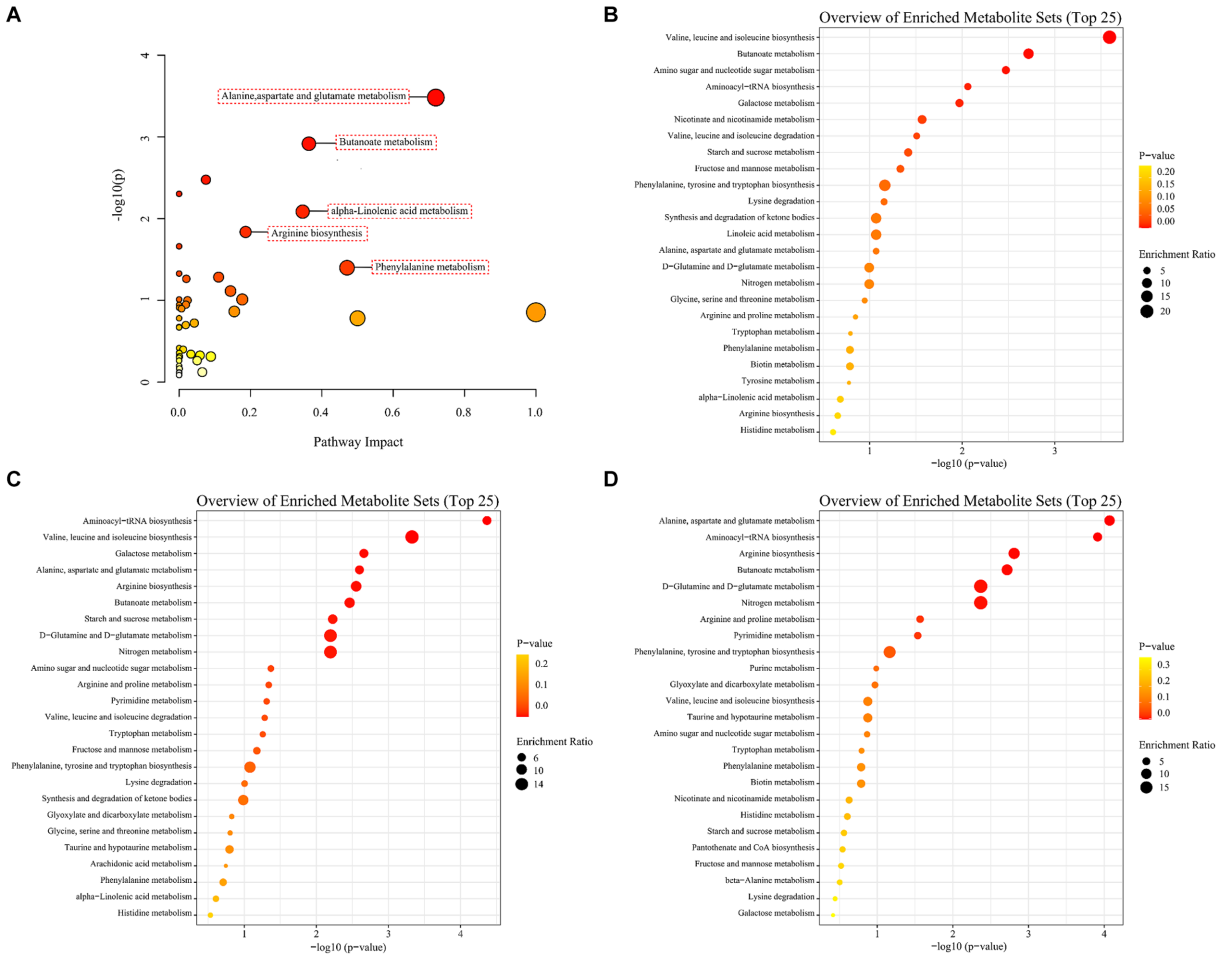

FIGURE 7
Enrichment analysis of metabolic pathways of differential metabolites in LSCK, **(A)** all differential metabolites; **(B)** 0D and 45D; **(C)** 0D and 90D; **(D)** 45D and 90D.

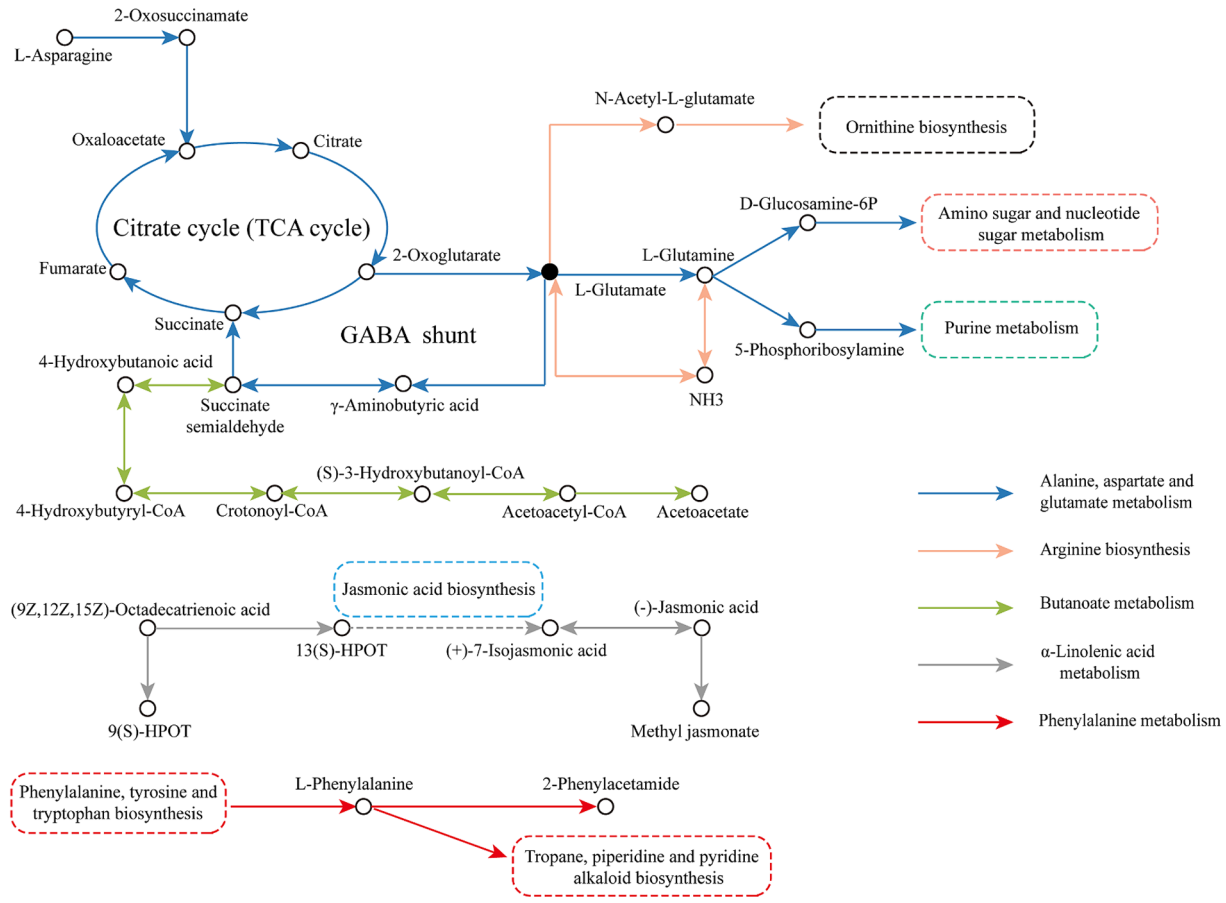

FIGURE 8
The major differential metabolic pathways in LSCK during fermentation.

which was then converted to oxaloacetate catalyzed by ω-amidase and subsequently entered the TCA cycle. 2-oxoglutarate was a product of the TCA cycle, which generated L-glutamate in the presence of glutamate synthase (NADH). Some of the L-glutamate could produce succinate through the γ-aminobutyrate shunt pathway and re-entered the TCA cycle. Part of the 2-oxoglutarate was converted to L-glutamine and catalyzed by phosphate aminotransferase and amidophosphoribosyl transferase to produce D-glucosamine 6-phosphate and 5-phosphoribosylamine, which participated in amino sugar and nucleotide sugar metabolism and purine metabolism, respectively. In the butanoate metabolism pathway, the L-glutamate generated by alanine, aspartate and glutamate metabolism was used as the raw material to produce the succinate semialdehyde through the amino acid metabolism pathway. Succinate semialdehyde was then reduced to 4-hydroxybutanoic acid by glyoxylate reductase and reacted with CoA ligase (ADP-forming) to form 4-hydroxybutyryl-CoA, which generated crotonoyl-CoA catalyzed by 4-hydroxybutanoyl-CoA dehydratase. Crotonoyl-CoA was then transformed to (S)-3-hydroxybutanoyl-CoA catalyzed by enoyl-CoA hydratase, which finally entered the ketone body biosynthesis pathway to produce the final product acetoacetate. In the arginine biosynthesis pathway, 2-oxoglutarate produced by TCA cycle was used as a raw material to generate L-glutamate. A part of L-glutamate was catalyzed by glutamate dehydrogenase (NADP+) to generate NH3, which was then converted to L-glutamine under the catalysis of glutamine synthetase. The other L-glutamate was catalyzed by glutamate N-acetyltransferase/amino-acid N-acetyltransferase to synthesize N-acetyl-L-glutamate and entered the ornithine biosynthesis. In the α-linolenic acid metabolism pathway, α-linolenic acid was used to generate 9(S)-HPOT and 13(S)-HPOT under the catalysis of lipoxygenase. 13(S)-HPOT entered the jasmonic acid biosynthesis pathway to produce (+)-7-isojasmonic acid, which was subsequently converted to (−)-jasmonic acid, and produce the final product methyl jasmonate catalyzed by the jasmonate O-methyltransferase. In the phenylalanine metabolism pathway, L-phenylalanine produced by phenylalanine, tyrosine and tryptophan biosynthesis was utilized as the raw material to produce 2-phenylacetamide catalyzed by L-phenylalanine oxidase. While, some of the L-phenylalanine directly participated in the tropane, piperidine and pyridine alkaloid biosynthesis.

L-glutamic acid is central to amino acid metabolism. Besides the described pathways above, L-glutamic acid is also involved in the arginine and proline metabolism, histidine metabolism, D-amino acid metabolism, glutathione metabolism, ornithine biosynthesis, and other important metabolic pathways. In the butanoate metabolism pathway, L-glutamic acid is converted to γ-amino butyric acid (GABA), a four-carbon nonprotein amino acid, which is a major neurotransmitter in the mammalian central nervous system. GABA-rich foods exhibit a variety of pharmacological functions, such as antihypertensive and antidepressant (48). GABA levels increased

significantly in the earlier fermentation period (0–45 days) and decreased remarkably in the later fermentation period (45–90 days), which was probably due to the glutamate decarboxylation reaction catalyzed by the enzyme glutamate decarboxylase, produced by microorganisms. This enzyme exhibits relatively higher activity at a low pH condition. Microorganisms such as *Lactobacillus lactis* in the earlier fermentation period produce a large number of organic acids, enhancing GABA shunt activity. However, in the later stages, some of the organic acids might be consumed as a carbon source, while GABA was also used to synthesize acetoacetate, an intermediate product in the butanoate metabolism pathway (49). Methyl jasmonate, the final product in the α-linolenic acid metabolism pathway, increased significantly during fermentation. Methyl jasmonate can activate α-linolenic acid metabolism, raising α-linolenic acid concentration and enhancing the activities of lipoxygenase, allene oxide synthase, and allene oxide cyclase (50).

4 Conclusion

In this study, the non-volatile metabolites of a novel low-salt kohlrabies industrially fermented for 0 day, 45 days, and 90 days were analyzed by LC-MS/MS coupled with multivariate statistical analysis. A total of 202 differential non-volatile metabolites were identified among the A1 (0D–45D), A2 (0D–90D), and A3 (45D–90D) (VIP >1, $p < 0.05$, Log2FC >1). The differential non-volatile metabolites were mainly amino acids, peptides, and analogues, fatty acyls, organic acids and derivatives, and carbohydrates and carbohydrate conjugates. Furthermore, a total of 17 major differential non-volatile metabolites were screened based on Venn diagrams analysis, and a total of five relevant metabolic pathways were obtained from metabolic pathway analysis for the first time. Optimization of the LSCK processing and utilization of nutritional and functional non-volatile metabolites in LSCK will be investigated in the future. The microbial diversity and succession will be investigated in the future as well as the mechanisms on the formation of the non-volatile metabolites in LSCK regulated by microorganisms through metagenomics and other techniques. This work reveals the non-volatile metabolites of LSCK by LC-MS/MS-based metabolomics for the first time and provides a theoretical basis for flavor regulation of the industrially fermented LSCK.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

XJ: Formal analysis, Methodology, Writing – original draft. XW: Writing – original draft. HC: Investigation, Writing – original draft. DL: Methodology, Writing – original draft. BD: Formal analysis, Writing – original draft. LA: Formal analysis, Writing – original draft. JY: Formal analysis, Writing – original draft. XN: Data curation, Formal analysis, Supervision, Writing – review & editing. ZZ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1450789/full#supplementary-material

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Chitosan and its derivatives regulate lactic acid synthesis during milk fermentation

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Introduction: The influence of chitosan's physicochemical characteristics on the functionality of lactic acid bacteria and the production of lactic acid remains very obscure and contradictory to date. While some studies have shown a stimulatory effect of oligochitosans on the growth of Lactobacillus spp, other studies declare a bactericidal effect of chitosan. The lack and contradiction of knowledge prompted us to study the effect of chitosan on the growth and productivity of *L. bulgaricus* in the presence of chitosan and its derivatives.

Methods: We used high molecular weight chitosan (350 kDa) and oligochitosans (25.4 and 45.3 kDa). The experiment was carried out with commercial strain of *L. bulgaricus* and the low fat skim cow milk powder reconstituted with sterile distilled water. After fermentation, dynamic viscosity, titratable acidity, pH, content of lactic acid, colony forming units, chitosan and oligochitosans radii were measured in the samples. Fermented dairy products were also examined using sodium dodecyl sulfate electrophoretic analysis, gas chromatography-mass spectrometry and light microscopy.

Results and discussion: The results of the study showed that when *L. bulgaricus* was cultured in the presence of 25.4 kDa oligochitosans at concentrations of 0.0025%, 0.005%, 0.0075% and 0.01%, the average rate of LA synthesis over 24 hours was 11.0×10^{-3} mol/L/h, 8.7×10^{-3} mol/L/h, 6.8×10^{-3} mol/L/h, 5.8×10^{-3} mol/L/h, respectively. The 45.3 kDa oligochitosans had a similar effect, while the average rate of lactic acid synthesis in the control sample was only 3.5×10^{-3} mol/L/h. Notably, 350 kDa chitosan did not affect the rate of lactic acid synthesis compared with the control sample. Interestingly, interaction of chitosan with *L. bulgaricus* led to a slowdown in the synthesis of propanol, an increase in the content of unsaturated and saturated fatty acids, and a change in the composition and content of other secondary metabolites. The quantity of *L. bulgaricus* in a sample with 0.01% chitosan exceeded their content in the control sample by more than 1,700 times. At the same chitosan concentration, the fermentation process was slowed down, increasing the shelf life of the fermented milk product from 5 to 17 days while maintaining a high content of *L. bulgaricus* (6.34×10^6 CFU/g).

KEYWORDS

oligochitosan, lactic acid, lactobacilli, fatty acids, propanol, benzaldehyde, secondary metabolites, glucosamine monomers

1 Introduction

A significant amount of scientific research has been dedicated to improving the quality and functional value of food products over the last few decades, as functional products can enhance people's quality of life and health compared to conventional foods (1, 2). One of the most common groups of functional food products includes fermented dairy products, which are recognized as the most popular and extensively produced and consumed dairy products worldwide (3, 4). In the production of fermented dairy products, the production of lactic acid by bacteria of the genera *Lactobacillus* and *Bifidobacterium genera* is commonly employed (5). Among lactic acid bacteria, *L. bulgaricus* represents the most widespread in Central, Eastern, and South-Eastern Europe, commonly used in the preparation of fermented dairy products (6). During the growth of *L. bulgaricus* in milk and dairy products, the bacteria produce beta-β-galactosidase (E.C.3.2.1.23), which catalyzes the fermentative cleavage of milk lactose (β-D-galactopyranosyl-(1→ 4)-D-glucose) into glucose and galactose, followed by the homofermentation of these carbohydrates into lactic acid as the sole or main end product (7, 8).

The cultivation of lactic acid bacteria usually leads to the production of functional additives such as macro- and micronutrients and dietary fiber (9, 10). Among them, natural poly- and oligosaccharides are of particular interest and value, as they can be used not only as thickeners and gelling agents but also as prebiotics that stimulate the growth of beneficial microorganisms and prolongs their activity in the digestive system (11, 12). Considering this, it is important to note that recent works are increasingly studying Chitosan as polysaccharides with specific properties (13–15).

Chitosan is a partially or fully deacetylated chitin—poly-(1→ 4)-β-D-N-acetylglucosamine. It is produced industrially by the deacetylation of chitin from crab, shrimp, or insect shells and is widely used in pharmaceuticals, food, and cosmetic compositions due to its wide range of antimicrobial activities against bacteria, molds, and yeasts (16–19). As an artificial biopolymer, chitosan is characterized by its molecular weight and degree of deacetylation (%) (20, 21). Based on molecular weight, chitosan can be divided into three categories: low molecular weight (molecular weight $<$ 150 kDa), medium molecular weight (molecular weight $<$ 700 kDa), and high molecular weight (molecular weight $>$ 700 kDa) (22).

High molecular weight chitosan possesses longer molecular chains with the availability of more hydroxyl groups (23). There is also a higher possibility that there are more amino groups, although the number of amino groups is determined by the degree of deacetylation (24). High molecular weight, high degree of polymerization, and, as a consequence, a high number of inter- and intra-molecular hydrogen bonds inside the polymer chains determine the relatively low solubility of High molecular weight chitosan and limit its application in some products due to high viscosity (25). However, specific properties of high molecular weight chitosan found wide application in development of active films for food packaging application (26). Medium molecular weight chitosan is soluble in weak acid solutions. This limits their usage compared to the antimicrobial capacity of acid- and water-soluble chitosan with different degrees of deacetylation and viscosities (22). Low molecular weight chitosan is a linear amino polysaccharide with high nitrogen content. It is a weak base with deprotonated amino groups as nucleophiles that is able to form hydrogen bonds between molecules and has highly reactive groups for crosslinking and chemical activation (27).

Low molecular weight chitosan forms salts with organic and inorganic acids, has chelating and complex properties, and exhibits ionic conductivity as polyelectrolytes (pH $<$ 7) (28). In contrast to high and medium molecular weight chitosan, low molecular weight chitosan demonstrates considerable solubility in various media. However, it is characterized by an unpredictably wide distribution of molecular weight and degree of deacetylation, complicating the standardization of parameters for industrial applications (29–31). Therefore, to address this challenge in dispersing food systems such as milk, a promising avenue lies in the conversion of chitosan into oligomers with more consistent molecular sizes and improved solubility (32). These oligomers, termed oligochitosans, result from the profound depolymerization of chitosan. It is noteworthy that significant disparities exist in the physicochemical parameters and biological activity between chitosan and oligochitosans, accentuating the scientific interest in their comparative study within the realm of food technology applications (33–35).

Amidst its bioavailability, safety, and antimicrobial attributes, chitosan exhibits immunostimulatory and anti-angiogenic properties, mitigating the risk of neurodegenerative conditions while facilitating the regeneration of articular cartilage in osteoarthritis, and enhancing the bioavailability of glucosamine (36–38). In an acid media, chitosan has a high positive charge density due to the protonation of free amino groups and can interact with negatively charged cell walls (31, 39). Therefore, chitosan can potentially interact with the plasma membrane of lactic acid bacteria causing a stress and perturbation of the membrane walls and the death of the cells (40, 41). As a response, LAB can generate a number of adaptive reactions (42, 43) which may insert desirable or undesirable changes in the functionality of lactic acid bacteria and fermented dairy products.

Unfortunately, the influence of chitosan's physicochemical characteristics on the functionality of lactic acid bacteria and the production of lactic acid remains very obscure and contradictory to date. While some studies have shown a stimulatory effect of oligochitosans on the growth of *Lactobacillus* spp., which used added oligochitosans as nutrients (44, 45), others have described a bactericidal effect of chitosan (40, 42, 46). The lack and contradiction of knowledge prompted us to study the effect of chitosan on the growth and productivity of *L. bulgaricus* in the presence of chitosan and its derivatives. The aim of this work was therefore to study the effect of different concentrations of oligochitosans and chitosan on the production of lactic acid and secondary metabolites by *L. bulgaricus* during fermentation and long-term storage of skimmed cow's milk.

2 Materials and methods

2.1 Materials

Commercial strain of *L. bulgaricus* and the low fat skim cow milk powder (solubility−93%, moisture content−5%; fat content−1.5%; proteins−32%, and lactose−50 ± 3%) were purchased from official store of "Stavropolsky Dairy Plant" (Stavropol, Russia). The milk powder was reconstituted with sterile distilled water (50 ml) at temperature 30–35°C during 24 h at sterile conditions to the total solids concentration not <9.0%. The reconstituted skim milk solution was characterized by the following parameters: proteins−3.2%, lactose−5%, total fat−0.15%, and mineralization−0.7%, pH 5.9.

High molecular weight chitosan with 350 kDa molecular weight and 5.0% DD manufactured by Bioprogress Ltd. (Moscow, Russia). Two types of oligochitosans hydrochlorides with 25.4 kDa molecular weight, 1.0% degree of deacetylation and 45.3 kDa molecular weight, 1.5% degree of deacetylation were obtained from the initial chitosan according to the protocol of Berezin et al. (47). The obtained oligochitosans were analyzed in accordance with the requirements of the EU Pharmacopeia 9.0 for chitosan hydrochloride (48). Oligochitosans with a solubility in demineralized water Milli-Q > 99.95% formed a colorless and untroubled 1% solution in accordance with the tests on transparency and degree of opalescence, and degree of liquids coloring. The initial 1% chitosan solution was prepared by dissolving the sample in 1% lactic acid. The pH of the initial solution of the sample was adjusted to pH 5.0 by adding drops of 1M sodium hydroxide solution.

2.2 Milk sample preparation and fermentation

Reconstituted skim milk (100 mL) was mixed with a fixed amount (0.0025, 0.005, 0.075, and 0.01 g) of chitosan stock solution, and the mixture was pasteurized at 85°C for 5 min. After cooling to 43–45°C, 3 g of the commercial freeze-dried starter culture of *L. bulgaricus* was added to achieve a viable count of 10^5 CFU/mL in the sample. The sample was thoroughly mixed and incubated at 43–45°C for 17 days until the maximum titratable acidity was reached. Subsequently, the sample was stored at 4°C before being analyzed.

2.3 Fermented milk product analysis

2.3.1 Dynamic viscosity

After the storage, the dynamic viscosity of fermented dairy product was measured at 20°C using a Brookfield digital rotational viscometer DV-II+PRO (Brookfield Engineering Laboratories, Middleboro, MA, USA).

2.3.2 Titratable acidity and pH

During the fermentation, the milk sample (10 mL) was centrifuged at 6000 rpm (30 min) using MicroCL 17R centrifuge (Thermo FS, Waltham, MA, USA), and pH value of the supernatant was measured with analizator Expert-001 (Econix-Expert, Moscow, Russia). The titratable acidity of fermented dairy product was determined by titration of the supernatant with 0.1 N NaOH using phenolphthalein as indicator (49). Titratable acidity was expressed in percentages of lactic acid content (T, %) or as the volume of 0.1 M of sodium hydroxide consumed for the neutralization of 100 ml of fermented milk product (V_t, mL).

2.3.3 Content of lactic acid

Molar concentrations of lactic acid (mol/L) in the fermented milk supernatant were determined following the method described in the previous work (50). The molar concentrations of lactic acid (mol/L) were calculated following the equation:

$$LA(mol/L) = [V_t\ 0.1]/100; \quad (1)$$

where V_t–titratable acidity of the fermented milk supernatant.

2.3.4 Sodium dodecyl sulfate electrophoretic analysis

The analysis of the protein composition of fermented milk product was carried out after 17 days of storage using the method of sodium dodecyl sulfate (SDS) electrophoretic separation in polyacrylamide gel (20%) under denaturing conditions (DSN electrophoresis) in accordance with the generally accepted protocol (51).

2.3.5 Colony forming units counting

Colony count technique for CFU determination was used in accordance with ISO 15214 (1998-2021) after 10–1000-fold dilution of the fermented milk supernatant solution.

2.3.6 Microscopy

Sample supernatant was centrifuged at 15,000 rpm for 10 min using MicroCL 17R centrifuge (Thermo FS, Waltham, MA, USA) and filtrated using a 0.2 μm PVDF membrane. The morphology of lactic acid bacteria was observed after the membrane filtration. Samples was treated with methylene blue, destained by water washing and dried on air. Photographs were made and recorded using a BIOLAM light microscope (Scopica, Ekaerinburg, Russia).

2.3.7 Measuring the chitosan and oligochitosans radii

The dynamic light scattering method was used to measure the chitosan and oligochitosans molecules radii. Experiments on dynamic light scattering were carried out in a disposable micro-cuvette with a volume of 4 μl, using a detector located at an angle of 90° in the DynaPro Nanostar device (Wyatt Technology, Santa Barbara, CA, USA). The samples were filtered through a 0.2 μm nylon filter. The data was analyzed using the "Regulation fit" (multimodal) analysis method in the Dynamics software (Wyatt Technology, Santa-Barbara, CA, USA).

2.3.8 Analysis of secondary metabolites: gas chromatography-mass spectrometry

After 17 days of storage, a 10 mL sample of the fermented dairy product was centrifuged at 6,000 rpm for 30 min using a MicroCL 17R centrifuge (Thermo FS, Waltham, MA, USA), followed by lyophilization. The residual solid product (1 g) was then extracted twice with 70% ethanol (1:10 wt/v). The resulting extracts were combined and filtered through 0.25 μm PVDF membranes. Secondary metabolites were separated and analyzed using an Agilent 5975B gas chromatograph equipped with a mass selective detector (Agilent Technologies, Santa Clara, CA, USA). The separation of secondary metabolites was performed using a capillary column DB-5MS (5% phenyl methyl siloxane, J&W 122-5062). Identification of components in the mass spectra was conducted using a library of mass spectra NIST0.5A. Comparative semi-quantitative analysis of secondary metabolites was performed based on peak areas without using correction factors. The semi-quantitative content of secondary metabolites was calculated from the peak area without considering the peak of lactic acid and without using correction factors.

2.4 Statistical analysis

In order to compare the means of factor's levels, a one-, two-, or three-way analysis of variance (ANOVA) with subsequent Dunnett's test (comparing several treatments with a control), Student's *t*-test (matching the means of two groups) or Tukey's Honest Significant Difference (HSD) test (performing multiple pairwise comparisons) were applied. R functions aov, DunnettTest, *t*-test, TukeyHSD and DescTools packages were involved in the statistical analysis. Statistical differences between groups were set as significant at $p < 0.05$ level with correction for multiple pairwise comparisons. Correlation analysis was performed using Pearson's Criterion. Plots were created in Microsoft Office Excel (MS Corporation, Shadeland, IN, USA). The data in tables and graphs are shown as the mean ± the half-width of 95% confidence interval ($n = 3$).

3 Results and discussion

3.1 Cultivation of *L. bulgaricus* in the presence of chitosan and oligochitosans

The effect of oligochitosans of 25.4 kDa molecular weight, 1% degree of deacetylation, and 45.3 kDa molecular weight, 1.5% degree of deacetylation on the lactic acid fermentation process was studied in comparison with CH of 350 kDa molecular weight, 5.0% degree of deacetylation at *L. bulgaricus* cultivation. During cultivation, pH and titratable acidity of the culture liquid were measured in experimental and control samples every 4 h for 24 h. Based on the results of titratable acidity, the molar concentration and the rate of synthesis of lactic acid were calculated. Figure 1 shows changes in the pH and titratable acidity of a culture liquid containing 0.0025, 0.005, 0.0075, and 0.01% oligochitosans of 25.4 and 45.3 kDa. According to the data obtained, pH depends on the concentration of oligochitosans (Figures 1A, C). Notably, the maximum decrease in pH was observed at oligochitosans concentration of 0.0025% (Table 1). At the end of 24-h cultivation, pH of the experimental samples was lower than in control sample by 73% in 25.4 kDa oligochitosans, by 75% in 45.3 kDa oligochitosans, and by 1% in 350 kDa chitosan. At higher concentrations, pH less intense decreased in samples with oligochitosans, but particularly was not changed in samples with chitosan. Inversely dependent, the titratable acidity of the culture liquid decreased with the increase of oligochitosans concentration, but was higher than the titratable acidity of control samples (Figures 1B, D).

According to Table 1, at the same percentage concentration of oligochitosans and chitosan, their molar concentration in fermented dairy products differs significantly. However, the total content of glucosamine monomers included in their composition was the same. In the acidic environment, the oligochitosans amino groups are in a protonated state (52). Being positive charge, they can interact with negatively charged cell walls of lactic acid bacteria (53, 54). With an increase in the concentration of oligosaccharides in the experimental samples, the content of glucosamine monomers also increased. Consequently, the number of positively charged amino groups capable of interacting with lactic acid bacteria increased.

It was revealed that at 0.0025% concentration and 1.39×10^{-4} mol/L glucosoamine monomers, oligochitosans increase the synthesis of lactic acid by 3 times (25.4 kDa) and 2.7 times (45.3 kDa) relative to control sample. It should be noted that samples with chitosan, like samples with oligochitosans, contained 1.39×10^{-4} mol/L glucosoamine monomers at a concentration of 0.0025%. However, it was surprisingly found that at 0.0025% chitosan and 1.39×10^{-4} mol/L glucosoamine monomers, chitosan slowed down the synthesis of lactic acid by 0.98 times relative to the control. Higher concentrations of chitosan more intense affected lactic acid synthesis and consequently, fermented dairy product with chitosan had less lactic acid concentration than control sample.

On the other hand, at the highest concentration of oligochitosans (0.01 %), the content of glucosamine monomers reached 5.56×10^{-4} mol/L and lactic acid synthesis slowed down. However, the resulting lactic acid content in both oligochitosans groups was still higher than in the control sample. The rate of lactic acid synthesis is critically important in milk fermentation (55). Thus, dependence of the average rate of lactic acid synthesis on concentration of glucosamine monomers of oligochitosans and chitosan at 24 h-cultivation was analyzed and presented in Figure 2.

The analysis of the interaction of lactic acid bacteria with the positively charged amino groups of oligochitosans glucosamine monomers shows that with an increase in their concentration, the rate of lactic acid synthesis slows down (Figure 2). Theoretically, at a concentration of oligochitosans glucosamine monomers close to 10^{-3} mol/L, a process of deep inhibition of lactose metabolism and lactic acid synthesis is possible (56, 57). Similarly, an increase in the content of glucosamine monomers of 350 kDa chitosan leads to a reduction in lactic acid, as shown in Figure 2. According to Figure 1, pH and titratable acidity stabilize on the 20th h of cultivation in samples with 0.0025% oligochitosans. This indicates a slowdown in the synthesis of lactic acid by lactic acid bacteria. Therefore, it is critically important to understand the dynamics of lactic acid accumulation in the presence of oligochitosans. For this

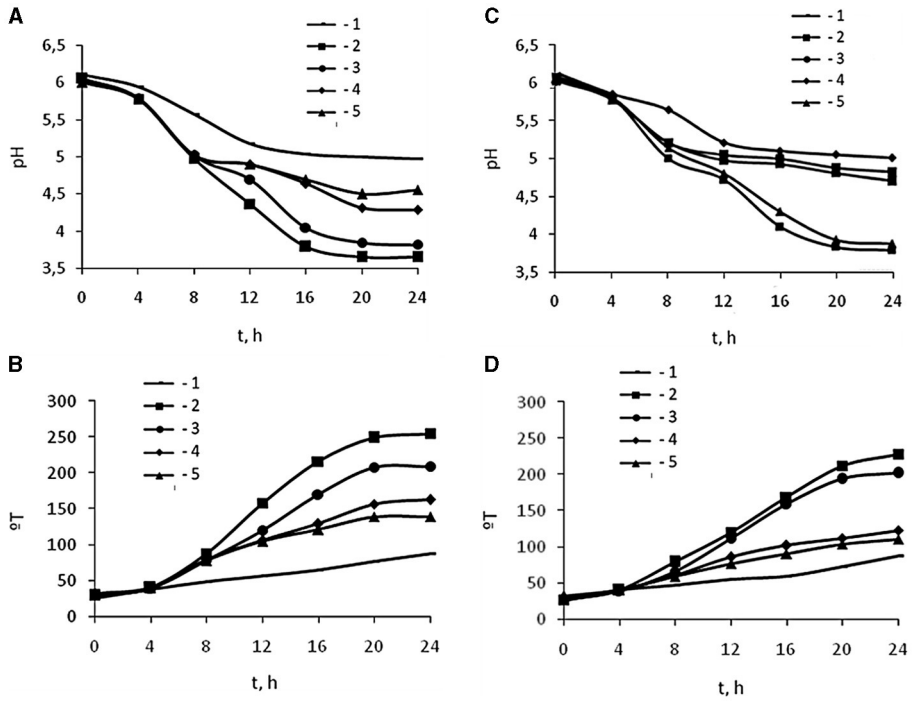

FIGURE 1
Effect 25.4 kDa **(A, B)** and 45.3 kDa **(C, D)** OCHs on active acidity pH **(A, C)** and titratable acidity °T **(B, D)** during 24 h cultivation (t, h) of *L. bulgaricus*: 1 – control sample, 2 – 0.0025 % OCHs, 3 – 0.0050 % OCHs, 4 – 0.0075 % OCHs, 5 – 0.01 % OCHs.

purpose, the rate of lactic acid synthesis was analyzed every 4 h of cultivation. The results obtained are presented in Figure 3.

Notably, the maximum rate of lactic acid synthesis was observed at 0.0025% 25.4 kDa oligochitosan on the 12th h of *L. bulgaricus* cultivation, which is 6.44 times higher than in the control sample. Similarly, on the 12th h of *L. bulgaricus* cultivation, the rate of lactic acid synthesis was the most intense, being 5.44 times higher than in the control sample. It should be pointed out that with an increase in oligochitosans concentration, the rate of lactic acid synthesis decreases and achieves the minimal value on the 24th h of *L. bulgaricus* cultivation. Simultaneously, the rate of lactic acid synthesis reached the maximum value only on the 24th h of *L. bulgaricus* cultivation.

Thus, the results obtained show that 25.4 and 45.3 kDa oligochitosans significantly influence the rate of lactic acid synthesis. The presence of 1.39×10^{-4} mol/L oligochitosan glucosamine monomers in the culture liquid leads to a significant decrease in pH, an increase in titratable acidity, and rapid synthesis and accumulation of lactic acid compared to the control sample. Such oligochitosan activity may be explained by the mechanism of exposure to *L. bulgaricus* through extracellular, intracellular, or both extracellular and intracellular effects (58–61).

The cell wall of *L. bulgaricus* can prevent the direct binding of lactic acid to the cell membrane components and impede intracellular effects (62). Transporting molecules through the barrier of the outer layer of the rigid cell wall occurs through several subtle mechanisms or via simple diffusion (63–66). The porosity of the cell wall and the pore size determine whether oligochitosans can pass through the bacterial cell wall (67). Pore sizes vary among different bacteria and fungi, ranging from 2–4 to 8 nm (68, 69). Therefore, the radii of hydrated oligochitosans were measured using the dynamic light scattering method. According to the data obtained, the average hydrodynamic radius was 2.5 nm for 25.4 kDa oligochitosan and 3.5 nm for 45.3 kDa oligochitosan. Consequently, due to their small sizes, oligochitosans can penetrate the pores of the lactic acid bacteria cell walls and interact with plasma membrane proteins. It is likely that oligochitosans induce stress in lactic acid bacteria cells, leading to adaptive reactions that result in deviations in physiological and biochemical processes (70, 71). For instance, based on the results presented in Figure 3, the presence of 0.0025% oligochitosans in the culture liquid stimulates the acceleration of lactose catabolism, leading to increased lactic acid synthesis and accumulation.

Likely, the reason for such changes is the reaction of lactic acid bacteria stress response caused by oligochitosans. The mechanism of stress response entails the metabolic changes necessary for the survival of lactic acid bacteria (72–74). Previous studies have demonstrated that oligochitosans and chitosan, upon interacting with eukaryotic cells, induce abiotic stress, triggering physiological protective reactions (75). These protective responses may include overexpression of genes associated with carbohydrate metabolism (76), among other reactions (77–79).

At high concentrations, oligochitosans cause not only intracellular but also extracellular effects. Gram-positive bacteria have a negative charge due to the presence of phosphate groups associated with teichoic acids in the cell wall structure (80, 81). With an increase in the concentration of oligochitosans molecules, an electrostatic interaction with negatively charged lactic acid

TABLE 1 Effect of OCHs and CH on LA fermentation parameters after 24 h of *L. bulgaricus* cultivation.

Index	Concentration of OCHs and CH, %				
	0 (control)	0.0025	0.005	0.0075	0.01
25.4 kDa, 1% DD OCH (glucosamine monomers 140/mol OCH)					
Molar concentration of OCH, mol/L	-	0.99×10^{-6}	1.98×10^{-6}	2.98×10^{-6}	3.97×10^{-6}
Glucosamine monomers content, mol/L	-	1.39×10^{-4}	2.78×10^{-4}	4.17×10^{-4}	5.56×10^{-4}
pH	5.00 ± 0.20^{A}	3.65 ± 0.10^{B}	3.82 ± 0.10^{B}	4.29 ± 0.10^{C}	4.56 ± 0.20^{C}
LA content, mol/L	0.085 ± 0.009^{A}	0.255 ± 0.02^{B}	0.209 ± 0.019^{C}	0.163 ± 0.015^{D}	0.139 ± 0.011^{E}
The average rate of LA synthesis, mol/L/h	$3.5 \times 10^{-3A,I}$	$11.0 \times 10^{-3B,I}$	$8.7 \times 10^{-3C,I}$	$6.8 \times 10^{-3D,I}$	$5.8 \times 10^{-3E,I}$
45.3 kDa, 1.5% DD OCH (glucosamine monomers 248/mol OCH)					
Molar concentration of OCH, mol/L	-	0.56×10^{-6}	1.12×10^{-6}	1.68×10^{-6}	2.24×10^{-6}
Glucosamine monomers content, mol/L	-	1.39×10^{-4}	2.78×10^{-4}	4.17×10^{-4}	5.56×10^{-4}
pH	5.00 ± 0.20^{A}	3.78 ± 0.10^{B}	3.87 ± 0.10^{B}	4.70 ± 0.20^{C}	4.82 ± 0.10^{C}
LA content, mol/L	0.087 ± 0.009^{A}	0.228 ± 0.025^{B}	0.202 ± 0.018^{B}	0.123 ± 0.011^{C}	0.111 ± 0.008^{C}
The average rate of LA synthesis, mol/L/h	$3.6 \times 10^{-3A,I}$	$9.5 \times 10^{-3B,I}$	$8.4 \times 10^{-3B,I}$	$5.1 \times 10^{-3C,II}$	$4.6 \times 10^{-3C,II}$
350 kDa, 5% DD CH (glucosamine monomers 1850/mol CH)					
Molar concentration of CH, mol/L	-	0.75×10^{-7}	1.50×10^{-7}	2.25×10^{-7}	3.00×10^{-7}
Glucosamine monomers content, mol/L	-	1.39×10^{-4}	2.78×10^{-4}	4.17×10^{-4}	5.56×10^{-4}
pH	5.00 ± 0.20^{A}	4.95 ± 0.20^{A}	4.99 ± 0.10^{A}	4.98 ± 0.10^{A}	5.01 ± 0.20^{A}
LA content, mol/L	0.090 ± 0.010^{A}	0.089 ± 0.009^{A}	0.082 ± 0.009^{B}	0.083 ± 0.009^{B}	0.075 ± 0.007^{C}
The average rate of LA synthesis, mol/L/h	$3.8 \times 10^{-3A,I}$	$3.7 \times 10^{-3A,II}$	$3.4 \times 10^{-3B,II}$	$3.4 \times 10^{-3B,III}$	$3.1 \times 10^{-3C,III}$

The values represent the mean ± SD (n = 3). Means without a common letter within the same column (I–III) and row (A–E) indicate significant difference at $p < 0.05$.

bacteria cell walls can occur (40, 82, 83). Cell walls are dynamic structures that undergo changes during replication, development, and age (84). This flexibility allows different molecules to pass through the cell wall (85). Consequently, ion immobilization of oligochitosans on the lactic acid bacteria cell wall can lead to a loss of flexibility, porosity, and alteration of its permeability (86, 87). The low permeability of the lactic acid bacteria cell walls leads to reduction of nutrient intake, resulting in a slowdown in the lactic acid synthesis and an increase in the pH of the culture liquid. This process, as depicted in Figures 1–3 and Table 1, depends on the molecular weight of oligochitosans and their concentration. Thus, two mechanisms of their action are realized depending on the concentration of oligochitosans. At low concentrations of oligochitosans, the acceleration of lactic acid synthesis occurs due to intracellular processes in *L. bulgaricus*. However, increasing the concentration of oligochitosans by four times leads to the realization of their extracellular effects, which slow down the metabolism of lactose and decrease the accumulation of lactic acid by 1.6 times at 25.4 kDa oligochitosan, or by 1.28 times at 45.3 kDa oligochitosan. The implementation of these two mechanisms of oligochitosans activity is multidirectional. As observed in Figure 2 and Table 1, the dominance of the mechanism of extracellular action of oligochitosans increases with an increase in the content of glucosamine monomers.

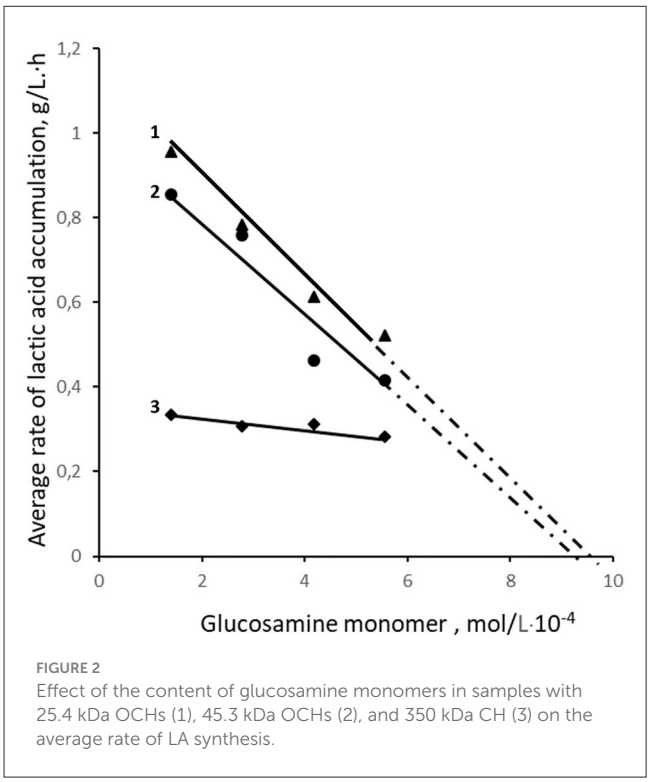

FIGURE 2
Effect of the content of glucosamine monomers in samples with 25.4 kDa OCHs (1), 45.3 kDa OCHs (2), and 350 kDa CH (3) on the average rate of LA synthesis.

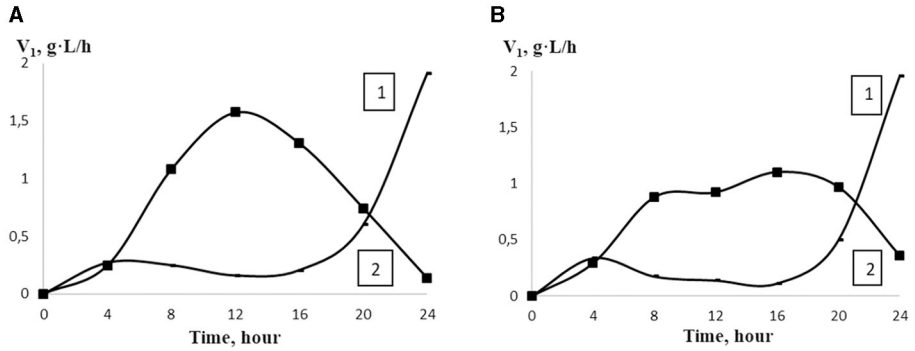

FIGURE 3
The rate of LA accumulation (V_1) in the control sample (1) and in the presence of 25.4 kDa OCHs **(A)** and 45.3 kDa OCHs **(B)** at concentration of 0.0025% (2) during 24 h cultivation of *L. bulgaricus* (t).

These observations are confirmed by the study of the effect of various concentrations of chitosan on the cultivation of *L. bulgaricus*. According to Table 1, the chitosan molecule contained 1850 glucosamine monomers and had an average hydrodynamic radius of 133.2 nm. Due to the large size of the chitosan molecules and the positively charged amino groups of glucosamine residues, this polysaccharide can interact with the cell walls of lactic acid bacteria (23). With an increase in pH and chitosan concentration, the density of its positive charge increases, and it effectively interacts with the negatively charged cell walls of *L. bulgaricus*. As can be seen from Table 1, at 0.0025% chitosan and oligochitosans, their molar concentrations differ, but the total content of glucosamine monomers remains the same (1.39×10^{-4} mol/L). However, at 0.0025% oligochitosans, interaction with *L. bulgaricus* leads to the realization of the intracellular mechanism of their action, which leads to an acceleration of lactic acid synthesis. At the same time, 0.0025% chitosan causes opposite effects in lactic acid bacteria, which slow down the catabolism of lactose and the lactic acid synthesis due to extracellular interaction. At 0.01% chitosan and oligochitosans, the total content of glucosamine monomers increases by 4 times and reaches 5.56×10^{-4} mol/L.

As a result, the total number of protonated amino groups of these polysaccharides increases, which leads to effective interaction with the lactic acid bacteria cell wall and inhibition of lactose catabolism. Moreover, the biochemical effects of oligochitosans and chitosan are unidirectional, which may indicate the same mechanism of their action due to extracellular effects. Thus, comparative studies of the effect of different concentrations of oligochitosans and chitosan on the cultivation of *L. bulgaricus* revealed two mechanisms of their action. At low concentrations, oligochitosans interact with the plasma membrane, which leads to an acceleration of metabolic processes in *L. bulgaricus*. At 0.01% oligochitosans and chitosan, they interact with the *L. bulgaricus* cell wall and affect the rate of lactic acid synthesis. This observation confirms the previously obtained results (88).

Thus, the results obtained expand the understanding of the mechanism of oligochitosans and chitosan effect on lactic acid bacteria. However, regarding fermented milk products, it is also critical to understand the oligochitosans and chitosan behavior toward lactic acid bacteria not only at cultivation but also at storage. Therefore, the next stage of the experiments represents the effect of these cationic polysaccharides on the metabolic processes of *L. bulgaricus* during long-term storage of fermented dairy products.

3.2 Effect of oligochitosans and chitosan on the metabolic processes of *L. bulgaricus* during long-term storage of fermented milk product

During a 17-day storage period of fermented dairy products containing oligochitosans, a slowdown in lactic acid fermentation was observed alongside a decrease in oligochitosans concentration. The interaction of oligochitosans with the plasma membrane of *Lactobacillus bulgaricus* affects intracellular processes related to lactose catabolism and cell division. Table 2 illustrates that at low concentrations of oligochitosans, the CFU value of *L. bulgaricus* were lower than in the control sample. This decrease in viable cells in the fermented dairy product led to a slowdown in the average rate of lactic acid synthesis after 17 days of storage. Consequently, there was a slight increase in LA content, maintaining low pH values compared to the control sample. At a concentration of 0.01% oligochitosans, where they interact with the *L. bulgaricus* cell wall, the synthesis of LA slows down, but a significant number of viable *L. bulgaricus* cells are preserved.

Based on the data presented in Table 2, it is evident that an increase in the concentration of chitosan leads to a reduction in the intensity of lactic acid fermentation. The most notable deceleration of lactose catabolism via homofermentative lactic acid fermentation occurred in the sample containing 0.01% chitosan. This decrease in lactose catabolism intensity in *L. bulgaricus* could be attributed to the interaction between chitosan and the bacterial cell wall. This external influence may disrupt the permeability of the cell wall to the substrate and the enzyme β-galactosidase (EC 3.2.1.23), responsible for lactose hydrolysis into glucose and galactose (89). Additionally, structural alterations in the cell wall might impede the active transport of lactose hydrolysis products into Lactobacillus cells (90). On the 17th day of storage, the *L. bulgaricus* content in the control sample of fermented milk product was measured at

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TABLE 2 Effect of OCHs and CH on pH and metabolism and content of *L. bulgaricus* in the fermented milk product at 17-days storing.

Index	Concentration of OCHs and CH, %				
	0 (control)	0.0025	0.005	0.0075	0.01
25.4 kDa, 1% DD OCH (glucosamine monomers 140/mol OCH)					
pH	3.80 ± 0.10^{A}	3.30 ± 0.10^{B}	3.66 ± 0.10^{A}	3.88 ± 0.10^{A}	4.35 ± 0.10^{B}
LA content, mol/L	0.121 ± 0.011^{A}	$0{,}037 \pm 0.001^{B}$	$0{,}017 \pm 0.001^{C}$	$0{,}034 \pm 0.002^{B}$	$0{,}015 \pm 0.002^{C}$
The average rate of LA synthesis, mol/L/h	$7.3 \times 10^{-3A,I}$	$2.2 \times 10^{-3B,I}$	$1.0 \times 10^{-3C,I}$	$2.0 \times 10^{-3B,I}$	$0.9 \times 10^{-3C,I}$
L. bulgaricus content, CFU/g	$3.73 \times 10^{3A,\alpha}$	$2.28 \times 10^{2B,\alpha}$	$2.46 \times 10^{2B,\alpha}$	$3.89 \times 10^{3A,\alpha}$	$4.65 \times 10^{4C,\alpha}$
45.3 kDa, 1.5% DD OCH (glucosamine monomers 248/mol OCH)					
pH	3.82 ± 0.10^{A}	3.48 ± 0.10^{B}	3.61 ± 0.10^{B}	3.91 ± 0.10^{A}	3.96 ± 0.10^{A}
LA content, mol/L	0.116 ± 0.01^{A}	0.043 ± 0.001^{B}	0.048 ± 0.001^{B}	0.070 ± 0.001^{C}	0.063 ± 0.001^{C}
The average rate of LA synthesis, mol/L/h	$6.8 \times 10^{-3A,I}$	$2.5 \times 10^{-3B,I}$	$2.8 \times 10^{-3B,II}$	$4.1 \times 10^{-3C,II}$	$3.7 \times 10^{-3C,II}$
L. bulgaricus content, CFU/g	$3.71 \times 10^{3A,\alpha}$	$2.6 \times 10^{2B,\alpha}$	$2.56 \times 10^{2B,\beta}$	$3.49 \times 10^{3A,\alpha}$	$4.89 \times 10^{4C,\alpha}$
350 kDa, 5% DD CH (glucosamine monomers 1850/mol CH)					
pH	3.82 ± 0.1^{A}	3.85 ± 0.1^{A}	3.87 ± 0.1^{A}	3.96 ± 0.1^{A}	4.04 ± 0.1^{A}
LA content, mol/L	0.113 ± 0.01^{A}	0.102 ± 0.009^{B}	0.104 ± 0.009^{B}	0.072 ± 0.007^{C}	0.063 ± 0.005^{C}
The average rate of LA synthesis, mol/L/h	$7.0 \times 10^{-3A,I}$	$6.0 \times 10^{-3B,II}$	$6.0 \times 10^{-3B,III}$	$4.2 \times 10^{-3C,II}$	$3.7 \times 10^{-3C,II}$
L. bulgaricus content, CFU/g	$3.69 \times 10^{3A,\alpha}$	$3.65 \times 10^{3A,\beta}$	$3.59 \times 10^{3A,\gamma}$	$5.3 \times 10^{5B,\beta}$	$6.34 \times 10^{6C,\beta}$

The values represent the mean ± SD (n = 3). Means without a common letter within the same column (I–III; α-γ) and row (A–C) indicate significant difference at $p < 0.05$.

3.69×10^3 CFU/g. Table 2 illustrates that the inclusion of chitosan resulted in an increase in *L. bulgaricus* content, reaching 3.65×10^3 CFU/g at 0.0025% chitosan, 3.59×10^3 CFU/g at 0.005% chitosan, 5.3×10^5 CFU/g at 0.0075% CH, and 6.34×10^6 CFU/g at 0.01% chitosan. Consequently, the fermented milk product sample containing 0.01% CH contained 1,700 times more *L. bulgaricus* than the control sample.

As can be seen from Tables 1, 2, the effect of oligochitosans and chitosan on the metabolic processes of *L. bulgaricus* is multidirectional. The acceleration of metabolic processes is realized due to intracellular processes of interaction of oligochitosans with lactic acid bacteria cells. Chitosan interacting with the lactic acid bacteria cell wall slows down metabolic processes. Based on the results obtained, it is logical to assume that due to the differences in the mechanism of action of oligochitosans and chitosan, they should significantly affect the composition and content of secondary metabolites during long-term storage of fermented milk product. In this regard, the relative content of secondary metabolites in the control and experimental samples was determined using GC-MS. A typical GC-MS chromatogram of a fermented milk product extract on the 17th day of storage is shown in Supplementary Figure 1. The chromatogram contains a profile of the metabolites that were analyzed and included in Table 3. Samples of fermented dairy products with 45.3 kDa oligochitosan were chosen due to the higher values of rate of lactic acid synthesis and lactic acid content in comparison with ones with addition of 25.4 kDa oligochitosan.

The analysis of the results shows that 0.0025% of 45.3 kDa oligochitosan accelerates the synthesis of propanol, leading to a 230% increase in its relative content compared to the control sample. Propanol metabolism is closely related to the synthesis of lactic acid (91), the content of which was 260% higher in this sample than in the control sample. The acceleration of lactic acid synthesis was accompanied by an increase in propanol synthesis, consistent with previously obtained results (92, 93). With an increase in oligochitosan concentration, the process of lactic acid synthesis slows down and the relative propanol content decreases. Another important component of secondary metabolites presented in Table 3 is benzoic acid. It was revealed, that different concentrations of oligochitosan had practically no effect on its synthesis compared to the control sample.

Fermented dairy products also contained a significant number of phenolic compounds: 3,4-dihydro-4,4-dimethyl-2H-1,2-benzisothiazine; 2-furanmethanol; 3,5-dimethyl-4-deuteroxymethyl-isoxazole; 4H-pyran-4-one, 3-hydroxy-2-methyl; 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one. Their total content in the control sample reached 54.68%. In the experimental samples of fermented dairy products with oligochitosan, the content of phenolic compounds ranged from 31.81% at a concentration of 0.0025% 45.3 kDa oligochitosans to 45.65% at a concentration of 0.005% 45.3 kDa oligochitosans. It is important to note that phenolic substances exhibit antioxidant properties and affect the sensory characteristics of fermented dairy products (94, 95).

Fatty acids have a significant role in shaping the sensory attributes of fermented dairy products. Saturated fatty acids made up 1.81% of the control sample, including methyl stearate (0.44%), heptadecanoic acid, methyl ester (0.89%), and heptadecanoic acid, 16-methyl-, and methyl ester (0.49%). Furthermore, the control sample had two unsaturated fatty acids, 8-octadecanoic acid, methyl ester, (E)—(0.66%) and 12-octadecenoic acid, methyl ester (0.63%), that were absent from the other samples. The control

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TABLE 3 Relative content of the main secondary metabolites in extracts from fermented dairy products containing various concentrations of OCHs and CH on the 17th day of storage.

Retention time	Substance, CAS, molecular formula	The relative content, %						
		Control	Concentration of 45.3 kDa OCH, %			Concentration of 350 kDa CH, %		
			0.0025	0.0075	0.01	0.0025	0.0075	0.01
5.64	2-Propanol, 000067-63-0, C_3H_8O	8.35	19.50	2.45	1.63	4.84	2.08	n.d.
5.66	3,4-Dihydro-4,4-dimethyl-2H-1,2-benzisothiazine, 2000114-14-7, $C_{49}H_{66}N_{10}O_{12}S$	2.55	3.95	n.d.	n.d.	n.d.	1.69	n.d.
5.72	2-Furanmethanol, 000098-00-0, $C_5C_6C_2$	n.d.	n.d.	n.d.	n.d.	n.d.	2.48	2.05
9.03	3,5-dimethyl-4-deuteroxymethyl-isoxazole, 2000024-72-1, $C_{12}H_{12}N_2O$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.77
9.90	4H-Pyran-4-one, 3-hydro-xy-2-methyl, 000118-71-8, $C_6H_6O_3$	10.17	0.91	1.21	1.22	0.99	2.35	8.85
10.33	Carbamic acid, butyl-, ethyl ester, 000591-62-8, $C_7H_{15}NO_2$	4.13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10.46	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, 028564-83-2, $C_6H_8O_4$	37.86	26.95	44.44	37.24	37.90	35.23	20.70
10.67	Benzoic acid, 000065-85-0, $C_7H_6O_2$	8.87	9.22	7.60	8.68	12.40	12.77	9.62
13.25	Pentadecanoic acid, 001002-84-2, $C_{15}H_{30}O_2$	n.d.	n.d.	n.d.	n.d.	n.d.	1.31	1.28
19.60	Hexadecanoic acid, methyl ester, 000112-39-0, $C_{17}H_{34}O_2$	0.89	0.69	0.73	1.26	0.60	1.14	0.90
19.95	n-Hexadecanoic acid, 000057-10-3, $C_{16}H_{32}O_2$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.91
21.28	8-Octadecenoic acid, methyl ester, (E)-, 026528-50-7, $C_{19}H_{36}O_2$	0.66	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
21.29	12-Octadecenoic acid, methyl ester, 056554-46-2, $C_{19}H_{36}O_2$	0.63	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
21.39	Methyl stearate, 000112-61-8, $C_{19}H_{38}O_2$	0.44	0.37	n.d.	n.d.	0.37	n.d.	n.d.
21.49	Heptadecanoic acid, 16-methyl-, methyl ester, 005129-61-3, $C_{19}H_{38}O_2$	0.49	n.d.	0.39	n.d.	n.d.	0.74	0.55
21.64	Cis-11-octadecenoic acid, 000506-17-2, $C_{18}H_{34}O_2$	n.d.	n.d.	n.d.	n.d.	n.d.	5.79	21.58
21.82	Octadecanoic acid, 000057-11-4, $C_{18}H_{36}O_2$	n.d.	n.d.	n.d.	n.d.	1.07	2.09	2.93
29.91	Cholest-5-en-3-ol, 2000683-53-6, $C_{27}H_{46}O$	1.34	0.98	1.22	2.67	1.25	2.47	1.35

sample of fermented dairy product had a total fatty acid level of 3.11%.

Variations in the content of 45.3 kDa oligochitosan led to notable alterations in the fatty acid synthesis in fermented dairy products. Table 3 shows that samples with an oligochitosan of 45.3 kDa were devoid of unsaturated fatty acids. Heptadecanoic acid, methyl ester ($C_{17}H_{34}O_2$), methyl stearate ($C_{19}H_{38}O_2$), and hepta-decanoic acid, 16-methyl-, methyl ester ($C_{19}H_{38}O_2$) are examples of saturated fatty acids. Their overall content was lower than that of the control sample (3.11%), although it did grow significantly with increasing OCH concentration, reaching 1.06% at 0.0025% oligochitosan, 1.15% at 0.0075% oligochitosan, and 1.26% at 0.01% oligochitosan. Saturated fatty acids have been demonstrated in the past to be a crucial growth factor for several lactic acid bacteria (9, 96).

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TABLE 4 Effect of 350 kDa CH on titratable acidity of fermented milk product during storage.

Storage time, days	Titrated acidity, °T				
	Concentration of 350 kDa CH, %				
	0 (control)	0.0025	0.005	0.0075	0.01
1	$90 \pm 3^{A,I}$	$89 \pm 2^{A,I}$	$82 \pm 2^{B,I}$	$83 \pm 1^{B,I}$	$75 \pm 1^{C,I}$
3	$117 \pm 2^{A,II}$	$110 \pm 3^{B,II}$	$106 \pm 1^{B,II}$	$104 \pm 3^{B,C,II}$	$100 \pm 4^{C,II}$
5	$140 \pm 4^{A,III}$	$132 \pm 3^{B,III}$	$118 \pm 3^{C,III}$	$110 \pm 2^{D,II,III}$	$103 \pm 2^{E,II}$
7	$157 \pm 3^{A,IV}$	$145 \pm 2^{B,IV}$	$132 \pm 3^{C,IV}$	$116 \pm 4^{D,III}$	$114 \pm 1^{D,III}$
9	$180 \pm 2^{A,V}$	$164 \pm 1^{B,V}$	$148 \pm 3^{C,V}$	$128 \pm 4^{D,IV}$	$126 \pm 4^{D,IV}$
11	$185 \pm 4^{A,V,VI}$	$170 \pm 3^{B,VI}$	$155 \pm 2^{D,VI}$	$138 \pm 2^{D,V}$	$129 \pm 4^{E,IV,V}$
13	$192 \pm 5^{A,VI,VII}$	$179 \pm 2^{B,VII}$	$161 \pm 4^{C,VI,VII}$	$142 \pm 5^{D,V,VI}$	$131 \pm 3^{E,IV,V}$
15	$198 \pm 4^{A,VII,VIII}$	$185 \pm 4^{B,VII,VIII}$	$164 \pm 2^{C,VII}$	$145 \pm 3^{D,VI}$	$132 \pm 4^{E,IV,V}$
17	$203 \pm 2^{A,VIII}$	$191 \pm 5^{B,VIII}$	$186 \pm 2^{B,VIII}$	$155 \pm 3^{C,VII}$	$138 \pm 4^{D,V}$

The values represent the mean ± SD (n = 3). Means without a common letter within the same column (I–VIII) and row (A–E) indicate significant difference at $p < 0.05$.

The main and secondary metabolite composition of the fermented milk product underwent considerable alterations as a result of the 45.3 kDa oligochitosan, according to the data shown in Tables 1–3. This is because the 45.3 kDa oligochitosan's intracellular mechanism of action causes biochemical alterations, which are responsible for the fermented milk product's low pH, high propanol and LA content, minimal saturated fatty acid content, and low *L. bulgaricus* content. Consequently, the consumer qualities of the fermented dairy product with 45.3 kDa oligochitosan are lost. Nevertheless, in light of the effects that have been found, oligochitosans may be utilized as a stimulant for lactic acid synthesis during lactic acid bacteria culture.

Unlike the 45.3 kDa oligochitosan, chitosan's effect on *L. bulgaricus* occurs via extracellular interaction. According to Tables 2, 3, with an increase in the chitosan concentration, the lactic acid synthesis slows down and the synthesis of propanol is prevented. Fermented dairy product containing 0.0025 and 0.0075% has a 140% higher content of benzoic acid relative to the control sample. It is well-known that a relatively high content of benzoic acid can prevent contamination of fermented milk products by yeast and other microorganisms (97–99). Table 3 shows that chitosan causes additional synthesis of some saturated fatty acids: pentadecanoic acid; hexadecanoic acid, methyl ester; n-hexadecanoic acid; methyl stearate; heptadecanoic acid, 16-methyl-, methyl ester; octadecanoic acid; as well as one unsaturated fatty acid—cis-11-octadecenoic acid. At 0.0025% chitosan, fermented milk product contained 2.04% saturated fatty acids and did not contain unsaturated acids. However, 0.0075% chitosan caused an increase in the content of saturated fatty acids to 5.28% and unsaturated by 5.79%. Fermented milk product with 0.01% chitosan had the highest content of fatty acids after 17 days of storage: 13.57% saturated fatty acids and 21.58% unsaturated fatty acid (cis-11-octadecanoic acid). As presented in Table 2, with an increase in the chitosan concentration, the content of *L. bulgaricus* increased as well. At the same time, according to Table 3, there was a significant increase in the content of fatty acids, which confirms their importance for *L. bulgaricus* growth. Thus, chitosan in interaction with *L. bulgaricus* causes metabolic shifts in the synthesis of secondary metabolites. As a result, lactic acid bacteria produce antifungal metabolites such as organic acids, phenolic compounds and a wide range of carboxylic acids and their esters (100, 101). These substances determine the high sensory characteristics of fermented milk product (102).

Furthermore, the shelf life of a fermented dairy product is a crucial indicator. Titratable acidity is seen to rise with an increase in storage duration. The titratable acidity of fermented dairy products, which should not be more than 140°T (49), determines their maximum shelf life. This value was attained in the control sample on the 5th day of storage and surpassed by 145% on the 17th day of storage (Table 4).

Experimental samples containing 0.0025, 0.005, 0.0075, and 0.01% chitosan reached the required titratable acidity levels on the 7, 9, 13, and 17th days of storage, respectively. Surprisingly, the optimal concentration of 0.01% chitosan maintained the titratable acidity of the fermented dairy product at 138°T on 17th day of storage. In general, this concentration increased the shelf life of the fermented dairy product by three times compared to the control sample. Additionally, lactic acid fermentation process was slowed down, while maintaining a high content of lactic acid bacteria and a low concentration of lactic acid.

An important sensory indicator of fermented milk product during storage is the clot density of the clot (103, 104). On the 17th day of storage, the viscosity of the control sample was 168 MPa×s, while the viscosity of the sample with 0.01% chitosan reached to 176 MPa×s. Thus, in the presence of chitosan, the liquefaction of the clot slowed down during storage. This is attributed to the fact that the experimental sample contained less lactic acid and maintained a relatively high pH value. Consequently, hydrolysis of milk proteins and destruction of casein micelles does not occur in experimental samples, as confirmed by SDS-electrophoretic examination (Supplementary Figure 2).

Further, in order to clarify the effect of chitosan on the form of LAB and confirm the absence of extraneous microflora in the samples, fixed preparations were obtained and studied after 17 days of storage. The results of microscopic studies are depicted in Figure 4.

Analysis of the microscopic preparations showed that interaction of *L. bulgaricus* with chitosan at concentrations of 0.0075 and 0.01% did not lead to a change in their rod shape. There were no foreign microflora in the samples examined. This

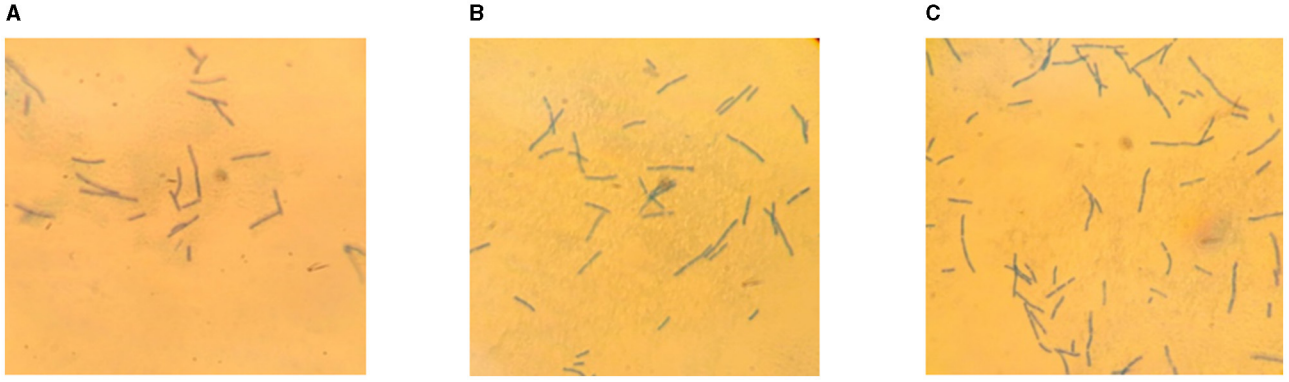

FIGURE 4
Microscopic preparations of fermented milk product: control sample **(A)**, 0.0075% CH **(B)**, 0.01% CH **(C)**.

may be due to the high content of secondary metabolites, such as benzoic acid, which prevent contamination of the product by other microorganisms (105).

Thus, the effective interaction of *L. bulgaricus* with chitosan is due to the presence in its 1850/mol structure of glucosamine monomers which, in an acidic environment, perform a multipoint ionic interaction with negatively charged teichoic acid molecules of the lactic acid bacteria cell wall. With increasing chitosan concentration, the process of lactose catabolism by *L. bulgaricus* and the accumulation of the lactic acid slows down. As a result of this process, the shelf life of the fermented milk product is significantly increased. At the same time 0.01% chitosan provided a high level of lactic acid bacteria and improved sensory characteristics of the product during 17 days storage. The concentrations of chitosan used did not give astringent taste to the fermented dairy product.

4 Conclusions

This study presents the results of a comparative study of the effect of oligochitosans and chitosan on the lactic acid fermentation process during cultivation of *L. bulgaricus* and long-term storage. The foundation of this study was the potential utilization of the ionic interaction between cationic polysaccharides and negatively charged *L. bulgaricus* cells to regulate lactic acid synthesis. Depending on the molecular weight, concentration, and molecules size of oligochitosans and chitosan effect on lactic acid synthesis due to intracellular or extracellular interactions during cultivation of *L. bulgaricus* and long-term storage of the fermented dairy product. When *L. bulgaricus* was cultured in the presence of 0.0025% 25.4 kDa oligochitosan, 45.3 kDa oligochitosan, and 350 kDa chitosan, their molar concentrations were 0.99×10^{-6}, 0.56×10^{-6}, and 0.75×10^{-7} mol/L, respectively. At the same time, the total content of glucosamine monomers in these samples was the same and amounted to 1.39×10^{-4} mol/L. After 24 h of cultivation, the lactic acid content in the fermented milk samples was 0.087 mol/L in the control sample, 0.255 mol/L in sample with 25.4 kDa oligochitosan, 0.228 mol/L in sample with 45.3 kDa oligochitosan, and 0.089 mol/L in sample with 350 kDa chitosan. At equal glucosamine monomers content, oligochitosans accelerate the synthesis of lactic acid, while chitosan has no such an effect compared to the control sample. These results indicate different mechanisms of action of oligochitosans and chitosan on the synthesis of lactic acid. With increasing concentrations of oligochitosans, a slowdown in the synthesis of lactic acid is observed. At the same time, its content remained higher than in the control sample. During long-term storage (17 days) of dairy product fermented with oligochitosan, further accumulation of lactic acid, decrease in pH and *L. bulgaricus* content occurred. The results obtained confirm that oligochitosans can be used as stimulators of lactic acid synthesis based on lactose containing substrates industrial fermentation using *L. bulgaricus* starter cultures.

Chitosan with molecular weight 350 kDa had the opposite effect on metabolic processes in fermented dairy product. With increasing chitosan concentration, lactic acid and propanol synthesis slowed down and the content of saturated and unsaturated fatty acids significantly increased. As a result, the titratable acidity decreased and the *L. bulgaricus* content increased. The result was an increase in the shelf life of the fermented dairy product. Chitosan provided low acidity of the product, high content of biologically active substances, increased *L. bulgaricus* content and better sensory characteristics compared to samples with addition of oligochitosans. The concentrations of chitosan used did not give astringent taste to the fermented dairy product. Further prospects are related to the study of the effect of 350 kDa chitosan on the technological processes of lactic acid bacteria various strains cultivation used in the production of commercial fermented dairy products.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

VK: Conceptualization, Data curation, Methodology, Project administration, Supervision, Writing – original draft. TH: Conceptualization, Formal analysis, Investigation,

Methodology, Resources, Validation, Visualization, Writing – original draft. AY: Methodology, Writing – original draft. MS: Investigation, Methodology, Validation, Writing – original draft. LA: Investigation, Writing – review & editing. IE: Conceptualization, Data curation, Resources, Writing – review & editing. AL: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. VT: Methodology, Resources, Writing – original draft. AN: Validation, Visualization, Writing – original draft. FA: Validation, Visualization, Writing – review & editing. AA-F: Formal analysis, Writing – review & editing. NA: Data curation, Formal analysis, Writing – review & editing. MS: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1441355/full#supplementary-material

SUPPLEMENTARY FIGURE 1
A typical GC-MS chromatogram of an extract from fermented dairy product on the 17th day of storage.

SUPPLEMENTARY FIGURE 2
SDS-electrophoregram of proteins of fermented dairy product on the 17th day of storage: control sample (5), 0.01% CH (4), 0.0075% CH (3), 0.0025% CH (2), original milk (1).

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Effect of fermentation, malting and ultrasonication on sorghum, mopane worm and *Moringa oleifera*: improvement in their nutritional, techno-functional and health promoting properties

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Background: Food processing offers various benefits that contribute to food nutrition, food security and convenience. This study investigated the effect of three different processes (fermentation, malting and ultrasonication) on the nutritional, techno-functional and health-promoting properties of sorghum, mopane worm and *Moringa oleifera*.

Methods: The fermented and malted flours were prepared at 35°C for 48 h, and for ultrasonication, samples were subjected to 10 min at 4°C with amplitudes of 40–70 Hz. The biochemical, nutritional quality and techno-functional properties of the obtained flours were analysed using standard procedures.

Results: Fermentation resulted in significantly lower pH and higher titratable acidity in sorghum and mopane worm (4.32 and 4.76; 0.24 and 0.69% lactic acid, respectively), and malting resulted in higher total phenolic content and total flavonoid content in sorghum (3.23 mg GAE/g and 3.05 mg QE/g). Ultrasonication resulted in higher protein and fibre in raw sorghum flour (13.38 and 4.53%) and mopane worm (56.24 and 11.74%) while raw moringa had the highest protein (30.68%). Biomodification by fermentation in sorghum led to higher water and oil holding capacity and increased dispersibility in the ultrasonicated samples. Ultrasonication of mopane worms led to higher water holding capacity, oil holding capacity and dispersibility. Lightness was found to be significantly higher in the fermented samples in sorghum and mopane worm. Raw moringa had the greatest lightness compared to the ultrasonicated moringa. Moringa had the most redness and browning index among all samples.

Conclusion: In this study, all the investigated processes were found to have caused variations in flours' biochemical, nutritional and techno-functional properties. Ultrasonication process was noteworthy to be the most efficient to preserve the nutritional value in sorghum, mopane worm and *M. oleifera* flours.

KEYWORDS

traditional processing, novel processing, nutritional benefits, underutilised, edible insect

1 Introduction

The world population has been reported to reach above 7.9 billion since 2022, and it is being predicted to slowly increase as the population increases (1). The population growth rate has been a concern due to its implications on resources, the environment, and social and economic systems. In recent decades, there has been an increasing awareness of the significance of a nutritious diet, prompted by concerns about the increase in obesity, diabetes, cardiovascular diseases, and other diet-related health issues (2). Several dietary trends have been reported (3, 4) from the adaptation of wholly plant-based diets to the inclusion of edible insects or flexitarian diets emphasising the consumption of fruits, vegetables, whole grains, and legumes. However, health issues, environmental concerns, and ethical considerations are the driving forces for this change (3).

Concerns about environmental sustainability have influenced the eating habits of individuals, hence there is a growing interest in consuming locally sourced and sustainably produced foods (2). Another challenging issue is malnutrition which remains a pressing issue in many nations, posing a significant risk factor for disease burden and mortality rates (1). The estimated 2 billion individuals that are affected by the detrimental effects of malnutrition extend far beyond physical health, impacting cognitive development and social skills (4, 5). Children who experience prolonged malnutrition may suffer from delayed physical growth and motor development, reduced intellectual quotient (IQ) and increased behavioural problems (5). Furthermore, the insufficient intake of essential nutrients such as proteins, vitamins, and minerals present a significant challenge for communities in developing countries, where undernutrition and related disorders are prevalent, which further highlights the necessity of consuming local indigenous crops and related products (4).

Sorghum is an indigenous crop consumed across Africa and India and has gained more consumer interests being a gluten-free alternative, high bioactive compounds, and fibre content (5). It is considered a valuable crop that can withstand drought-prone areas of Africa and India. Researchers have used sorghum as a whole grain or an additive to increase its value. Sorghum is known for its high phytochemical content, which contains phenols, tannins, anthocyanins, hydroxybenzoic acids, hydroxycinnamic acids, and other flavonoids (6). These phenolic compounds have a substantial influence on human health and provide antioxidant action (6). However, its protein and nutritional quality restrict its use as human food. Therefore, there is a need to include high-protein foods to sorghum-based foods to improve the protein content and digestibility. There is a variety of high-protein foods such as legume flour and whey protein. Recently, interests have moved away from more conventional food to edible insects such as cricket and mopane worms (4). These insects are high in protein and considered underutilised; despite their nutritional benefits they have not been significantly used in food products (7). Mopane worms (*Gonimbrasia belina*), are a unique and fascinating culinary delicacy deeply rooted in the traditional diets of certain African communities (8). The mopane worms have been a traditional staple food mostly in the Limpopo province in South Africa, Botswana and Zimbabwe. They have been reported to be a valuable food source due to their nutritional composition, especially in areas where conventional cattle production may be difficult (8). They make a substantial contribution to the nutritional needs by offering a well-balanced blend of protein (60–70%), lipids (15–20%), vitamins, and minerals (7).

Moringa plant (*Moringa oleifera*) is prioritised for its medicinal properties and several other uses, with the majority of its components are edible. Its defined seeds, blooms, and fruits (pods) provide pertinent nutrients and chemicals for feeding as characterised by (9). Moringa is an important plant in India, the Philippines, Ethiopia as well as Sudan though it popularly grown in the South, East and West Africa. *M. oleifera* leaf powder (MOLP) generated from the tree leaves; is widely acclaimed for its outstanding nutritional profile (10). A few studies have taken a methodical approach to the supplementation of food with MOLP, highlighting the usage of *M. oleifera* as a natural additive and as a functional food (11–13). The MOLP has been reported to have shown all the nine essential amino acids, hence it is considered a complete protein source (10). Hence blending MOLP with sorghum and mopane worm will be able to develop a food product that is not only nutritionally better but also environmentally sustainable and culturally suitable while also addressing various areas of food security and health.

Food processing plays a vital role in ensuring food safety, accessibility, and convenience while extending shelf life and bridging the gap between supply and demand (14). It can enhance the nutritional value, safety, and quality of food, despite concerns about additives in processed foods. Traditional food processing methods involve time-tested techniques like drying, fermenting, and salting, while novel food processing leverages modern technologies such as high-pressure processing, pulsed electric fields, ultrasonication and nanotechnology to enhance food quality, safety, and shelf life (14). The nutritional and health profile of sorghum, insects and moringa has been investigated mostly using fermentation and malting (15, 16). However, it appears that these approaches did not fully explore the comparison of the aforementioned processes and ultrasonication. Ultrasonication has been reported to activate enzymes naturally present in food by increasing enzymatic activity, which can have a positive effect depending on the specific enzymes involved (17). Ultrasonication activates naturally occurring enzymes in food by creating conditions that facilitate enzymatic reactions via cavitation, microstreaming, mechanical effects, thermal effects, and sonochemistry (18). For instance, it can activate enzymes responsible for contributing to nutritional quality or initiate enzymatic reactions that can lead to undesirable changes in food quality (17). It can also be used as an additional step to help reduce microbial load, therefore increasing the shelf life (18). The exact management of ultrasonication settings is required to obtain the optimum amount of enzyme activation while avoiding adverse effects.

In a study by Awobusuyi et al. (15), sorghum was blended with edible insect and in Mridula et al. (16) it was blended with sunflower and peanut flour to produce cookies with improved protein. Mridula et al. (18) found that cookies with a sorghum substitution level of up to 50% could be made using composite flours made of wheat and sorghum. Sorghum can be used to replace some of the wheat flour in

Abbreviations: ABTS, 2,2-Azinobis-(3-ethylbenzthiazolin-6-sulfonic acid); FMW, Fermented mopane worm; FRAP, Ferric ion reducing antioxidant power; FSF, Fermented sorghum flour; MSF, Malted sorghum flour; NaOH, Sodium hydroxide; OHC, Oil holding capacity; RMW, Raw mopane worm; RMO, Raw Moringa oleifera; RSF, Raw sorghum flour; SG, Sorghum grains; TFC, Total flavonoid content; TPC, Total phenolic content; TTA, Total titratable acidity; UMO, Ultrasonicated Moringa oleifera; UMW, Ultrasonicated mopane worm; USF, Ultrasonicated sorghum flour; WHC, Water holding capacity.

bread, cookies, and other snacks, according to some reported studies (19, 20). However, studies are limited on the use of sorghum and mopane worm blends into food products. As a result, this present study explored the effect of three different processes (fermentation, malting and ultrasonication) on the nutritional, functional and health-promoting properties of sorghum, mopane worm and *M. oleifera* to develop a health-promoting and value-added snack.

2 Materials and methods

2.1 Material

White raw sorghum grains (SG) were purchased from AGT (Krugersdorp, South Africa) and mopane worms (MW) were bought from a local market (Johannesburg, South Africa) in dry form and packaged in plastic containers. *M. oleifera* (MO) leave powder was procured from a health store (Dischem, Johannesburg, South Africa). All analytical-grade chemicals and reagents that were used during the experiment were purchased from Merck (Darmstadt, Germany) and other reputable suppliers.

2.2 Methods

2.2.1 Flour sample preparation of SG and MW

The SG and MW were removed from foreign components (stones, stalks), washed, and kept in airtight containers at room temperature until further usage. A portion (200 g) of cleaned SG (another portion reserved for malting) and MW were milled using Philips Mill HR2056/90 (Koninklijke Philips N.V., Eindhoven, Netherlands) into flour for fermentation and ultrasonication process. The milled SG and MW were then labelled as raw sorghum flour (RSF) and raw mopane worm (RMW), respectively. The moringa was purchased already in powder form (RMO) and was sieved to obtain a fine powder and the resulting flour was stored at 4°C in Ziploc bags for further analysis.

2.2.2 Processing of samples

2.2.2.1 Fermentation

A portion of the obtained flours of RSF and RMW was sieved through a 500 µm sieve (Analysette 3 Spartan, Fritsch GmbH, Idar-Oberstein, Germany). The processing method followed was the probiotic fermentation using freeze-dried culture (Lactic acid bacteria, CHN-22; Hansen Holding A/S, Horsholm, Denmark). The prepared RSF and RMW flour were fermented according to the procedure previously described by Kewuyemi et al. (21) to obtain a fermented sourdough. Triplicate fermentation was performed by combining 0.4 g of starter culture with 100 g flour and 200 mL of distilled water. The produced dough was fermented for 48 h in an incubator at 35°C. The sourdoughs were subsequently subjected to freezing at −20°C followed by freeze-drying (Telstar LyoQuest freeze dryer, Terrassa, Spain). The obtained samples were kept at 4°C in Ziploc bags for further analysis.

2.2.2.2 Malting

The malting process for RSF followed the method as outlined by Ojha. (22) with slight modifications Approximately 400 g of sorghum grains was washed with sterile distilled water and drained. The RSF was immersed in sterilised water at a ratio of 1:3 (w/v) at a temperature of 35°C for 48 h. The softened grains were then thoroughly washed, distributed evenly on a germination tray, covered in muslin fabric cloth and incubated at 27°C for 48 h. The grains were regularly moistened to ensure adequate hydration. Sprouted grains were later dried in a laboratory oven preheated at 50°C for 24 h. The dried grains were then milled using a laboratory miller (Platinum stand dry miller, KJ-1250, Castelfranco Veneto, Italy) and the resulting flour was sifted to obtain fine material using a sieve (500 µm) to produce a malted sorghum flour (MSF). The flour was kept in Ziploc bags at 4°C until analysis.

2.2.2.3 Ultrasonication

The ultrasonication of the samples was carried out using Misonix Ultrasonic Liquid Processor (FB705, Fisher Scientific) following a procedure previously described by Lohani & Muthukumarappan; Kingwascharapong (23, 24), with minor modifications. In 100 mL of distilled water, 30 g of the samples was reconstituted in a glass beaker. Samples were then subjected to 10 min of ultrasonication at 4°C with amplitudes of 70 Hz. The samples were ultrasonicated while in a water bath with ice (to prevent heat accumulation) until they reached 37°C. The obtained samples were transferred into 50 mL centrifuge tubes and closed tightly with the lid, frozen and freeze-dried. The samples were then stored in freezer bags for further analysis.

2.2.3 Analytical methods

2.2.3.1 Proximate composition

The proximate components, including crude protein, crude fat, ash content, and moisture, were determined using methods described by AOAC (25). Total carbohydrate was measured by difference, whereas total energy was computed using the Atwater factors.

2.2.3.2 pH and titratable acidity

The pH value was measured using a pH metre (HANNA, Woonsocket, United States) by immersing the pH probe into 10 mL of the sample. The reading was recorded as displayed on the screen (26). The amount of TTA was determined by titration of the supernatant with sodium hydroxide solution (NaOH, 0.1 N) to pH 8.3 (27). The TTA of the samples was expressed as % lactic acid according to Equations 1 and 2 below:

$$\%lactic\ acid = \frac{N\ x\ V\ x\ ME\ of\ \ lactic\ acid\ x\ 100}{Weight\ \ of\ \ sample\left(mL\right)} \quad (1)$$

Where:

N is the normality of the sodium hydroxide.

V is the volume of sodium hydroxide (mL) used to reach the titration end-point

$$ME\left(mili-equivalent\ \ of\ \ lactic\ acid\right) = \frac{Molecular\ \ weight\ \ of\ \ lactic\ acid}{1000} \quad (2)$$

2.2.3.3 Extraction of samples

The processed samples were extracted following the procedure described by Arouna (28) with some minor adjustments.

Approximately,0.25 g of the sample was dissolved in 5 mL of 70% aqueous methanol in a centrifuge tube. The resulting solution was sonicated (Scientech 704, Labotech, Johannesburg, South Africa) for 30 min and centrifuged (Eppendorf 5702R; Merck) for 10 min at 2500 rpm, 4°C. The recovered supernatants were placed into Eppendorf tubes and kept at a temperature of 4°C for further analysis.

2.2.3.4 Total phenolic content

According to Moyo et al. (29), the Folin–Ciocalteu method was used to determine the TPC of the samples. In a 96-well microplate, 10 µL of extract was taken in triplicates, followed by 50 µL of Folin–Ciocalteu reagent. After 3 min in the dark, approximately 50 µL of 7.5% Na_2CO_3 was added. The plate was then covered with aluminium foil, and the absorbance was measured at 750 nm using a microplate reader (iMark, Biorad, South Africa). The results were expressed as mg gallic acid equivalents (GAE)/g, with gallic acid as standard.

2.2.3.5 Total flavonoid content

To evaluate total flavonoid content, 10 µL of the extract was pipetted onto a microplate with 30 µL of 2.5% $NaNO_2$ and let to stand for 5 min, as per the method by Moyo et al. (29). The next step was to add 30 µL of 1.25% $AlCl_3$ and 100 µL of 2% NaOH. A microplate reader was used to test the sample's absorbance at 450 nm. The data were presented as mg quercetin equivalents (QE)/g, with quercetin as the standard.

2.2.3.6 ABTS [2,2-Azinobis (3-ethyl-Benzothiazone-6-sulfonic acid)]

The assay was performed based on the method by Kewuyemi et al. (21). The radical scavenging capacity was measured by using ABTS+ radical cation. Following the addition of 180 µL of ABTS solution and 5 min of incubation in the dark, 20 µL extract was pipetted onto a microliter plate. The solution's absorbance was determined using a microplate reader (BioTek, Agilent Technologies, South Africa) set to 750 nm. The findings were presented as µM Trolox equivalents (TE)/g sample using Trolox as the reference solution.

2.2.3.7 Ferric ion reducing antioxidant power

The FRAP test followed the procedure reported by Kewuyemi and Adebo (30). The solutions required for the experiment contained acetate buffer (300 mM, pH 3.6), diluted HCl (40 mM), 2,4,6-Tripyridyl-S-triazine (TPTZ, 10 mM), and freshly made ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$, 20 mM). Pipette 240 µL of FRAP working solution (at 37°C) into each microplate well, then add 10 µL of Trolox solution (standard solution 0 to 1 mM). In another set of wells, 10 µL of methanolic extracts were pipetted, while the control contained 75% ethanol. The plate was then incubated at 37°C for 30 min (Model: 222/227, Scientific Manufacturing CC, Cape Town, South Africa), and the resulting reaction mixture was read at 593 nm on a microplate smartReader (Accuris Instruments, United Sttaes). The FRAP of the extracts was presented as a millimolar of Trolox equivalent per gram (mM TE/g).

2.2.3.8 Oil holding capacity

The oil holding capacity (OHC) and water holding capacity (WHC) of flours are important properties for in-process handling and as a result, impact customer preference for the finished product. For example, a high WHC is ideal for improved thickening capacity, hydration, and satiety, but a moderate degree of OHC is optimal for in-process fat holding, taste retention, and palatability (31). The sample's absorption of oil was examined based on the method described by Ohizua et al. (32), with minor adjustments. In a clean 15 mL centrifugal tube, 1 g of sample flour was combined with 10 mL oil and shaken to make a homogenous mixture. The tube was then centrifuged for 20 min at 1100 × *g* after standing at ±23°C for an hour. The amount of free oil was used to calculate the oil absorption capacity percentage.

2.2.3.9 Water holding capacity

The water holding capacity was determined based on the procedure (33). About 0.5 g of each sample and 20 mL of distilled water were properly mixed before being put into centrifugal tubes. The mixture was centrifuged for 10 min at 700 × g at 4°C (Eppendorf 5702R, Merck, Darmstadt, Germany). The recovered supernatant was then dried at room temperature ± 24°C to achieve consistent mass. The dry material will be treated as a solid mass in the supernatant.

2.2.3.10 Dispersibility

Dispersibility was estimated using the reported method by Ohizua et al. (32) of the flour samples. About 10 g of each sample was added to the pre-dried 100 mL cylinder and was filled with distilled water until 100 mL mark. The obtained mixture was stirred and left to stand for 3 h and the dispersibility of the flours was expressed as percentage.

2.2.3.11 Colour profile

The colour characteristics (L*, a*, b*, and ΔE) of the flours were measured using a dual system consisting of a measuring head and a data processor (CR-410 and DP-400 Ver. 1.20, Konica Minolta, Inc., Tokyo, Japan). Prior to analysing the flour samples, the device was calibrated using a white tile (refractive index was mentioned). The flour samples were analysed for their lightness (L*), redness/greenness (a*), yellowness/blueness (b*), and overall colour differences (ΔE*) (21). In addition, the browning index was determined using Equations 3 and 4 below:

$$BI = \frac{\left[100\left(y - 0.31\right)\right]}{0.17} \tag{3}$$

$$y = \frac{\left(a* + 1.75L*\right)}{\left(5.645L* + a* - 3.012b*\right)} \tag{4}$$

where BI = browning index, L = lightness, a = redness, b = yellowness.

2.2.4 Statistical analysis

All generated data for each analysis was performed on one-way analysis of variance software (IBM SPSS, ver. 26.0, New York, United States) at $p < 0.05$. The results were shown as mean ± standard deviation (SD). The unsupervised principal component analysis

(PCA; SIMCA 18, Umetrics, Umea, Sweden) was used to visualise grouping the patterns and also to identify the outliers in data sets.

3 Results and discussion

3.1 Effect of processing (fermentation, malting and ultrasonication) on proximate composition

The proximate composition of food is usually used for the estimation of the quantitative analysis of food substance which includes ash, moisture, fibre, fat, protein and total carbohydrates (34). The macronutrients of the samples are presented in Table 1, and it can be observed that malted sorghum flour (MSF) had the highest moisture content while ultrasonicated sorghum flour (USF) had the lowest moisture content. The fermented sorghum flour (FSF) significantly decreased as compared to raw sorghum flour (RSF). Raw mopane worm (RMW) reported the highest moisture content as compared to ultrasonicated mopane worm (UMW) which had the lowest moisture content. Ultrasonicated *M. oleifera* (UMO) reported the highest moisture content while raw *M. oleifera* (RMO) had the lowest moisture content. Mopane worm flour was observed to have more ash content compared to all the other samples that were investigated in the current study. These findings are consistent with what is reported by Kewuyemi & Adebo (30), where no significant difference between the processes in all samples was observed.

The USF, UMW and UMO showed significant increases in fibre content as compared to other processed samples and raw samples. MSF was reported to have decreased with no significant difference with RSF. As expected, RMW had the highest protein content among all the samples reported, with UMW (56.24%) being the highest. This can be due to ultrasonication creating mechanical vibrations when applied to food, disrupting the structure of the food matrix. This disruption causes the release of proteins that are attached to food particles or cellular structures. As a result, more proteins become accessible in the liquid phase, hence boosting the total protein content (35). The same trend is also observed in sorghum but not in MO and this could be due to the disruption of sensitive cells in plants. Proteins contain complex molecules that are susceptible to mechanical and chemical stress therefore denaturing or degrading the proteins, resulting in protein loss rather than an increase (36).

Among all the samples studied, RSF contained less amount of fat as expected and MW had the highest fat content. This is due to mopane worms feeding mainly on mopane tree leaves that are high in lipids, namely unsaturated fatty acids like oleic and linoleic acid. As a result, the caterpillars absorb lipids from their food, which contributes to their high-fat content (37). Mopane worms usually store their energy as fats rather than carbohydrates hence the carbohydrates for RMW it ranged from 0.58–6.02% which was the lowest in all samples. Fat offers a more concentrated and efficient energy reserve than carbohydrates, making it a favoured storage type, especially in insects that undergo substantial growth and developmental changes. Grains have been reported to contain more carbohydrates than other food. The carbohydrates recorded for RSF ranged from 68.62–74.64% with USF being the highest and no significant difference was observed between FSF and USF as well as in energy levels. The MO samples showed the lowest energy levels 329.13–334.63 kcal.

3.2 Effect of processing (fermentation, malting and ultrasonication) on biochemical properties

The fermentation process significantly ($p \leq 0.05$) decreased pH in FSF (4.32) and FMW (4.76) as observed in Table 2 compared to the control, malting and ultrasonication. With MO, the UMO had the lowest pH (5.57) compared to the RMO. As expected, when the pH decreased, the TTA correspondingly increased as well. The highest TTA observed in Table 1 for RSF was the FSF with the lowest pH while the lowest recorded was seen in RSF (0.07% lactic acid) which had the highest pH (6.23). The same trend was also observed in RMW as well as in RMO. According to Van Kerrebroeck et al. (38), the biomodified flour's pH and acidity are influenced by both endogenous (enzymes and bacteria) and external variables (dough yield, temperature, and time). Similar trends were also observed in the study by Kewuyemi et al. (21).

The ultrasonicated samples had the highest pH as compared to fermented samples. This is because, during fermentation, bacteria

TABLE 1 Proximate composition and energy content of raw, fermented, malted and ultrasonicated sorghum, mopane worm and moringa flours.

Sample	Ash (%)	Moisture (%)	Protein (%)	Fibre (%)	Fat (%)	Carbohydrate (%)	Energy (kcal/g)
RSF	1.53 ± 0.00^{a}	9.93 ± 0.02^{h}	12.77 ± 0.03^{ab}	3.73 ± 0.01^{a}	3.35 ± 0.17^{b}	68.69 ± 0.16^{f}	363.48 ± 0.75^{c}
MSF	1.49 ± 0.00^{a}	10.39 ± 0.18^{i}	12.46 ± 0.05^{a}	3.69 ± 0.01^{a}	3.35 ± 0.12^{b}	68.62 ± 0.01^{f}	361.87 ± 1.27^{c}
FSF	1.77 ± 0.01^{b}	3.24 ± 0.05^{b}	13.02 ± 0.05^{b}	4.23 ± 0.00^{b}	3.35 ± 0.05^{b}	74.39 ± 0.05^{g}	388.27 ± 0.39^{de}
USF	1.83 ± 0.01^{c}	2.80 ± 0.06^{a}	13.38 ± 0.13^{c}	4.53 ± 0.07^{c}	2.82 ± 0.03^{a}	74.64 ± 0.10^{g}	386.50 ± 0.25^{d}
RMW	10.34 ± 0.02^{f}	7.29 ± 0.04^{e}	51.59 ± 0.35^{f}	9.19 ± 0.02^{d}	15.57 ± 0.20^{e}	6.02 ± 0.26^{c}	388.98 ± 0.86^{e}
FMW	11.15 ± 0.02^{g}	5.91 ± 0.03^{d}	52.39 ± 0.25^{g}	11.28 ± 0.02^{f}	15.60 ± 0.34^{e}	3.66 ± 0.44^{b}	387.21 ± 1.95^{de}
UMW	11.10 ± 0.01^{g}	5.14 ± 0.12^{c}	56.24 ± 0.18^{h}	11.74 ± 0.05^{g}	15.20 ± 0.06^{e}	0.58 ± 0.35^{a}	387.52 ± 0.21^{de}
RMO	7.88 ± 0.01^{e}	8.63 ± 0.09^{f}	30.68 ± 0.16^{e}	10.00 ± 0.02^{e}	4.13 ± 0.48^{c}	38.68 ± 0.27^{e}	334.63 ± 2.63^{b}
UMO	7.46 ± 0.08^{d}	9.13 ± 0.00^{g}	29.32 ± 0.32^{d}	13.86 ± 0.01^{h}	4.64 ± 0.19^{d}	35.59 ± 0.21^{d}	329.13 ± 0.83^{a}

RSF, raw sorghum flour; FSF, fermented sorghum flour; MSF, malted sorghum flour; USF, ultrasonicated sorghum flour; RMW, raw mopane worm; FMW, fermented mopane worm; UMW, ultrasonicated mopane worm; RMO, raw *Moringa oleifera*; UMO, ultrasonicated *Moringa oleifera*. Values in brackets are standard deviations of the respective means with different superscripts which are significantly different ($p \leq 0.05$) per column.

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TABLE 2 pH, TTA, TPC, TFC and antioxidant activities of raw, fermented, malted and ultrasonicated sorghum, mopane worm and moringa flours.

Sample	pH	TTA (% lactic acid)	TPC (mg GAE/g)	TFC (mg QE/g)	ABTS (mM TE/g)	FRAP (mM TE/g)
RSF	6.23 ± 0.01^{i}	0.07 ± 0.06^{a}	0.79 ± 0.01^{a}	1.27 ± 0.03^{a}	11.68 ± 0.01^{b}	5.73 ± 0.02^{g}
MSF	5.29 ± 0.03^{c}	0.18 ± 0.06^{c}	3.23 ± 0.02^{e}	3.05 ± 0.03^{d}	28.38 ± 0.01^{d}	1.19 ± 0.02^{b}
FSF	4.32 ± 0.01^{a}	0.24 ± 0.11^{d}	1.10 ± 0.06^{b}	1.70 ± 0.03^{b}	18.71 ± 0.02^{c}	5.43 ± 0.02^{f}
USF	6.14 ± 0.01^{h}	0.17 ± 0.06^{b}	1.63 ± 0.03^{c}	2.17 ± 0.02^{c}	10.02 ± 0.02^{a}	3.79 ± 0.02^{e}
RMW	5.76 ± 0.01^{f}	0.49 ± 0.17^{h}	3.63 ± 0.02^{f}	6.98 ± 0.02^{e}	31.64 ± 0.01^{g}	3.10 ± 0.03^{d}
FMW	4.76 ± 0.01^{b}	0.69 ± 0.06^{i}	6.18 ± 0.03^{g}	8.84 ± 0.01^{g}	32.49 ± 0.01^{i}	0.41 ± 0.03^{a}
UMW	5.86 ± 0.01^{g}	0.42 ± 0.06^{g}	3.09 ± 0.03^{d}	8.57 ± 0.03^{f}	32.27 ± 0.02^{h}	2.61 ± 0.03^{c}
RMO	5.71 ± 0.01^{e}	0.30 ± 0.06^{f}	21.47 ± 0.03^{i}	74.79 ± 0.01^{i}	29.67 ± 0.03^{e}	55.30 ± 0.03^{i}
UMO	5.57 ± 0.01^{d}	0.29 ± 0^{e}	11.60 ± 0.03^{h}	74.02 ± 0.02^{h}	30.87 ± 0^{f}	44.12 ± 0.02^{h}

TTA, titratable acidity; TPC, total phenolic content; TFC, total flavonoid content; ABTS, 2,2-Azinobis-(3-ethylbenzthiazolin-6-sulfonic acid); FRAP, Ferric ion reducing antioxidant power; RSF, raw sorghum flour; FSF, fermented sorghum flour; MSF, malted sorghum flour; USF, ultrasonicated sorghum flour; RMW, raw mopane worm; FMW, fermented mopane worm; UMW, ultrasonicated mopane worm; RMO, raw *Moringa oleifera*; UMO, ultrasonicated *Moringa oleifera*. Values in brackets are standard deviations of the respective means with different superscripts which are significantly different ($p \leq 0.05$) per column.

TABLE 3 Physical properties of raw, fermented, malted and ultrasonicated sorghum, mopane worm and moringa flours.

Sample	WHC (g/g)	OHC (g/g)	Dispersibility (%)
RSF	1.94 ± 0.70^{a}	2.25 ± 0.01^{bc}	86.83 ± 0.29^{f}
MSF	2.12 ± 0.36^{ab}	2.11 ± 0.35^{b}	86.00 ± 0^{e}
FSF	2.50 ± 0.32^{b}	2.53 ± 0.32^{cd}	87.43 ± 0.12^{g}
USF	2.29 ± 0.06^{ab}	2.44 ± 0.05^{bcd}	87.87 ± 0.23^{h}
RMW	2.58 ± 0.01^{bc}	2.62 ± 0.01^{d}	80.33 ± 0.29^{c}
FMW	2.53 ± 0.01^{b}	2.54 ± 0.01^{cd}	81.00 ± 0^{d}
UMW	3.11 ± 0.28^{c}	3.20 ± 0.32^{e}	85.80 ± 0.35^{e}
RMO	3.76 ± 0.07^{d}	1.21 ± 0^{a}	70.73 ± 0.23^{a}
UMO	2.66 ± 0.07^{bc}	1.26 ± 0.01^{a}	73.23 ± 0.40^{b}

WHC, water holding capacity; OHC, oil holding capacity; RS, raw sorghum; FS, fermented sorghum; MS, malted sorghum; US, ultrasonicated sorghum; RMW, raw mopane worm; FMW, fermented mopane worm; UMW, ultrasonicated mopane worm; RMO, raw *Moringa oleifera*; UMO, ultrasonicated *Moringa oleifera*. Values in brackets are standard deviations of the respective means with different superscripts which are significantly different ($p \leq 0.05$) per column.

metabolise glucose and other chemicals in the food substrate. As a result of this metabolic process, they create organic acids such as lactic acid, acetic acid, and citric acid. These organic acids are acidic and help to reduce the pH of fermented foods. This reduction in pH limits the growth of spoilage microbes and diseases causing pathogens thereby preserving the quality of food (21). Ultrasonication, on the other hand, is a mechanical process that disrupts cells, emulsifies chemicals, and aids in extraction. While ultrasonication can change the physical features of food, such as texture or particle size, it has no direct impact on the chemical content or pH of the meal. As a result, ultrasonicated meals seldom undergo pH changes like fermented foods (39).

Food processing has been reported to increase the nutritional and health-promoting properties of food (40). Table 2 shows the TPC, TFC and antioxidant activities of the flour samples. As observed, malting had the highest TPC (3.23 mg GAE/g), TFC (3.05 mg QE/g) and ABTS (28.38 mM TE/g) among all the investigated processes in sorghum and reported the lowest in FRAP (1.19 mM TE/g). These alterations may be due to the increased release or enzymatic production (i.e., proteolysis) of soluble bioactive molecules with a higher potential to scavenge free radicals (41). The same trend was also observed in RMW, fermented RMW had the highest TPC (6.18 mg GAE/g), TFC (8.84 mg QE/g) and ABTS (32.49 mM TE/g) but reported the lowest in FRAP (0.41 mM TE/g). As expected, MO had the highest TPC, TFC and FRAP among all the other samples. However, the composition of the insoluble proteolytic products and non-phenol components produced in the samples might impact the considerable reduction ($p < 0.05$) of FRAP (0.41 mM TE/g) in FMW (41).

3.3 Effect of processing (fermentation, malting and ultrasonication) on physical properties and colour

The effect of processing of SF, MW and MO on physical properties and colour are presented in Tables 3, 4. The significance of fermented, malted and ultrasonicated flours for post-processing mostly depends on their excellent physical properties (42). The FSF (2.50%) showed a significant WHC increase ($p \leq 0.05$) as well as in OHC (2.53) while UMW showed a significantly higher WHC and OHC (3.11 and 3.20 g/g, respectively). Among all the investigated samples, RMO had the highest WHC (3.76 g/g) but showed the lowest OHC (1.26 g/g). This is because many plant tissue components such as polysaccharides, proteins, and pectin, are hydrophilic, which means they prefer water. These chemicals can absorb and hold water within the plant's cells and tissues (43). The FSF and USF had a significantly higher ($p \leq 0.05$) dispersibility (87.43 and 87.87%) as compared to other samples with MO being the lowest. According to Eke-Ejiofor (44), a high dispersibility can enhance the better reconstruction of starch in water to give a fine and constituent paste.

Colour profiling is a key indicator of food quality and customer preferences. Table 4 represents the colour profiling of the investigated samples. The study found that RMO and UMO samples exhibited significantly higher ($p \leq 0.05$) redness (10.51–3.82) and browning index (77.71–84.60), but lower total colour difference (24.06–32.12).

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TABLE 4 Colour attributes of raw, fermented, malted and ultrasonicated sorghum, mopane worm and moringa flours.

Sample	L*	a*	b*	ΔE*	BI
RSF	77.25 ± 0.09^{g}	3.19 ± 0.01^{b}	12.65 ± 0.04^{c}	50.71 ± 0.08^{g}	20.55 ± 0.10^{b}
MSF	77.99 ± 0.03^{h}	4.43 ± 0.02^{e}	12.85 ± 0.11^{d}	51.56 ± 0.06^{h}	21.81 ± 0.18^{c}
FSF	79.09 ± 0.02^{i}	3.64 ± 0.02^{c}	12.47 ± 0.04^{b}	52.46 ± 0.03^{i}	20.18 ± 0.07^{a}
USF	74.34 ± 0.12^{f}	3.62 ± 0.02^{c}	11.58 ± 0.07^{a}	47.64 ± 0.13^{f}	20.17 ± 0.09^{a}
RMW	58.43 ± 0.10^{d}	2.69 ± 0.01^{a}	24.29 ± 0.05^{i}	39.22 ± 0.05^{e}	55.65 ± 0.29^{e}
FMW	58.55 ± 0.05^{e}	2.66 ± 0.09^{a}	23.85 ± 0.01^{h}	39.02 ± 0.01^{d}	54.25 ± 0.20^{d}
UMW	42.57 ± 0.01^{b}	3.58 ± 0.02^{c}	17.89 ± 0.06^{e}	23.71 ± 0.05^{a}	59.36 ± 0.28^{f}
RMO	46.36 ± 0.01^{c}	10.51 ± 0.01^{f}	23.33 ± 0.02^{g}	32.12 ± 0.05^{c}	84.60 ± 0.13^{h}
UMO	38.99 ± 0.03^{a}	3.82 ± 0.03^{d}	20.23 ± 0.05^{f}	24.06 ± 0.05^{b}	77.71 ± 0.10^{g}

L*, lightness; a*, redness; b*, yellowness; ΔE, total colour difference; BI, browning index; RSF, raw sorghum flour; FSF, fermented sorghum flour; MSF, malted sorghum flour; USF, ultrasonicated sorghum flour; RMW, raw mopane worm; FMW, fermented mopane worm; UMW, ultrasonicated mopane worm; RMO, raw *Moringa oleifera*; UMO, ultrasonicated *Moringa oleifera*. Values in brackets are standard deviations of the respective means with different superscripts which are significantly different ($p \leq 0.05$) per column.

In contrast, raw, fermented, and malted red sorghum flour (RSF) samples showed significant increases in lightness (74.34–79.09), while RMW as well as FMW had a higher yellowness (24.29 and 23.85 respectively). The differences are likely influenced by the seed coat colour of sorghum grains and the natural colour of mopane worms. The UMW had a significant decrease in lightness (42.57) but had an increased redness (3.58).

The maximum redness of the UMW might have led to its browning index of 59.36. The higher browning index indicates the extent of browning impacted by ultrasonication. In other words, the RMW ultrasonication improved its redness and browning index and a lesser intensity of yellowness and reduced lightness. Plants exhibit a higher amount of redness in colour profile than other types of food due to the presence of pigments including anthocyanins, lycopene, and betacyanins, as well as environmental and genetic influences (45). This might have resulted in the MO's highest redness and browning index, and consequently having the lowest lightness. The colour profile results also showed that malting increases the lightness of MSF (77.09) compared to USF (74.34). The observed differences are partly related to the earlier reported low USF's TPC (1.63 mg GAE/g; Table 1), suggesting that the leaching of polyphenol constituents during malting steps improved the lightness of MSF.

3.4 Principal component analysis

An unsupervised modelling technique (PCA) was used to understand and highlight the groupings of the samples in this study, based on the investigated parameters. This allowed for an exploratory statistical analyses and descriptive assessment of the data showing trends and relationships as depicted in Figures 1A–D. The first two principal components (PCs), PC1 and PC2 explained 49.3 and 25.6% of the variation (total of 74.9%). Figure 1A shows a distinct separation of sorghum (to the left of the plot), moringa (to the top right) and mopane worm (to the bottom left). This is in alignment to the inherent composition and characteristics of these food sources, reflected in the obtained data on Tables 1–4.

The groupings also reflect the unique groupings of the substrates investigated in the current study: sorghum (being a cereal), moringa (being a medicinal plant) and mopane (being an edible insect). While still retaining the clusters, Figure 1B shows that the processes (fermentation, malting and ultrasonication) affected the properties of the samples. As observed in Figure 1D, PC1 separated more of the processed samples to the left while the unprocessed samples were shifted to the right. Most the processed samples were clustered in the lower part of the PC1 as compared to the unprocessed. The identified separation and clusters could be due to biochemical activities that occurred during the fermentation, malting, and ultrasonication processes.

4 Conclusion

The present study explored traditional (fermentation and malting) and novel (ultrasonication) processes to improve the quality properties of sorghum, mopane worm and *M. oleifera* flours. The processes were found to have caused variations in the biochemical, nutritional and functional properties of the investigated flours. As expected, fermentation of flours led to low acidity levels and higher TTA than other processes. Higher TPC and TFC were observed in malting corresponding in higher antioxidant (ABTS) for sorghum while for mopane worms it was observed in fermentation then raw moringa had the highest TPC and TFC. The ultrasonication of RSF and RMW resulted in higher contents of ash, protein and fibre. The biomodification by fermentation in sorghum led to higher WHC and OHC and increased dispersibility in ultrasonication. In mopane worms, ultrasonication led to higher WHC, OHC and dispersibility. Lightness was greater in the fermented samples of RSF and RMW while raw MO had the greatest lightness than UMO. Among all samples, MO had the most redness and browning index. Ultrasonication has proven to be more effective in improving the nutritional value in the samples, followed by fermentation. The blend of bioprocessed flours with various nutritional and health properties can help address the challenges of malnutrition by developing finished products from these flours. It can be recommended to further investigate the technological features of the processed flours to help compose the formulations.

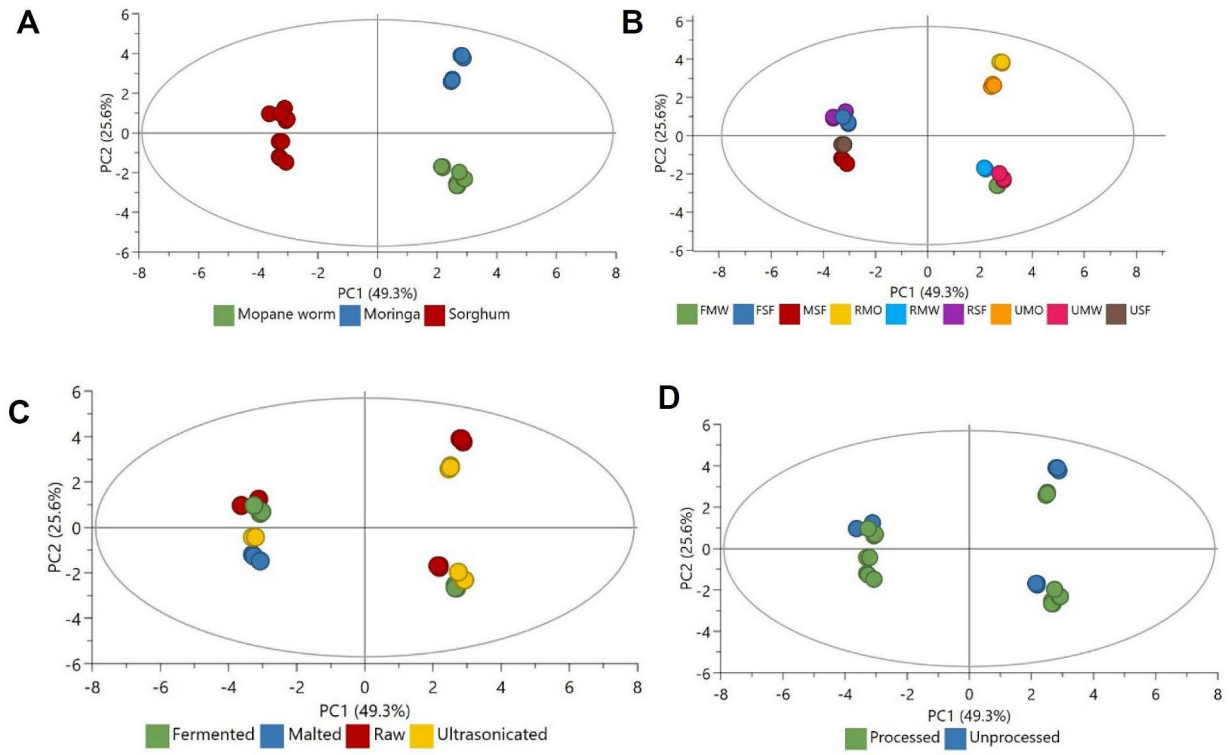

FIGURE 1
PCA score plots showing distribution and separation. **(A)** Relationship of mopane worm, moringa and sorghum, **(B)** relationship of the differently processed samples, **(C)** relationship of raw, fermented, malted and ultrasonicated, **(D)** relationship of processed and unprocessed.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

MM: Writing – original draft, Writing – review & editing, Formal analysis. OA: Writing – review & editing, Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision. JW: Writing – review & editing, Methodology, Project administration, Supervision. ML: Writing – review & editing, Formal analysis, Methodology. XF: Writing – review & editing, Methodology, Project administration, Supervision. JG: Writing – review & editing. TM: Writing – review & editing, Methodology, Project administration, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Impact of dehulling, germination and fermentation on the bioactive and functional properties of grey pea flour

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Introduction: Grey pea is a largely overlooked legume in the Nordic countries, and its potential uses in various food products remain unexplored. It is a nutrient-rich crop with low environmental impact, making it an attractive option for sustainable and nutritious plant-based alternatives.

Objectives: To investigate the impact of dehulling, germination, and fermentation on the bioactive (polyphenol content and antioxidant capacity) and functional characteristics (water absorption index, water solubility index, water and oil binding capacity, emulsifying properties and gelation concentration) of grey pea flour. Additionally, protein content and pasting properties (temperature, peak viscosity, trough viscosity, breakdown, final viscosity, and setback) were measured.

Methods: Dehulling was performed using a runner disk sheller. Germination was carried out for 24 and 48h at ambient temperature, and fermentation was conducted for 8h at 43°C using a starter culture.

Results: The results indicate that dehulling did not significantly affect functional properties and gelling capacity ($p = 0.297$ for oil absorption capacity, $p = 0.5$ for emulsion activity, and $p = 0.607$ for emulsion stability), but it resulted in a notable decrease in total polyphenol content (TPC) and antioxidant capacity (TAC). Conversely, 48h of germination increased TAC measured by two methods: FRAP (19%) and DPPH (30%). This process increased through viscosity by 1.2-fold, while it did not significantly affect the water absorption index (WAI), water solubility index (WSI), or the emulsifying properties of grey pea flour. Fermentation significantly improved TPC ($p<0.001$ for whole grey peas and $p = 0.004$ for dehulled grey peas), with a TPC increase of up to 67% in fermented dehulled pea flour. TAC measured by both methods, showed significant increases, ranging from 35 to 104%. However, fermentation reduced emulsifying and pasting properties, as indicated by the peak, through and final viscosity, which may be desirable only for certain food products. Further, germination and fermentation showed significant increases in protein content, by 4 and 8%, respectively.

Conclusion: Fermented grey pea flour exhibited enhanced bioactive characteristics, while 48-h germination positively impacted pasting properties. Overall, these processes led to changes in both the bioactive and functional properties of grey pea flour, creating opportunities for the use of these flours in a wide array of food products.

KEYWORDS

antioxidant capacity, fermentation, functional properties, germination, grey pea, polyphenols

1 Introduction

Grey pea, a subgroup of the peas (*Pisum sativum L.*) and pulses family, was once a staple food in the Nordic countries used in various dishes from soup to bread. However, its popularity declined from the 19th century until today (1). Nowadays, grey pea is regaining attention due to its status as a nutrient-rich crop, it has high protein, fiber and mineral content. Besides its nutritional properties, grey peas can be sustainably cultivated, improving soil fertility through nitrogen-fixing properties and intercropping with cereals like oats, thus increasing farm biodiversity. Tidåker et al. (2) showed that the environmental impact of cultivation of grey pea was lower than that of beans. Thus, the growing awareness and interest in improving overall health and minimizing the environmental impact of dietary choices has led to more attention toward the consumption of pulses. Recent trends in Sweden regarding the healthy eating and consumption of sustainable and locally sourced foods as well as increasing interest in a more plant-based diet have promoted the consumption of domestically grown pulses such as grey peas (3). It is believed that its cultivation can be expanded and it has the potential to reduce dependency on imported soybean in the future (4). However, up to date the consumption of grey peas is still limited, within one of the limitations is the lack of information on the functional properties of these peas along with information on how processing alters the nutritional and bioactive compounds in peas.

Several processing methods, including dehulling, cooking, roasting, germination, and fermentation, are employed to mitigate antinutrient levels, enhance palatability, and improve sensory acceptance of pulses and pulse flours. These treatments not only increase the bioavailability of nutrients in pulses but have the potential to enhance their functional properties (3, 5). Dehulling is a process of loosening and removal of the fibrous seed coat (6). This process improves the appearance, cooking quality, and palatability of the pulses as well as enhancing their digestibility (7). Additionally, dehulling produces higher quality flour without visual specks. Germination is a traditional and cost-effective process that enhances the nutritional and functional properties of pulses. During germination structural elements are altered and novel bioactive compounds are synthesized which improves digestibility, stability, and nutritional profile of the grains (8). Moreover, sensory properties may improve during germination by reducing the beany flavor through the activation of endogenous enzymes and the conversion of starch into simpler sugars (9). Fermentation is an ancient food technology in which a population of microorganisms is utilized for biological conversion of complex substrates into simpler compounds (10). The fermentation process can be beneficial due to the elimination and decrease of antinutritional factors such as phytic acid, trypsin, and chymotrypsin inhibitors, thus improving the nutritional quality and protein digestibility of pulses (11).

Relatively extensive work has been undertaken investigating the effects of germination and fermentation on nutritional and antinutritional compounds in pulses. However, there is limited information on the effect of processing methods, such as germination and fermentation, on the production of bioactive compounds, i.e., polyphenols. Pulses are sources of phenolic compounds such as phenolic acid, flavonoids, isoflavones and tannins. The specific types and amounts of these phenolic compounds which may exist in free, esterified or bound forms differ depending on the type and genotypes of pulse (12). Fermentation has been shown to increase bioactive phenolic compounds in some pulses and legumes, resulting in greater antioxidant activities. Moreover, conjugated phenolic compounds can be converted into their free forms during fermentation enhancing their bioavailability and health benefits (13). Furthermore, changes in functional properties of grey pea flour during processing are not sufficiently reported. Functional properties play a crucial role in developing new food products and determining the behavior of food during manufacturing, processing, storage, and consumption (14). It was also reported that functional properties of food ingredients can enhance processing efficiency (15). For example, fermentation of sorghum flour improved functional properties such as emulsifying capacity and stability (16). Other authors noted that yeast fermentation of rice reduced the hot paste viscosity (17), highlighting the relevance of investigating changes in functional properties of flours due to processing, which can have a great impact on the use of these ingredients in the food industry.

Therefore, this study aimed at investigating the effect of dehulling, germination, and fermentation on protein content, total phenolic content and antioxidant capacity of grey pea flour. Moreover, physical, functional, and pasting properties of the untreated and treated flours were studied. This study will pave the way toward creating new food ingredients with improved health benefits and functional properties. The information on the raw and processed grey pea flour will shed light on the most suitable way to diversify the use of grey peas in the food industry.

2 Materials and methods

2.1 Materials

Dried grey peas (variety retrija) were purchased from Nordisk Råvara (Stockholm, Sweden). Starter culture Lyofast VSAB1 3UC/100 L (SACCO starter cultures, Kemikalia AB, Skurup, Sweden) was used for fermentation of grey peas. All reagents used were of analytical grade. Sodium hydroxide (VWR Chemicals, Leuven, Belgium) used for acidity. Folin–Ciocalteu reagent, sodium carbonate, gallic acid, TPTZ (1,3,5-tri(2-pyridyl)-2,4,6-triazine), sodium acetate, DPPH (2,2-diphenyl-1-picrylhydrazyl), and Trolox ((+/−)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were purchased from (Sigma-Aldrich, Stockholm, Sweden). Ferric chloride ($FeCl_3 \cdot 6H_2O$) was purchased from (Fluka, Hanover, Germany).

2.2 Methods

2.2.1 Processing of grey peas

Dehulling, germination, and fermentation were the methods used in processing grey peas. All the processes were conducted in duplicate. A flowchart of the processing steps is presented in Figure 1.

Dehulling of the unprocessed grey peas was performed using a runner disk sheller (Streckel and Schrader, Hamburg, Germany). Grey peas, either whole or dehulled were milled using a hammer mill

Abbreviations: DPPH, 2,2-diphenyl-1-picrylhydrazyl; dwb, Dry weight basis; EA, Emulsion activity; ES, Emulsion stability; FRAP, Ferric reducing antioxidant power; LGC, Least gelation concentration; OAC, Oil absorption capacity; TAC, Total antioxidant activity; TPC, Total phenolic content; WAC, Water absorption capacity; WAI, Water absorption index; WSI, Water solubility index.

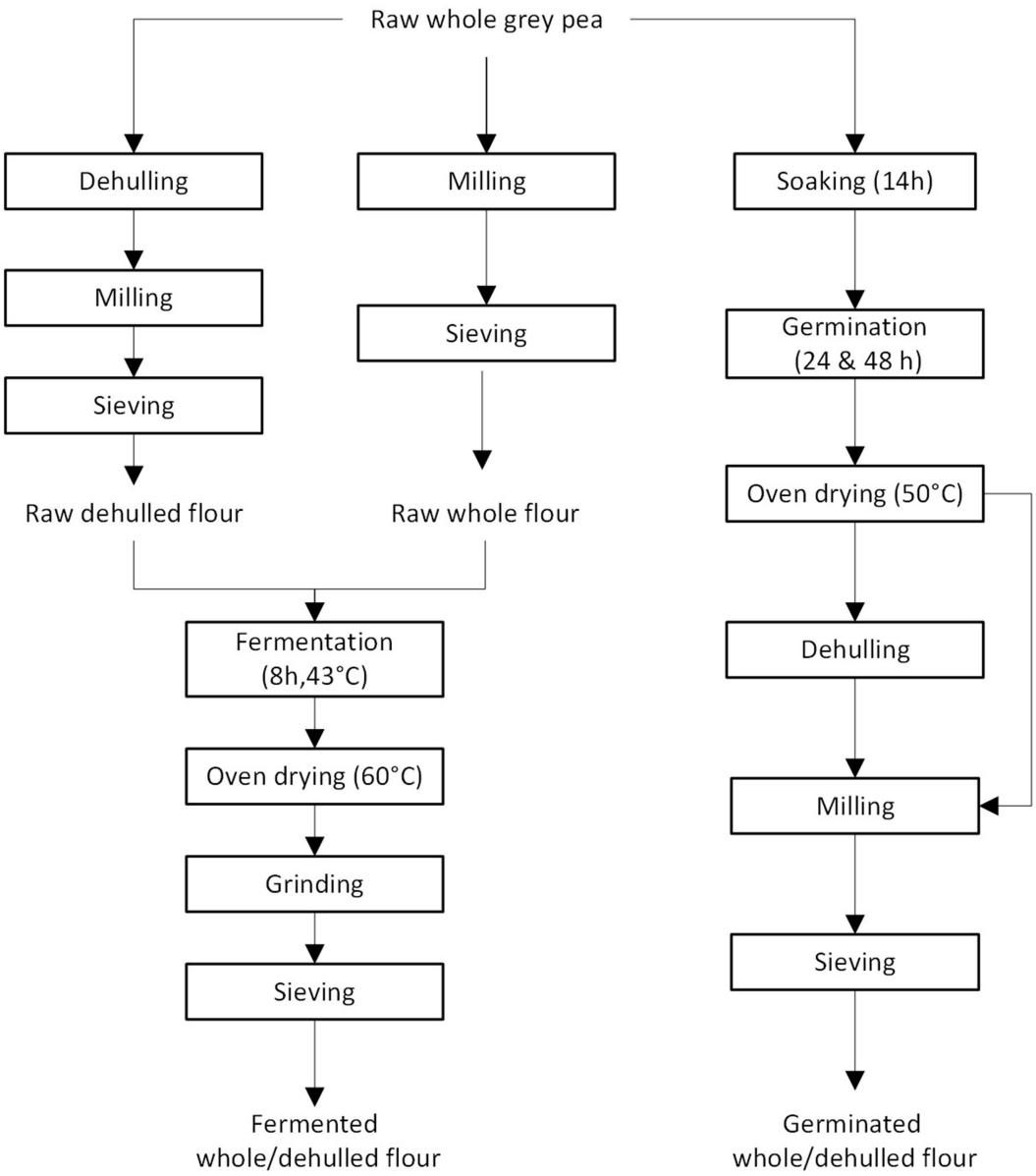

FIGURE 1
Flowchart of processes and conditions used in dehulling, germination and fermentation of grey peas.

(Laboratory mill 120, Perten Instruments AB, Stockholm, Sweden) and sifted through a 500 µm sieve. The flour was weighed into small portions, packed in the vacuum bags, and stored at 4°C for further analysis or processing.

Germination was conducted in whole grey peas following the method described by Ferawati et al. (3) with slight modification. In this study, the peas were first washed and then soaked in tap water (1:3 w/v) for 14 h at room temperature (~20°C), rather than under controlled temperature conditions in an incubator. Soaked grey peas were put between layers of wet tissue paper and germinated under ambient laboratory conditions for 24 and 48 h. The germinated seeds were divided into two batches, one to be used as whole and in the second batch, the peas were dehulled. The germinated peas (whole and dehulled) were dried in oven at 50°C (Termaks, TS4057, Bergen, Norway) and then milled to obtain the germinated pea flours.

Fermentation of grey pea flours (whole and dehulled) was conducted following the method standardized in our previous studies (18). Briefly a suspension of grey pea flour was prepared with distilled water (1:2 w/v) and inoculated with the starter culture Lyofast VSAB and incubated at 43°C for 8 h (Termaks, TS4057, Bergen, Norway). Lyofast VSAB is a commercial starter culture that consists of selected strains of *Streptococcus thermophilus* added with probiotic strains of *Lactobacillus acidophilus* and *Bifidobacterium animalis ssp. lactis* with an optimum growth temperature of 43°C. It is mainly used to produce fermented vegetable drinks and dairy alternatives like vegan yoghurt; however, it has shown some potential in its applications fermenting plant-based food. To control the development of fermentation, samples were taken every 2 h to measure the pH and total acidity. After fermentation, the fermented slurries were dried at 60°C in an oven (Termaks, TS4057, Bergen, Norway).

2.2.1.1 pH and total titratable acidity (TTA)

The pH and total acidity were measured at every 2 h intervals of fermentation until the end of fermentation (8 h). pH was measured in duplicate by the method described by Nuobariene et al. (19). Briefly 10 g of sample was suspended in 90 mL distilled water and stirred for 4 min and then the pH was recorded with a pH meter (Mettler Toledo, Greifensee Switzerland). The total acidity was determined by titration with 0.1 N sodium hydroxide (20). Briefly, a 30 mL of aliquot of the homogenized sample prepared for pH measurement was taken and titrated with 0.1 N NaOH, using phenolphthalein as an indicator. The titration continued until a faint pink color persisted for 30 s.

2.2.2 Protein analysis

The protein content of the grey pea flour samples, before and after processing, was measured using the dynamic flash combustion method (modified Dumas method) as described by Krotz et al. (74). 0.25 mg of dried sample was weighed and placed in the protein analyzer equipment (Thermo Scientific™ Flash™, EA 1112 series, MA, USA), the nitrogen to protein conversion factor of 6.25 was used. All the results are presented on a dry weight basis (dwb). The moisture content of grey pea flour was determined using the AOAC method (22). 5 g of flour samples were dried in the oven at 105°C (Termaks, TS4057, Bergen, Norway) until a constant weight was obtained. All analyses were conducted by duplicate.

2.2.3 Total phenolic content (TPC)

Extraction of phenolic compounds was performed according to the method of Xu and Chang (75). Flour samples (0.5 g) were extracted with 5 mL aqueous acetone 50% (v/v) for 3 h at room temperature at 300 rpm (IKA, KS 130 basic, Staufen, Germany). The extraction was followed by keeping the samples for 12 h in the dark. The samples were then centrifuged at 3000 rpm for 10 min (Centrifuge 5804R, Eppendorf, Hamburg, Germany) and the supernatants were transferred to new tubes. 5 mL of extraction solvent was added to the residues and the extraction procedure was repeated. The two extracts were combined and stored at 4°C in the dark until analysis.

The TPC was determined in the extracts using the method of Xu and Chang (75). 50 µL of the extract, 3 mL of distilled water, 250 µL of Folin–Ciocalteu's reagent, and 750 µL of 7% Na_2CO_3 were mixed and incubated at room temperature. After 8 min, 950 µL of distilled water was added to the mixture and left for 2 h at room temperature. The absorbance was measured at 765 nm using a spectrophotometer (PerkinElmer, LAMBDATM Bio+, MA, USA) with distilled water as blank. TPC results in dwb were calculated and expressed as gallic acid equivalents (mg of GAE/g sample).

2.2.4 Total antioxidant capacity (TAC)

The extraction procedure was followed according to the method of Sulaiman et al. (24). Briefly 2 g of flour samples were extracted with 25 mL of 70% (v/v) acetone for 24 h in room temperature using a shaker set at 200 rpm (Model GFL 3005, Delitzsch, Germany). The extraction was carried out under light protected conditions (25). Then samples were filtered through Whatman No.1 filter paper and filtrate stored at−20°C until analysis.

TAC was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) methods. TAC was expressed as Trolox equivalent (TE)/g of sample. The DPPH method performed according to Ruiz-Torralba et al. (26). 250 µL of DPPH solution were added to 25 µL of extracted sample and deionized water were added to reach 10 mL final volume. The absorbance was measured at 515 nm. The FRAP method was conducted by the procedure described by Benzie and Strain (27). 25 µL of the acetone extract samples were mixed with 900 µL of freshly prepared FRAP solution and the final volume was adjusted to 10 mL with deionized water. Absorbance was measured at 593 nm.

2.2.5 Water absorption index (WAI) and water solubility index (WSI)

WAI and WSI of untreated and treated grey pea flour samples were determined following procedures described by Du et al. (28). 2.5 g of flour were suspended in 30 mL distilled water in a pre-weighed centrifuge tube and cooked in a water bath for 30 min at 70°C. After cooling to room temperature, the mixture was centrifuged at 3000 × g for 20 min (Centrifuge 5804R, Eppendorf, Hamburg, Germany). The supernatant was transferred into pre-weighed aluminum containers to determine its solid content by evaporating the supernatant in an oven at 105°C overnight. The sediment was weighed. WAI and WSI were calculated using Equations 1, 2:

$$\mathrm{WAI}\left(\mathrm{g}/\mathrm{g}\right)=\frac{\text{Weight of sediment}}{\text{Weight of flour sample}} \quad (1)$$

$$\mathrm{WSI}\left(\frac{g}{100g}\right)=\frac{\text{Weight of dissolved solids in supernatant}\times 100}{\text{Weight of flour sample}} \quad (2)$$

2.2.6 Color characteristics

Color measurements of flour samples were carried out using a portable spectrophotometer (Konica Minolta CM-700d/CM-600d, Tokyo, Japan). The recorded parameters were L*, a* and b*. The L*value indicates lightness ranging from 0 (dark) to 100 (light). The a* value represents green-red spectrum with positive number indicating redness and negative numbers indicating green color. The b* value represents the yellow-blue spectrum, with positive numbers indicating yellow color (29).

2.2.7 Functional properties

2.2.7.1 Water absorption capacity (WAC)

WAC was determined using the method described by Ferawati et al. (3). 3 g of sample were dispersed in 25 mL distilled water in a pre-weighed centrifuge tube and stirred every 5 min for 30 min and then centrifuged at 3000 × g for 25 min (Centrifuge 5804R, Eppendorf, Hamburg, Germany). After centrifugation, the supernatant was decanted and the excess moisture was removed by drying the samples in an oven (Termaks, TS4057, Bergen, Norway) at 50°C for 25 min. The tube was then reweighed, and the WAC was expressed as grams of water bound per gram of the sample on a dwb.

2.2.7.2 Oil absorption capacity (OAC)

OAC was measured using the method described by Kaur and Singh (30). 0.5 g of sample were dispersed in 6 mL corn oil in a pre-weighed centrifuge tube and stirred for 1 min and left for 30 min before being centrifuged at 3000 × g for 25 min (Centrifuge 5804R,

Eppendorf, Hamburg Germany). After centrifugation, the oil layer was removed, and the tube was inverted for 25 min to drain excess oil before being re-weighed. The OAC was expressed as grams of oil bound per gram of the sample on a dwb.

2.2.7.3 Emulsion activity (EA) and emulsion stability (ES)

Emulsifying properties of flours were determined according to the method of Ferawati et al. (3). 3.5 g of flour sample were homogenized at 19000 rpm for 30 s in 50 mL distilled water. Then 25 mL peanut oil were added, and the mixture was homogenized again for 30 s. Another 25 mL of peanut oil were then added, and the mixture was homogenized for 90 s. The emulsion was evenly divided and transferred into two 50 mL centrifuge tubes and centrifuged at 1100 × g for 5 min. To determine emulsion stability the same method as described above was used to prepare the samples. The emulsified samples were heated for 15 min at 85°C in a water bath. Then cooled and centrifuged at 1100 × g for 5 min. EA and ES were calculated using Equation 3:

$$\text{Emulsion activity and stability}\left(\text{EA,ES,\%}\right) = \frac{\text{Volume of emulsified layer}}{\text{Total volume of emulsion}} \times 100 \quad (3)$$

2.2.7.4 Least gelation concentration (LGC)

The least gelation concentration was determined following the method described by Ferawati et al. (3). Suspension of grey pea flour samples at concentrations of 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20% (w/v) were prepared and heated for 60 min in a boiling water bath. The test tubes were cooled immediately under cold running water and further cooled at 4°C for 2 h. The least gelation concentration is the concentration at which the samples did not fall or slip when the test tube was inverted.

2.2.8 Pasting properties

The pasting properties of treated and untreated grey pea flours were studied by using a rapid visco analyzer (RVA, Perten 4,500, Stockholm, Sweden). A suspension of 3.5 g flour in 25 g of distilled water was prepared, adjusted to compensate for 14% moisture basis correction of the sample. The measurement protocol included 1 min of mixing, stirring, and warming up to 50°C at 160 rpm followed by 222 s of heating up to 91°C, 150 s of holding at 91°C, and then 228 s of cooling back down to 50°C, at the same rate as the heating. From the pasting curve, pasting temperature, peak viscosity, trough viscosity, breakdown, final viscosity, and setback were measured.

2.3 Statistical analysis

Processing trials were conducted in duplicate, further, untreated and treated samples were analyzed also in duplicate ($n = 2$), duplicate processing trials, duplicate analyses. Results are reported as mean ± standard deviation (SD). Paired *t*-tests were conducted to evaluate differences between whole and dehulled peas for each parameter under each treatment (i.e., TPC in fermented whole peas vs. TPC in fermented dehulled peas). One-way ANOVA followed by post-hoc Tukey analyses were used to determine significant differences between the reported parameters as a function of the type of treatment (i.e., comparison of TPC in raw, dehulled, germinated and fermented grey pea flour). Pearson correlations were computed to evaluate the associations between TAC obtained by two methods and the results of TPC in grey peas after each process. The level of significance was set at $p < 0.05$, statistical analyses were carried out using SPSS Statistics software version 26 (SPSS Inc., IBM Corporation, Armok, USA). Unpaired t-test was computed, to investigate differences on pH and lactic acid produced during fermentation, using GraphPad Prism 9 (GraphPad Software, Boston, MA, USA).

3 Results

3.1 Effect of fermentation on pH and acidity

The effect of fermentation on pH and total acidity of whole and dehulled grey pea flour is shown in Figures 2,3. Initial pH for whole and dehulled grey pea flour was recorded as 6.29 ± 0.02 and 6.52 ± 0.02, respectively. At the end of fermentation, pH dropped to 4.33 in whole flour, and to 4.5 in dehulled flour. Lactic acid content increased from 0.04 to 0.076% in whole flour and to 0.084% in dehulled flour. The

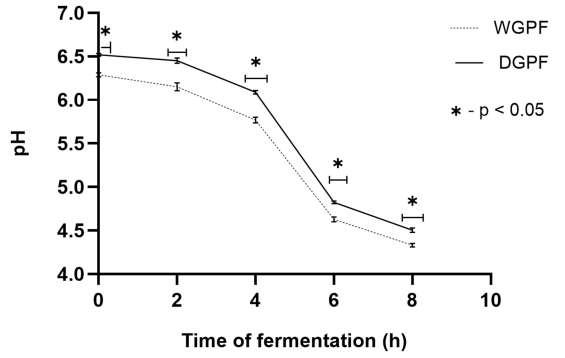

FIGURE 2
Changes in pH in whole and dehulled grey pea flour during fermentation. Significant differences are shown by * at $p < 0.05$ level. WGPF, whole grey pea flour; DGPF, dehulled grey pea flour.

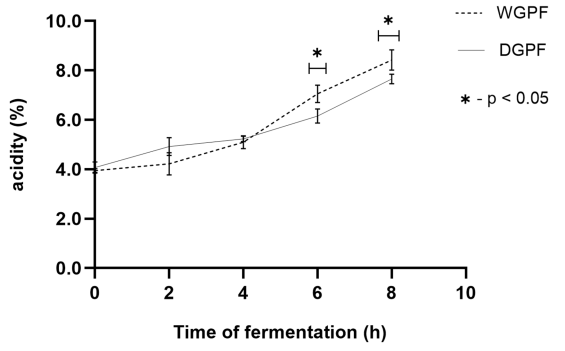

FIGURE 3
Changes in lactic acid content in whole and dehulled grey pea flour during fermentation. Significant differences are shown by * at $p < 0.05$ level. WGPF, whole grey pea flour; DGPF, dehulled grey pea flour; TTA, total titrable acidity.

results for pH showed significant differences between whole and dehulled flour at every 2 h intervals.

3.2 Processing effect on protein, TPC and TAC

The results for the moisture and protein content determination are shown in Table 1. Moisture content decreased in the fermented and 24 h germinated samples while the 48 h germinated sample retained nearly the same moisture level as raw flour. The protein content of flour ranged from 22.5 to 25.8%, with the raw whole flour having the lowest and the fermented dehulled flour having the highest protein content. The protein content changed significantly in dehulled treated flours compared to dehulled raw flour, after 24 h germination $p = 0.003$, after 48 h germination $p = 0.002$ and after fermentation $p < 0.001$.

Total phenolic content of raw and treated grey pea flour is expressed as gallic acid equivalents (mg of GAE/100 g sample). The results are presented in Table 1. Significant differences were found in the TPC after treatment methods. Fermented whole flour showed the highest TPC with 227 mg GAE/100 g sample, 1.6-fold higher than raw whole pea flour while raw dehulled grey pea flour had the lowest TPC at 35 mg GAE/100 g sample. Dehulling led to a significant reduction ($p < 0.001$) in the TPC of raw flours, which resulted in a 4-fold decrease of TPC in dehulled pea flour.

The TAC results using two different methods, FRAP and DPPH, are presented in Table 1. In both methods fermented whole peas had the highest TAC value with 204 ± 4.7 and 398 ± 4.1 mgTE/100 g in FRAP and DPPH, respectively, while the lowest value was observed in raw dehulled peas with 53.6 ± 1.8 mgTE/100 g in FRAP method and 110.8 ± 7.4 mgTE/100 g in DPPH method. Dehulling significantly decreased TAC in both methods. Regarding germination, a significant increase was observed only in 48 h germinated flours in both flours made of whole and dehulled peas. Additionally, a significant correlation was found between TPC and TAC using both methods with $r = 0.810$ for TPC vs. TAC-FRAP and $r = 0.898$ for TPC vs. TAC-DPPH.

3.3 Water absorption index (WAI) and water solubility index (WSI)

Table 2 presents the results for the WAI and WSI of raw and treated grey pea flours. The WAI ranged from 2.89 to 3.33 (g/g), with raw dehulled flour having the highest value. No significant difference was observed in WAI between raw whole flour and other treatments as well as between raw dehulled flour and other dehulled treatments. Raw dehulled grey pea flour had the highest WSI with 24.50 (g/100 g). However, this value was not significantly higher than those obtained for 24 and 48 h dehulled germinated grey pea flours.

3.4 Color characteristics

Hunter color values (L*, a*, b*) of flour samples are shown in Table 2. Dehulling affected the hunter color values in all flour samples compared to their whole flour. Fermented whole flour showed the lowest L* and b* value, while having the highest a* value.

TABLE 1 Moisture, protein content, total phenolic content and antioxidant capacity of raw, germinated and fermented grey pea flour, whole and dehulled.

	Raw		Germinated 24 h		Germinated 48 h		Fermented	
	Whole	Dehulled	Whole	Dehulled	Whole	Dehulled	Whole	Dehulled
Moisture (%)	10.6 ± 0.01^{aC}	9.4 ± 0.1^{bZ}	7.9 ± 0.55^{aB}	7.0 ± 0.36^{bY}	10.5 ± 0.52^{aC}	10.0 ± 0.71^{bAZ}	5.9 ± 0.25^{aA}	5.8 ± 0.00^{aX}
Protein (%)	22.5 ± 0.34^{aA}	24.6 ± 0.29^{bX}	23.7 ± 0.21^{aBC}	25.7 ± 0.39^{bY}	23.4 ± 0.52^{aB}	25.8 ± 0.09^{bY}	24.3 ± 0.15^{aC}	25.8 ± 0.25^{bY}
TPC (mgGAE/100 g)	142 ± 0.03^{aC}	35 ± 0.07^{bX}	74 ± 0.04^{aA}	45 ± 0.18^{aX}	116 ± 0.05^{aB}	65 ± 0.22^{bX}	227 ± 0.01^{aD}	107 ± 0.10^{bY}
TAC-FRAP (mgTE/100 g)	151.5 ± 6.4^{aA}	53.6 ± 1.8^{bX}	146.2 ± 4.2^{aA}	56.3 ± 1.9^{bXY}	180.9 ± 2.2^{aB}	62.4 ± 3.7^{bY}	204.1 ± 4.7^{aC}	80.4 ± 3.8^{bZ}
TAC-DPPH (mgTE/100 g)	194.9 ± 6.9^{aA}	110.8 ± 7.4^{bX}	203.2 ± 4.0^{aA}	111.6 ± 5.7^{bX}	253.4 ± 3.7^{aB}	151.8 ± 3.9^{bY}	398.3 ± 4.1^{aC}	271.4 ± 9.4^{bZ}

For each parameter (each row), superscripts lowercase letters (a, b) indicate differences due dehulling, for raw, germinated (24 or 48) and fermented flours. Superscripts uppercase letters indicate differences due to processing (i.e., germination or fermentation), note that letters ABC denote differences due processing for whole pea flour and letters XYZ denote differences due processing for dehulled pea flour. Results are presented as mean ± SD and significant differences were computed at $p < 0.05$. TPC, total phenolic content; TAC-FRAP, total antioxidant activity by FRAP; TAC-DPPH, total antioxidant activity by DPPH.

TABLE 2 Water absorption index (WAI), water solubility index (WSI) and color characteristic of raw, germinated and fermented grey pea flour, whole and dehulled.

		Raw		Germinated 24 h		Germinated 48 h		Fermented	
		Whole	Dehulled	Whole	Dehulled	Whole	Dehulled	Whole	Dehulled
WAI (g/g)		2.89 ± 0.37^{aA}	3.33 ± 0.12^{bX}	3.30 ± 0.07^{aA}	3.07 ± 0.05^{bX}	3.13 ± 0.03^{aA}	3.15 ± 0.20^{aX}	3.29 ± 0.10^{aA}	3.13 ± 0.15^{bX}
WSI (g/ 100 g)		19.90 ± 0.38^{aB}	24.50 ± 0.50^{bY}	20.00 ± 0.73^{aB}	23.90 ± 0.50^{bY}	18.80 ± 0.73^{aB}	23.70 ± 0.38^{bY}	8.40 ± 0.56^{aA}	11.00 ± 0.23^{bX}
Hunter color values	L*	82.20 ± 0.57^{aBC}	88.37 ± 1.04^{bYZ}	83.83 ± 0.62^{aC}	89.20 ± 0.59^{bZ}	81.89 ± 1.18^{aB}	87.10 ± 0.17^{bY}	70.98 ± 0.56^{aA}	84.31 ± 0.21^{bX}
	a*	1.52 ± 0.07^{aB}	1.21 ± 0.06^{bY}	0.82 ± 0.14^{aA}	0.46 ± 0.02^{bX}	1.08 ± 0.21^{aA}	0.65 ± 0.16^{bX}	3.67 ± 0.16^{aC}	1.88 ± 0.08^{bZ}
	b*	14.91 ± 1.22^{aB}	18.15 ± 0.26^{bY}	12.50 ± 0.66^{aA}	15.42 ± 0.22^{bV}	12.05 ± 0.44^{aA}	16.69 ± 0.04^{bX}	11.45 ± 0.42^{aA}	20.19 ± 0.25^{bZ}

For each parameter (each row), superscripts lowercase letters (a, b) indicate differences due dehulling, for raw, germinated (24 or 48) and fermented flours. Superscripts uppercase letters indicate differences due to processing (i.e., germination or fermentation), note that letters ABC denote differences due processing for whole pea flour and letters XYZ denote differences due processing for dehulled pea flour. Results are presented as mean ± SD and significant differences were computed at $p < 0.05$.

3.5 Functional properties

It can be seen in Table 3, that most of the functional properties of grey pea flour had changed by different processing methods. The WAC of different flours ranged from 0.84 to 1.54 gwater/g DM and the fermented whole grey pea flour showed a significantly higher WAC than other flours. The OAC of raw and treated flours ranged from 0.81 to 1.06 g oil/g DM, with the 48 h germinated whole flours having the highest and the dehulled fermented flours having the lowest capacity to absorb oil. Germination did not significantly affect EA and ES. Fermentation resulted in a poor EA (3.49 ± 0.47% and 3.25 ± 0.24% in whole and dehulled flour respectively) and ES (4.62 ± 0.49% in whole and 4.36 ± 0.57% in dehulled flour), while significantly improved the WAC (1.54 ± 0.01 and 1.24 ± 0.04 g water/g DM in whole and dehulled flour respectively). Highest ES (57.64 ± 2.36%) was found in 48 h whole germinated flour. When looking at the effect of dehulling, significant improvements were not found compared to the whole flour in treated and untreated dehulled grey pea flours. The LGC values ranged from 10 to 12%. The LGC for both whole and dehulled flour remained consistent across different treatments indicating that dehulling, germination or fermentation did not impact the gelling capacity of grey pea flour.

3.6 Pasting properties

The results of the rapid visco analyzer for the raw and treated flours are presented in Table 4. The pasting temperature of flours was not significantly affected by the type of processing method except for the fermented dehulled flour sample, in which higher pasting temperature was observed (86.93 ± 0.03°C). Peak viscosities of flour were affected by both dehulling and processing methods. Dehulling increased peak viscosity in raw and treated samples. The highest peak viscosity was observed in 48 h germinated samples (1,061 ± 2.12 cP in whole and 1,308 ± 2.12 cP in dehulled flour) while fermentation resulted in lowest peak viscosity (291 ± 2.12 and 633 ± 1.41 cP in whole and dehulled flour respectively). The lowest breakdown value was found for 24 h germinated dehulled flour (5.50 ± 0.71 cP). In contrast the highest breakdown values were observed for 48 h germinated and fermented dehulled flours with 80 ± 0.00 and 82 ± 2.82 cP, respectively. In general, higher viscosities were obtained with different treatment methods except for the fermentation process, which on contrary caused a decrease in viscosities and resulted in the least paste stability. Additionally, 48 h germination had greater impact on viscosities compared to 24 h germination.

4 Discussion

4.1 Effect of fermentation on pH and total acidity

Fermentation resulted in a decrease in the pH and increase in lactic acid content for both whole and dehulled flour. These changes can be attributed to microbial activity, particularly the dominance of lactic acid bacteria, which degrade carbohydrates and acidify the products. It is reported that lactic acid produced during fermentation

TABLE 3 Functional properties of raw, germinated and fermented grey pea flour, whole and dehulled, results are presented in means ± SD in dry matter (DM).

	Raw		Germinated 24 h		Germinated 48 h		Fermented	
	Whole	Dehulled	Whole	Dehulled	Whole	Dehulled	Whole	Dehulled
WAC (g water/ g)	1.13 ± 0.02^{aC}	0.84 ± 0.05^{bX}	0.95 ± 0.01^{aA}	0.84 ± 0.01^{bX}	1.09 ± 0.02^{aB}	0.97 ± 0.01^{bY}	1.54 ± 0.01^{aD}	1.24 ± 0.04^{bZ}
OAC (g oil/ g)	0.94 ± 0.03^{aB}	0.90 ± 0.04^{aXY}	0.98 ± 0.07^{aBC}	0.96 ± 0.02^{aY}	1.04 ± 0.02^{aC}	1.06 ± 0.07^{aZ}	0.83 ± 0.01^{aA}	0.81 ± 0.03^{aX}
EA (%)	49.28 ± 0.92^{aB}	49.26 ± 0.47^{aY}	48.30 ± 0.48^{aB}	48.65 ± 0.24^{aY}	48.04 ± 0.38^{aB}	48.65 ± 0.24^{aY}	3.49 ± 0.47^{aA}	3.25 ± 0.24^{aX}
ES (%)	53.79 ± 1.29^{aBC}	54.10 ± 2.00^{aY}	51.70 ± 3.44^{aB}	52.79 ± 2.96^{aY}	57.64 ± 2.36^{aC}	54.44 ± 5.03^{aY}	4.62 ± 0.49^{aA}	4.36 ± 0.57^{aX}
LGC (%)	$12\%^{a}$	$12\%^{a}$	$10\%^{a}$	$10\%^{a}$	$10\%^{a}$	$10\%^{a}$	$12\%^{a}$	$12\%^{a}$

For each parameter (each row), superscripts lowercase letters (a, b) indicate differences due dehulling, for raw, germinated (24 or 48) and fermented flours. Superscripts uppercase letters indicate differences due to processing (i.e., germination or fermentation), note that letters ABC denote differences due processing for whole pea flour and letters XYZ denote differences due processing for dehulled pea flour. Results are presented as mean ± SD and significant differences were computed at $p < 0.05$.WAC, water absorption capacity, OAC, oil absorption capacity, EA, emulsion activity, ES, emulsion stability, LGC, least gelation concentration.

TABLE 4 Pasting properties of raw, germinated and fermented grey pea flour, whole and dehulled.

	Raw		Germ 24 h		Germ 48 h		Fermented	
	Whole	Dehulled	Whole	Dehulled	Whole	Dehulled	Whole	Dehulled
Pasting temperature (°C)	76.93 ± 0.53^{aA}	76.87 ± 0.53^{aX}	77.58 ± 0.53^{aA}	76.85 ± 0.49^{bX}	76.55 ± 0.07^{aA}	76.15 ± 0.63^{aX}	76.83 ± 0.53^{aA}	86.93 ± 0.03^{bY}
Peak viscosity (cP)	871 ± 13.43^{aB}	942 ± 8.48^{bY}	878 ± 1.42^{aB}	934 ± 4.24^{bY}	$1{,}061 \pm 2.12^{aC}$	$1{,}308 \pm 2.12^{aZ}$	291 ± 2.12^{aA}	633 ± 1.41^{bX}
Trough viscosity (cP)	848 ± 15.55^{aB}	930 ± 4.95^{aY}	863 ± 1.41^{aB}	928 ± 4.95^{bY}	$1{,}025 \pm 1.41^{aC}$	$1{,}228 \pm 2.12^{aZ}$	241 ± 0.70^{aA}	551 ± 4.24^{bX}
Breakdown (cP)	23.5 ± 2.12^{aAB}	11.5 ± 3.53^{aX}	15 ± 0.00^{aA}	5.50 ± 0.71^{bX}	36 ± 0.70^{aBC}	80 ± 0.00^{aY}	50 ± 1.41^{aC}	82 ± 2.82^{aY}
Final viscosity (cP)	$1{,}369 \pm 2.82^{aB}$	$1{,}434 \pm 9.89^{bY}$	$1{,}312 \pm 2.12^{aB}$	$1{,}414 \pm 13.44^{aY}$	$1{,}415 \pm 4.94^{aB}$	$1{,}677 \pm 3.53^{aZ}$	463 ± 4.94^{aA}	893 ± 2.12^{bX}
Setback (cP)	521 ± 12.72^{aC}	503 ± 4.94^{aZ}	449 ± 3.53^{aB}	486 ± 8.49^{aZ}	389 ± 3.53^{aB}	449 ± 1.41^{aY}	222 ± 4.24^{aA}	342 ± 6.36^{bX}

For each parameter (each row), superscripts lowercase letters (a, b) indicate differences due dehulling, for raw, germinated (24 or 48) and fermented flours. Superscripts uppercase letters indicate differences due to processing (i.e., germination or fermentation), note that letters ABC denote differences due processing for whole pea flour and letters XYZ denote differences due processing for dehulled pea flour. Results are presented as mean ± SD and significant differences were computed at $p < 0.05$.

with lactic bacteria acidifies the products, inhibiting spoilage bacteria and thus ensuring its preservation, a key factor in its popularity within the food industry (31).

Interestingly, alkaline fermentation of legumes has been previously reported, with the increase in pH attributed to protein degradation by *Bacillus* spp. During this process, sources of carbon and nitrogen are used by the bacteria to produce ammonium hydroxide and ammonia, resulting in high pH values and the characteristic odor of alkaline fermented foods (i.e., natto, douchi), which are mainly produced from soybeans (32). In contrast, in this study, the use of lactic acid bacteria for the fermentation of grey pea resulted in a decrease in pH due to increased acidity from lactic acid production. The selection of raw materials, starter cultures and fermentation conditions are key parameters influencing the course of fermentation and the characteristics of the final fermented product. Additionally, lactic bacteria may enhance sensory characteristics of fermented foods by generating desirable aroma components and reducing off-flavors, contributing to the overall improvement in taste and quality (33). The highest pH reduction occurred between 4 and 6 h fermentation process, this is likely the time needed to adapt fermentation condition by endogenous microbes (34). Fermentation of both raw and dehulled grey pea flour showed similar trends in acidity during fermentation with no significant difference between the two flours at the end of fermentation. Therefore, dehulling does not appear to affect fermentation outcomes in terms of acidity.

4.2 Moisture and protein content

Higher moisture content in 48 h germinated sample compared to 24 h germination may be due to increased water uptake by grey pea seeds in order to carry out the metabolic processes during germination, which resulted in more hydrated cells within the seeds, similar trend was shown for germination of chickpeas flour (35). It is important to monitor the moisture content in flours, as higher moisture levels can impact the food product's characteristics including physical appearance, texture, taste, weight. Additionally, moisture content affects factors such as shelf-life, freshness, quality and resistance to bacterial contamination.

Dehulling significantly increased protein content of raw and processed grey pea flour. This is because seed coat (hull) of pulses contains little to no protein. Removing the hull increases the concentration of the endosperm, thereby proportionally increasing the protein content in the dehulled seed (36, 37). The findings of this research are in agreement with the results obtained by Wang et al. (21) in various lentils varieties and by Pal et al. (36) in horsegram pulses. The results of this study showed a 5.3% increase in protein content in whole pea flour during 24 h germination and 4% after 48 h germination. It has been suggested that during germination, hydrolytic activities of the enzymes increased due to breakdown of proteins. The relative increase of protein in fermented grey peas can be attributed to the natural increment in bacterium biomass and the conversion of the inorganic nitrogen to organic nitrogen. This effect combined with the reduction of carbohydrates in the form of sugars consumed by bacteria during fermentation, may contribute to the higher protein content in the fermented products (38). Similar increase in the protein content (4.47%) of germinated grass pea flour was reported by Lakshmipathy et al. (39). Other authors have also reported an increase in protein during fermentation of pigeon pea 3.67% after 1 day and 9.63% after 5 days. These pigeon peas were boiled and dehulled and underwent natural fermentation (40).

4.3 Total phenolic content (TPC)

Dehulling significantly decreased the TPC in raw flour and this decreasing trend was observed in all treatments (ranging from 40.84–75.35%). Singh et al. (41) noted that the seed coat (hull) of pulses which acts as a protective layer for the cotyledons contains high concentrations of phenolic compounds. Consequently, dehulling of pulses removes substantial amounts of polyphenols. The reduction in TPC observed in germinated whole flour could be due to the increased activity of polyphenol oxidase and other catabolic enzymes. Additionally, the activation of enzymes during germination leads to hydrolysis of various components including phenolic compounds (42). Guajardo-Flores et al. (43) reported a 58.33% decrease in raw and 10% increase in 5-day germinated dehulled black bean flour. Similarly, Lakshmipathy et al. (39) observed a 14.4% reduction in TPC for dehulled and a 15.34% increase in 48 h germinated grass pea flour. The TPC reduction in raw dehulled grey pea flour in this study was higher (75.35%) than the one in the mentioned studies. During germination of whole grey pea flour TPC decreased by 47.98 and 18.3% for 24 and 48 h, respectively, while in dehulled grey pea flour TPC increased by 28.75 and 85.71% for 24 and 48 h germination, respectively. Similarly, Navyashree et al. (44) reported TPC reduction (47.72%) in 48 h germinated white finger millet. As germination time increased the TPC was also increased (from 74 ± 0.04 to 116 ± 0.05 mgGAE/100 g in whole and from 45 ± 0.18 to 65.022 mgGAE/100 g in dehulled flour). Similar results were obtained for chickpea flour from 130.41 ± 2.67 to 245.25 ± 2.61 mgGAE/100 g with germination time from 12 to 48 h (35). A significant increase in TPC in fermented samples (59.86% in whole and 205% in dehulled) might be due to the degradation of polymeric phenolic compounds by proteolytic enzymes into simpler, more biologically active compounds which are then released as soluble phenolic compounds. Additionally, fermentation can loosen the lignocellulosic matrix, resulting in the release of phenolic compounds from an inaccessible state (11, 45). Çabuk et al. (11) reported 88% increase in TPC in pea protein concentrate after 9 h fermentation with *L. plantarum*. Additionally, increases of 84.9 and 90.6% in TPC were observed after 48 h of fermentation with *L. plantarum* and natural fermentation of soybean flour, respectively, as reported by Fernandez-Orozco et al. (46).

4.4 Total antioxidant capacity (TAC)

The antioxidant capacity was measured through two different methods, the FRAP assay which assesses metal reducing ability in the presence of antioxidant and DPPH assay which is based on antioxidant ability to scavenge free radical (47). Dehulling significantly decreased antioxidant activity in raw flour by 64.62% (FRAP) and 43.15% (DPPH) and this decreasing trend was observed across all treatments likely due to hull removal. Phenolic compounds which are concentrated in seed coat are closely correlated to antioxidant capacity. Consequently, removing the hull which contains high amount of these phenolic compounds results in a decrease in

antioxidant capacity (43). The results are aligned with findings of Nelom et al. (48) where TAC reduction varied from 55.72 to 67.76% for dehulled cowpea when TAC was measured through DPPH method. Lower TAC reduction (13.08%) was reported by Lakshmipathy et al. (39) for grass pea flour using DPPH assay. The effect of germination on increasing antioxidant capacity could be explained by synergistic effect with phenolic compounds. Additionally enzymatic reaction during germination can enhance TAC due to formation of phenolic compounds from seed coats and cotyledons (49). In this study TAC in 48 h germinated whole flour increased by 19.4 and 30% using FRAP and DPPH methods, respectively. TAC increase (16.45%-DPPH) due to germination was reported by Lakshmipathy et al. (39) when grass pea was germinated for 48 h. Mao et al. (50) also reported 8.87 to 24.79% increase for different 72 h germinated chickpeas varieties using FRAP and 7.66 to 168% increase using DPPH assays. In this study, antioxidant activity improved after fermentation by 34.72 and 50% using FRAP, 104.36 and 145.4% using DPPH of whole and dehulled grey pea flour, respectively. It has been argued that microbial hydrolysis occurring during fermentation increases phenolic compounds and flavonoids. This hydrolysis may cause that bound phenolics are converted into free forms, resulting in a higher antioxidant level (51). In addition, Fermentation causes the structural disintegration of plant cell walls, resulting in the release of diverse antioxidant compounds (52). Okechukwu (53) reported that the DPPH antioxidant activity of ethanolic extract of naturally fermented pigeon pea at room temperature for 7-days increased from 0.810 to 1.014 mg/mL. The results in Table 1 indicate a correlation between TPC and TAC. However, this relation is complex and involves various factors. For instance, other substances can contribute to antioxidant properties beyond phenolic compounds. Moreover, Different analytical methods for assessing antioxidant capacity can lead to varying results.

4.5 Water absorption index (WAI) and water solubility index (WSI)

There was an increase (14.18 and 8.3%), however not significant, in WAI in 24 and 48 h germinated samples, respectively, compared to whole raw flour, which may be due to more hydrophilic sites exposed to water after germination. According to Du et al. (28) WAI is related to the hydrophilicity and gelation capacity of starch and protein as biomacromolecules in flour. Similar increase in WAI in 24 h germinated faba bean flour (2.79 to 3.13) was reported by Kumar et al. (54). Similar increase was found in grey pea whole flour after fermentation (2.89 to 3.29). Onweluzo and Nwabugwu (55) suggested that during fermentation process high molecular weight proteins and carbohydrates are hydrolyzed into smaller and more soluble components, thereby increasing WSI. However, a significant decrease (57.79 and 55.1%) in the WSI was observed in fermented grey pea flour. These findings are in agreement with results obtained by Toor et al. (56) who reported a decrease in WSI in fermented chickpea (3%) and pigeon pea (9%) flours. This reduction in WSI could be attributed to utilization of soluble compounds by microorganisms for their growth as discussed by Ilowefah et al. (17). The higher protein content might also explain the elevated WSI observed in dehulled fermented flour. There was also observed a significant increase in WSI in all dehulled samples, since WSI, is related to the presence of soluble molecules, dehulling step might have resulted in the increased solubility of grey pea flour by eliminating the effect of the seed coat on solubility, which is mainly composed of insoluble fiber.

4.6 Color characteristics

Color is a crucial characteristic for consumer appeal, particularly when incorporating as an ingredient in the final product (57). The higher L* value in dehulled flours ranging from 84.31 to 89.20 indicated a visually lighter color. This could be attributed to the reduction of phenolic and chlorophyl due to seed coat removal (58). This is in line with the TPC results that were significantly lower in dehulled samples. The observed increase, although not significant, in the lightness (L* value) of germinated flour may be attributed to the dissociation of colored pigments during the soaking process (44). It was also indicated that during germination of whole flour, non-enzymatic browning occurs due to the transfer of color pigments from seed coat to endosperm (35). Thus, the lightness of germinated flour can fluctuate based on the stages of soaking and germination. The reduction in a* and b* values in germinated flour could be attributed to changes in carbohydrates and protein hydrolysate (57). Similarly Lakshmipathy et al. (39) reported a reduction in a* and b* values in germinated grass pea flour. In fermentation the color variations could be attributed to the degradation of pigments (56). Since all treatment methods involve a heating step, heat treatment can impact each of the color values to some extent.

4.7 Functional properties

4.7.1 Water absorption capacity (WAC) and oil absorption capacity (OAC)

WAC of the flours is associated with the presence of hydrophilic components (30). Different protein conformations and hydrophilic carbohydrates fractions in flours contribute to variations in WAC (59). In this study, there was a significant reduction in WAC in both raw (25.66%) and treated dehulled flour which could be attributed to the removal of seed coat. The fibers present in hull bind and hold water and their absence leads to a decreased ability of the flour to retain water (60). Similar results were reported for dehulled grass pea flour (11.16%) by Lakshmipathy et al. (39). Lower WAC in germinated flour compared to whole raw flour (15.93 and 3.54% in 24 and 48 h germination respectively) may result from the reduction of hydrophilic points due to enzymatic degradation of starch and fiber as seen in germinated black chickpea flour by Kumar et al. (57). Conversely Ferawati et al. (3) reported an increase in WAC in 24 and 48 h germinated grey pea. An increase in WAC in 48 h germinated dehulled flour which was attributed to the enhancement of water binding sites resulting from macromolecules modification (61). Fermentation caused an increase in WAC (36.28 and 47.62% in whole and dehulled flour respectively), in previous studies it was observed an increase in WAC in fermented chickpeas and red beans suggesting that microbial protease enzymes breakdown peptide bonds during fermentation, leading to an increase in hydrophilic groups in proteins and low molecular weight proteins (62, 63).The OAC is influenced by the

binding of lipids to the hydrophobic amino acid side chains and their availability on the protein surface. The observed improvement (10.63 and 17.77%) in the OAC of 48 h germinated whole and dehulled flour, respectively, could be due to the changes in the conformation of the protein molecules which may have resulted in more exposure of the non-polar residues from the interior to the surface and increase in the surface availability of these hydrophobic amino acids (60). The decrease or increase of OAC during fermentation depends on the surface availability of hydrophobic amino acids as fat droplets bind with non-polar molecules. Therefore, any changes in protein molecular structure can result in an increase or decrease on OAC (56).

4.7.2 Emulsifying properties

Emulsifying properties of pulse flours are generally assessed by two parameters, emulsifying activity (EA) and emulsifying stability (ES). Germination is thought to enhance EA and ES through the dissociation and partial unfolding of polypeptides, exposing hydrophobic amino acid sites. This exposure enhances the hydrophobic interactions between peptide chains and lipid droplets, significantly boosting the availability of protein volume and surface area (39). However, in this study germination did not improve emulsifying properties of grey pea flour. This observation aligns with findings by Ferawati et al. (3) where a similar decrease was obtained in germinated grey pea. This reduction could be attributed to changes in protein concentrations or alteration of hydrophobicity/hydrophilicity ratio and structural constraints of the proteins. These factors could affect protein's ability to unfold and form a film around dispersed oil droplets (64).

Fermented flour showed a significant decrease in EA by approximately 93% and ES by around 92%. This reduction is attributed to the increase in hydrophobicity which affects protein's ability to migrate to the oil–water interface. This migration is crucial for lowering interfacial tension and facilitating emulsion formation and unhydrolyzed proteins (65). In addition, during fermentation the concentration of water-soluble protein decreases affecting the emulsifying properties of fermented flour. EA of pea flour decreased to 3.39 m^2/g after fermentation with *L. rhamnosus* L08 (66).

4.7.3 Least gelation concentration (LGC)

Although, dehulling process contributed to higher protein concentration, potentially improving the formation of the three-dimensional network, no significant difference in LGC of whole and dehulled flours was obtained in this study. This could be attributed to the fact that the increase in protein concentration facilitates gelation due to more intermolecular interaction during heating. Alternatively, the complex carbohydrate present in the seed coat may have interfered with the formation of a continuous network of molecules suggesting that their impact on the final gelation was not substantial enough to significantly affect the LGC (67, 68).

4.8 Pasting properties

Pasting properties are crucial for various applications in the food industry, as they are influenced by the presence of starch, protein, amylase activity and amylose/amylopectin ratio in the flour (35, 69). The pasting temperature did not change significantly in germinated flour. The increase in viscosities of dehulled flour samples might be due to a higher proportion of starch compared to fibrous whole flours (70). Similar result was observed in dehulled chickpea and faba bean flour by Teferra et al. (70). For 48 h germinated samples the increase in viscosity can be related to changes in the ratio of amylose /amylopectin due to starch degradation during germination. Increased interaction of starch granules with amylolytic enzymes can result in lower amylose content, which happens due to the breaking of intact cell walls during germination (71). The increase in peak viscosity for germinated flour was attributed to starch granules swelling resulting from protein and fiber matrix loosening (61). Breakdown viscosity decreased in 24 h germinated flour (36.17 and 52.17% in whole and dehulled flour respectively), while it increased in 48 h germinated flour. Lower breakdown viscosity indicates good paste stability and strong shearing resistance. Final viscosity improved significantly in 48 h germinated dehulled flour. Setback viscosity decreased for both 24 and 48 h germinated flour indicating a high retrogradation tendency. These results are similar to findings for 48 h germinated bambara groundnut flours where peak viscosity, trough viscosity, breakdown viscosity, final viscosity and setback viscosity increased by 2, 1.46, 54.28, 3.99 and 19.24%, respectively (61). The decrease in breakdown, setback and final viscosity in 24 h germinated flour could be attributed to the degradation of starch granules and hydrolysis of amylopectin and amylose by enzymes during germination, which can lead to less entanglement between the chains (72).

In the fermented flour pasting viscosities decreased by 66.55, 71.58, 66.14 and 57.39% for peak, trough, final and setback viscosity, respectively, compared to raw flour with the exception of breakdown value which increased by 112% in whole flour. Li et al. (73) investigated the effect of yellow pea flour fermentation with five lactic acid bacteria strains on pasting properties. The pasting properties of fermented yellow pea flour were significantly lower compared to raw flour. For instance, yellow pea fermented with *Lactobacillus acidophilus* ATCC 43121 for 18 h showed 6.09, 1.91, 29.05, 6.63% and 22.42% reduction in peak, trough, breakdown, final and seatback viscosity, respectively. Having compared and discussed the pasting properties of grey pea flour samples, it is worth mentioning that pasting properties of flours are not only affected by starch but also non-starch components such as proteins, fat, and fiber and their interactions with starch can influence the performance of flours during pasting (23). Lower pasting properties obtained through fermentation may be favorable for certain applications in the food industry, where lower tendencies to retrograde are favorable, for examples in the formulation of soups and sauces, since they can experience loss of viscosity and precipitation due to retrogradation (30). A better understanding of interactions at the molecular level is required to better understand these results.

5 Conclusion

In conclusion, various processing methods—such as dehulling, germination, and fermentation—played crucial roles in modifying the bioactive and functional properties of grey pea flour. Germination and in particular, lactic fermentation significantly enhanced bioactive properties as measured by TPC and TAC. The main findings of this research indicate that fermentation of grey peas notably increased TPC and improved total antioxidant capacity

as measured by both the FRAP and DPPH methods. Additionally, protein content showed increase following germination and fermentation of whole and dehulled grey pea flour. The impact of processing on functional properties varied; in some cases, such as germination, functional properties like pasting improved, while in others, such as fermentation, they were reduced. Depending on the intended purpose and desired properties of the final product, where grey peas are considered a major component, the obtained results provide a solid foundation for selecting a suitable processing method for grey peas and their successful incorporation into food formulations. Furthermore, the results could have policy implications by encouraging industries and farmer to increase grey pea production and processing.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

AA: Conceptualization, Formal analysis, Writing – review & editing, Investigation, Methodology, Writing – original draft. YS: Formal analysis, Validation, Writing – review & editing. OB: Conceptualization, Project administration, Resources, Visualization, Writing – review & editing. YG: Conceptualization, Methodology, Resources, Supervision, Writing – review & editing. CL: Conceptualization, Data curation, Formal analysis, Software, Supervision, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Koji amazake produced by double saccharification contains more isomaltose and modifies the gut microbiota in mice

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Koji amazake, which is made from rice and rice *koji* (a product of *Aspergillus oryzae*), is a traditional Japanese beverage that has glucose as its main component. It also contains isomaltose, which has been reported to have various functionalities related to gut health. In the present study, we attempted to produce *amazake* with a higher concentration of isomaltose without using any additives by focusing on the saccharification step of rice *koji* production as a means of creating new value for *amazake.* Two types of rice *koji* that were obtained at different fermentation time points were used, and we changed the saccharification process from the usual one step of saccharification to two steps of saccharification using a different type of *rice koji* for each step. The *amazake* made by double saccharification (DSA) contained 20 times more isomaltose than the commercial *amazake* products. In an *in vivo* study, oral administration of the DSA modified the cecal microbiota in mice. Moreover, changes were seen in the abundances of several gut microorganisms, such as *Anaerotignum lactatifermentans, Muribaculum intestinale*, and *Parabacteroides merdae*. These findings indicate that our novel method may be useful for producing *amazake* with a high isomaltose content that may have health benefits in humans.

KEYWORDS

koji amazake, Duncaniella, double saccharification, gut microbiota, mouse, Isomaltose, Muribaculum

1 Introduction

Washoku, which means traditional Japanese cuisine, was registered in 2013 on the intangible cultural heritage list by the United Nations Educational, Scientific, and Cultural Organization. As the Japanese government has emphasized, the health benefits of *washoku* have been gaining much attention not only in Japan, but also around the world (1). *Washoku* often contains many and various fermented foods (2). Most Japanese fermented foods contain *koji*, which is a grain, such as rice, wheat, and/or soybeans, fermented with a non-pathogenic fungus, i.e., *Aspergillus oryzae* or *Aspergillus luchuensis* (3); these fungi produce enzymes for the saccharification of the starch present in the grains (4).

Amazake is a drink that is one of the simplest traditional Japanese fermented products prepared using *koji*. There are two types of *amazake* that differ depending on the raw material used: *koji amazake* and sake-cake *amazake*. *Koji amazake* is made from rice and rice *koji* (*A. oryzae* products), and its manufacturing process consists of two main steps: preparation of the rice *koji* and saccharification. To make rice *koji*, brown rice is polished into white rice, gently washed in water, steeped, and steamed. The spores of *A. oryzae* are then inoculated onto the steamed rice. Next, the rice *koji* is mixed with water, and placed in a tank set at 50°C to 60°C for the saccharification (5).

The main component of *koji amazake* is glucose, which is produced from rice by breaking down rice starch; this is performed by an amylase secreted by *A. oryzae*. As a result, *koji amazake* mainly contains glucose rather than sucrose and fructose, which are found in many other beverages. Furthermore, various oligosaccharides are produced by transglycosylation, such as maltose (Glc(α1-4)Glc) and isomaltose (Glc(α1-6)Glc) (5). In particular, isomaltose has been gaining much attention as an alternative to sucrose due to its potential health benefits, such as its low glycemic index and slow hydrolysis (6–11), and probiotic potential (12, 13).

Accordingly, we believe that *amazake* with a high concentration of isomaltose may have more health benefits for humans and increase the value of *amazake* on the market. In a previous investigation to improve the quality of *koji amazake*, Oguro et al. (14) studied the relationships between temperature, saccharification, and oligosaccharide production, including isomaltose; they reported that modifications to the saccharification conditions resulted in only small changes to the concentration of isomaltose.

In this study, we focused on the rice *koji*-making process to find a way to increase the value of *amazake*, and established a method that significantly increased the isomaltose concentration in *amazake*. In the production of *amazake*, it is common to use one type of rice *koji* and to perform one saccharification step. However, in this study, we used two types of rice *koji* that were obtained at different fermentation time points, and saccharification was carried out in two steps using the two types of obtained rice *koji,* one for each saccharification step. The isomaltose concentration in the *amazake* produced using double saccharification (double-saccharification *amazake;* DSA) was increased as a result, which is a novel finding. Furthermore, administration of the DSA to mice induced changes in the gut microbiota. Here, we introduce our new two-step saccharification method for producing DSA, and report on the potential of DSA to confer health benefits, which is expected to increase the value of *amazake*.

2 Materials and methods

2.1 Starter *koji*

The starter *koji* used in this study (*koji* No. 1 and No. 2) were purchased from Akita Konno Co., Ltd. (Akita, Japan). *Koji* No. 1 is often used for making bean *koji*, and *koji* No. 2 is specifically used for making rice *koji* for sake. The starter *koji* strains were stored at 4°C until used for the experiments.

2.2 Preparation of rice *koji* and saccharification for making *amazake*

To examine the effects of different fungal strains on saccharification and the fermentation time, we separately used two types of starter *koji* (*koji* No. 1 and No. 2) to make rice *koji*. For making rice *koji*, we used *Akita Komachi* rice that was produced in 2023. The rice was gently washed in water, steeped overnight, and steamed for 1 h at normal pressure. Subsequently, the steamed rice was cooled to approximately 35°C, mixed with the *koji* (0.35 g/kg), and fermented in a bath maintained at 32°C during fermentation (for 42 h). At 18 and 25 h of fermentation, the rice *koji* samples were mixed by hand to break up the clumps that had formed and as a method to help decrease the temperature to the appropriate temperature range. The temperature of the rice *koji* was controlled during the fermentation process as follows: the temperature was 30°C at the start, then it was gradually increased to 38°C by 18 h, at which point the sample was mixed, then kept at 33°C until 25 h, at which point the sample was mixed again, and kept at 30°C until 42 h. The rice *koji* was sampled at 18, 25, and 42 h with the samples prepared using starter *koji* No. 1 referred to as samples X-1, Y-1, and Z-1, respectively, and those prepared using starter *koji* No. 2 referred to as samples X-2, Y-2, and Z-2, respectively. These samples were stored at-30°C until used in the experiments. For the experiments, 50 g of the obtained X, Y, or Z was mixed with an equal amount of water and incubated at 50°C for 6 h for the saccharification to make *amazake*.

2.3 Saccharification

For saccharification with the addition of maltose, 10 g of maltose was dissolved in 40 g of water. Subsequently, 40 g of each sample was mixed with the maltose solution and incubated at 50°C for 6 h, then sampled for the analysis of the sugar levels.

The DSA was prepared using the rice *koji* samples X-1 and Z-2 as follows: 50 g of sample X-1 was mixed with 50 g of water and incubated at 50°C for 6 or 24 h; subsequently, the obtained saccharification sample (60 g) was mixed with sample Z-2 (40 g) and incubated at 50°C for 6 or 30 h.

2.4 Analysis of the sugar concentrations in *amazake*

In addition to the DSA prepared in this study, commercial *amazake* products were also examined. For the glucose analysis, the *amazake* samples were diluted two-fold with distilled water and centrifuged at 1,870 ×*g* for 10 min at 4°C. Aliquots of the obtained supernatants were diluted and analyzed for glucose using a glucose analyzer GA05 (A&T Corporation, Kanagawa, Tokyo). The amount of maltose or isomaltose in each sample was analyzed by high-performance liquid chromatography (HPLC) using a WATERS1525 (Nihon Waters K.K., Tokyo, Japan). In brief, 2 g of sample and 2 mL of 50% ethanol were mixed and sonicated for 30 min. After extracting the saccharides from each sample, the supernatant that was obtained by centrifugation at 1,870 ×*g* for 10 min at 4°C was filtered through a 0.45-μm filter to remove the impurities. Then, each sample was

analyzed by HPLC with a Shodex Asahipak NH2P-50 4E column (4.6 mm I.D. × 150 mm) at 35°C to quantify the maltose or isomaltose. The eluent was 70% acetonitrile, and the flow rate was set at 0.8 mL/min. The sugars in the samples were detected with a Refractive Index Detector.

2.5 Determination of the nutrient composition of DSA

Determination of the nutrient composition of the DSA was outsourced to LSI Medience Corporation (Tokyo, Japan). One-hundred grams of DSA was prepared as described above, and samples were sent to LSI Medience Corporation.

2.6 Mice and oral administration of the DSA

C57BL/6 mice (7 weeks old, female) were purchased from Japan SLC (Shizuoka, Japan). The mice were housed under controlled temperature and light conditions with *ad libitum* access to a standard diet (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and sterile water. The mice were acclimatized for a week, then randomly allocated into two experimental groups (N = 6 each): a phosphate-buffered saline (PBS) control group and a DSA group. Each mouse was orally administered PBS or a homogenate of DSA (200 μL/mouse) for 14 consecutive days (Days 1 to 14); the homogenate of DSA was prepared by centrifugation of the DSA at 1,400 × *g* for 5 min. On day 14, the mice were euthanized, and the cecal contents were collected.

2.7 16S rRNA gene sequencing

DNA was extracted from the cecal content samples using a fecal collection kit, and the 16S V3–V4 region of the DNA was amplified according to the methods of a previous report (15). The DNA was sequenced using the Illumina MiSeq platform (Illumina, San Diego, CA, USA) and a MiSeq Reagent Kit v3 (Illumina). Quantitative Insights into Microbial Ecology version 2 (Qiime2) bacterial flora analysis software (16) was used for microbiota analysis. Qiime2view was used to analyze the diversity and changes in the intestinal flora at the family and species levels based on the resulting qzv files. The α-diversity was analyzed using the observed features (amplicon sequence variants; ASVs) and Shannon diversity. The β-diversity was analyzed using the Jaccard and Bray-Curtis dissimilarities. In addition, multiple comparison tests using the Tukey–Kramer method were performed to analyze the intestinal bacteria that were significantly different between the DSA and PBS groups at the species level.

2.8 Statistical analysis

Statistical analysis was performed using Prism software (version 7; GraphPad Software, San Diego, CA, USA). Outliers were identified using the ROUT method ($Q = 2\%$) and omitted before further statistical analysis. Statistically significant differences were assessed by two-tailed ordinary one-way analysis of variance followed by Tukey's multiple comparisons test when statistical significance ($p < 0.05$) was indicated. Results are presented as the mean ± standard error (SE).

3 Results

3.1 The effects of double saccharification on the concentration of isomaltose in *amazake*

Figure 1 shows a scheme of the manufacturing steps for DSA. The nutritional information on the DSA is shown in Table 1. The rice *koji* was sampled at three different time points, i.e., at 18 h (samples X), 25 h (samples Y), and 42 h (samples Z) (Figure 1A). The concentrations of glucose, maltose, and isomaltose in each sample were measured to examine the relationship between the fermentation time and the concentration of each saccharide. The concentration of glucose was the lowest in samples X (18-h time point) regardless of the type of *koji* used (*koji* No. 1 or No. 2; Figure 2A). The concentration of maltose was highest in samples X (Figure 2B). The concentration of isomaltose did not differ significantly between the samples, but it was slightly higher in sample X-1 than in the other samples (Figure 2C). Considering that isomaltose is mostly formed from maltose through the transglycosylation activity of α-glucosidase, a saccharification test was performed with the addition of maltose to examine which sample had the highest transglycosylation activity. The concentration of isomaltose increased when maltose was added, and was highest in samples Z from the longest time point, especially in sample Z-2 (Figure 2D). To confirm which sample had the highest maltose content and to examine how the concentration of maltose depended on the time of saccharification, sample X-1 was saccharified for 24 h. The concentration of maltose increased until 6 h, and remained stable thereafter. In contrast, the concentration of glucose continued to increase until 24 h (Figure 2E). A second saccharification test was conducted to examine how the time of the first saccharification influenced the concentration of isomaltose in *amazake* samples produced using sample X-1 saccharified for 6 or 24 h. In both *amazake* samples, the level of isomaltose was approximately 20% at 30 h after the start of the second fermentation (Figure 2F). When the concentration of isomaltose in the DSA was compared to those in commercial products, we found that the DSA contained significantly more (almost 20 times more) isomaltose than the commercial products (Figure 2G).

3.2 DSA altered the diversity of the cecal microbiota

After 2 weeks of oral administration of PBS or DSA in mice, the cecal contents were collected and analyzed by next-generation sequencing, and the α-diversity was examined. There was a significant difference in the observed features between the PBS and DSA groups (Figure 3A), while the Shannon diversity index showed no significant increase in the α-diversity in the two groups (Figure 3B). Figures 3C,D show the Bray-Curtis and Jaccard β-diversity analysis results for the mouse cecal contents. The principal coordinate analysis results showed that the oral administration of DSA altered the gut microbiota (Figures 3C,D). The

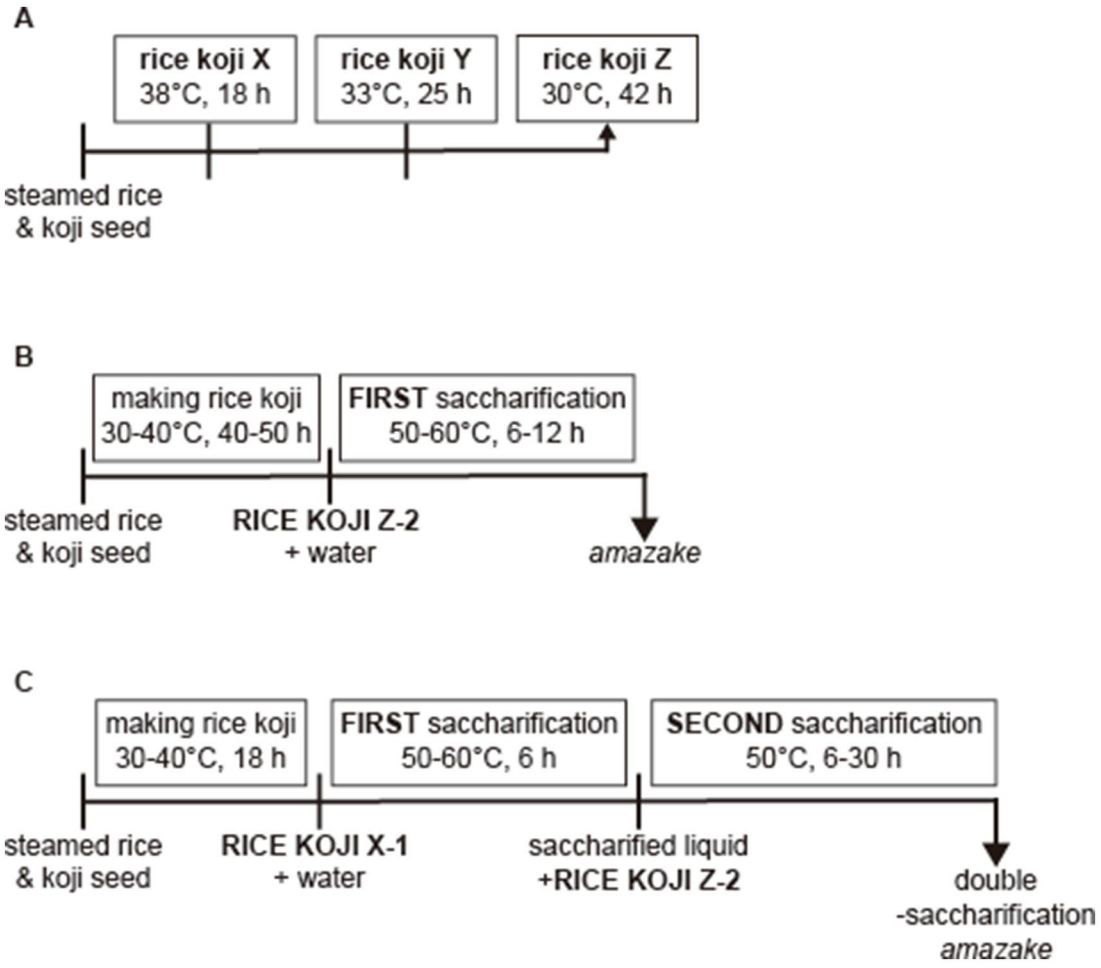

FIGURE 1
Scheme of the manufacturing of *amazake*. The schedule for rice *koji* sampling (A), the manufacturing steps for *amazake* (B), and for DSA (C).

TABLE 1 Nutritional information on the DSA.

General ingredients	Per 100 g
Energy	218 kcal
Moisture	46.00 g
Protein	3.50 g
Fat	0.50 g
Carbohydrates	49.90 g
Ash	0.10 g
Sodium	1.00 mg

results of the taxonomic analysis are presented in Figure 4A as a bar plot (via QIIME2) showing the bacterial composition (at the family level) of the cecal content in each animal of each group. The significance of differences was investigated. The differences in the gut microbiota due to the administration of DSA were examined by linear discriminant analysis (LDA) at the species level from the LDA effect size (LEfSe) analysis at the species level. Three bacteria were extracted (Figure 4B), and a comparison of the relative abundances showed that the abundance of *Anaerotignum lactatifermentans* was decreased, and those of *Muribaculum intestinale*, and *Parabacteroides merdae* were increased in the DSA group when compared to the PBS group (Figures 4C,D).

4 Discussion

It is already known that the concentration of isomaltose in *amazake* is not affected by the temperature or the time of saccharification (14). Therefore, in this study, we focused on the rice *koji*-making process, which is an important step in manufacturing *amazake*, for increasing the concentration of isomaltose.

In the present study, we used a common rice *koji* production method: rice *koji* production was completed in 42 h with mixing to break up clumps at 18 h and 25 h, and the temperature conditions were strictly controlled. However, numerous other methods also exist, and they differ between sake or miso breweries and stores. In the present study, the rice *koji* obtained at 18, 25, and 42 h was used as rice *koji* samples X, Y, and Z, respectively, and the concentrations of glucose, maltose, and isomaltose were measured after 6 h of saccharification (Figures 2A–C). When we compared all samples, the glucose concentration was lowest in the sample from the earliest time point in the rice *koji* making process, which were samples X. However, the

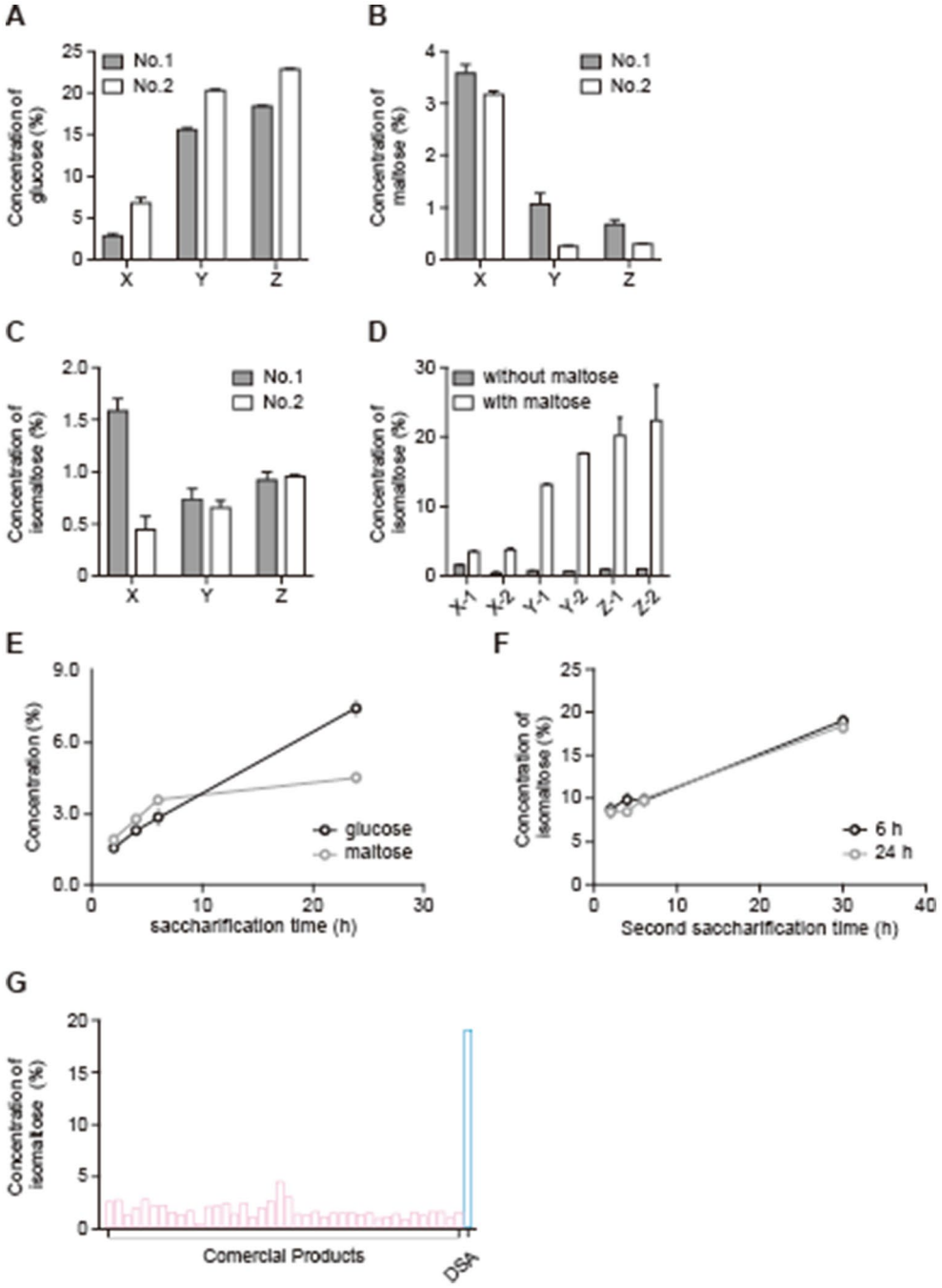

FIGURE 2
Variations in the concentration of each sugar due to the use of different starter *koji* and fermentation times, and increases in the isomaltose concentration in *amazake* due to double saccharification. Concentrations of glucose (A), maltose (B), and isomaltose (C) during *koji* production, concentrations of isomaltose during *koji* production with maltose addition (D), concentrations of glucose and maltose during the first fermentation (E), concentration of isomaltose during the second fermentation (F) using DSA, and comparison of isomaltose concentration in commercial *amazake* with that in DSA-produced *amazake* (G). Data are shown as the mean ± SE. $*p < 0.05$.

concentration of maltose was the highest in samples X. Glucose is the main component in *amazake*, and it is derived from rice starch. Rice starch is first broken down by the α-amylase and glucoamylase secreted by *koji* mold, resulting in maltose and glucose (5). The produced maltose is also broken down to glucose by the α-glucosidase secreted by *koji* mold. Therefore, the concentrations of these saccharides were affected by the degree of mycelial growth and the amounts of enzymes present. Indeed, samples X showed the lowest glucose level and the highest maltose level, likely because the activity of maltase, which degrades maltose, was low. In fact, the activity of α-glucosidase and maltase has been shown to increase with the fermentation time (17, 18); thus, it is likely that the activity of these enzymes was lower in samples X since the fermentation time was shorter than for samples Y and Z. However, we did not examine the

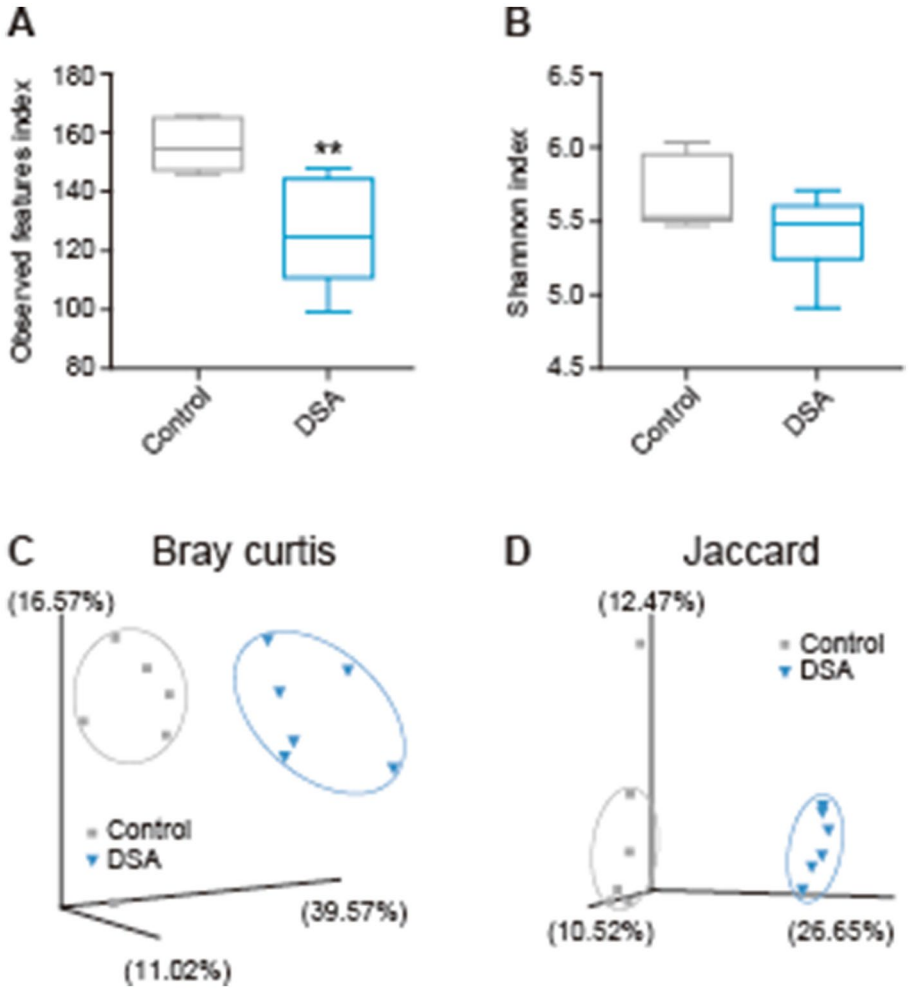

FIGURE 3
Variations in the diversity of the intestinal microbiota in mice administered DSA. Observed features index (A), Shannon index (B), and β-diversity analyzed using Bray-Curtis (C) and Jaccard (D) dissimilarities. $N = 6$. Data are shown as the mean ± SE. $^{**}p < 0.01$.

enzyme activities in the present study, and they will need to be analyzed in the future to confirm this. In addition, the concentration of isomaltose was similar in all samples, although it was slightly higher in sample X-1. It is known that isomaltose is commonly synthesized from maltose and maltooligosaccharides through hydrolytic and transfer reactions by α-glucosidase with transglycosylation activity, especially when maltose is present at a high concentration (19). In other words, a low concentration of maltose in samples may cause the maltose to become glucose through a hydrolytic reaction.

According to these results and the fact that a high concentration of maltose is needed to produce isomaltose, we conducted a saccharification test with the addition of maltose. We found that the addition of maltose increased the concentration of isomaltose (Figure 2D). Moreover, it has been reported that the production of enzymes in *koji* increases with the fermentation time (17), which might explain why samples Z showed the highest concentration of isomaltose: samples Z likely contained the most α-glucosidase and possibly the highest transglycosylation activity.

In the previous investigation, it was revealed that a high concentration of maltose and the transglycosylation activity of α-glucosidase were necessary for increasing the content of isomaltose in *amazake*. Thus, we considered that it may be possible to increase the isomaltose content of *amazake* without the use of additives if two saccharification steps were conducted using sample X-1 for the first step and sample Z-2 for the second step (Figures 1, 2). Regardless of the first saccharification time (6 or 24 h), the effect of the second saccharification using sample Z-2 provided a significantly higher concentration of isomaltose when compared to that of the commercial *amazake* products. The reason no difference was seen between the 6 and 24 h time points in the first saccharification is likely because the concentration of maltose was similar in the two samples (Figures 2E,F).

In conclusion, we succeeded in finding a new method of *amazake* production for increasing the concentration of isomaltose without the use of additives. Our *amazake* contained approximately 20 times more isomaltose than the commercial products (Figure 2G). However, the concentration of essential enzymes, such as α-amylase, glucoamylase, and α-glucosidase, as well as their transglycosylation activities, were not checked when the two saccharification steps were performed. Also, in this study, sample X-1 was used for the first saccharification and sample Z-2 was used for the second saccharification, but the details of how the two kinds of *koji* samples interact remain unclear. Therefore, further experiments are needed in the future.

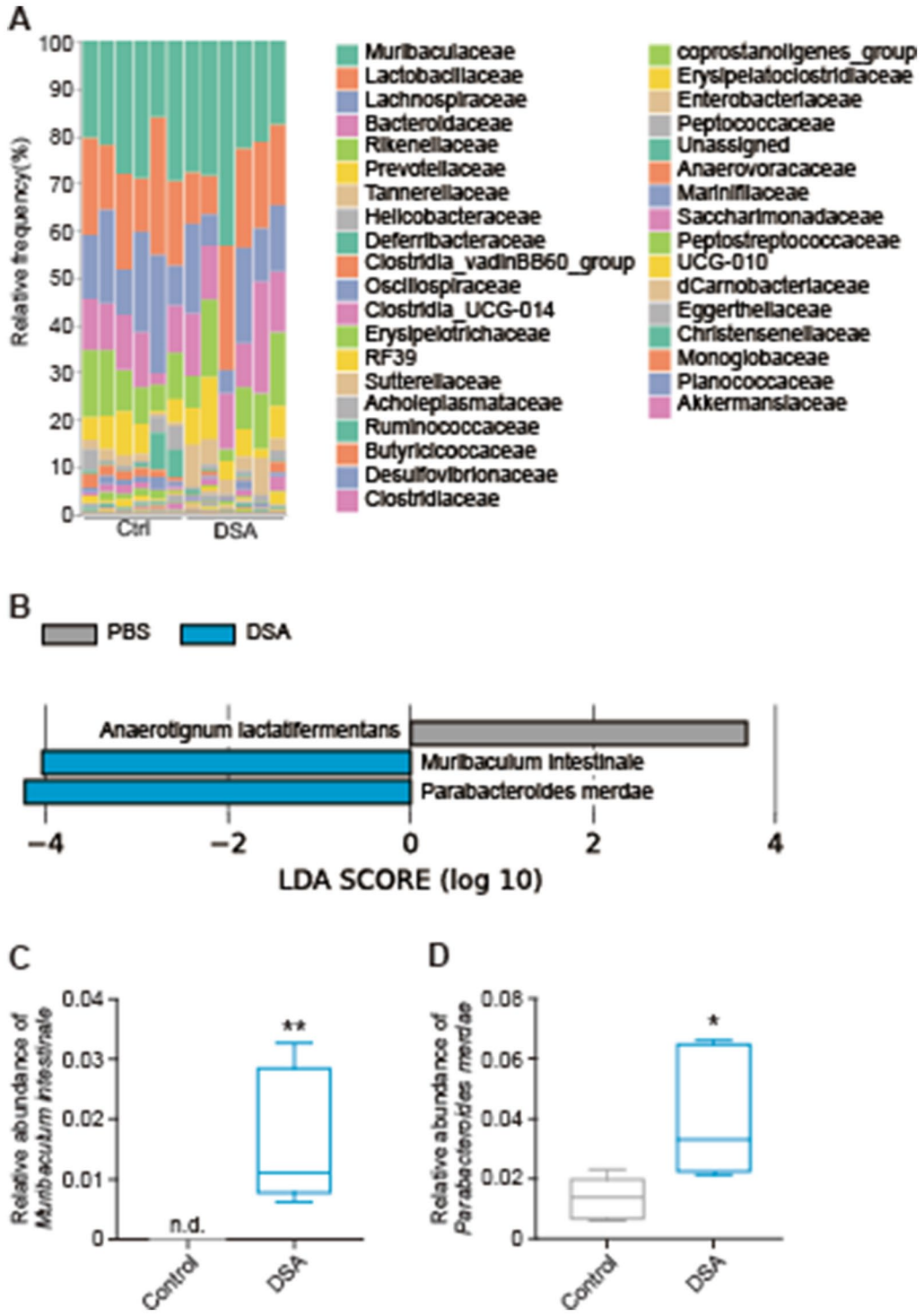

FIGURE 4
Composition of the gut microbiota and the relative abundances of several bacteria affected by DSA administration. A bar plot **(A)**, bacteria with significant differences in the ASVs at the species level **(B)**, the relative abundances of *M. intestinale* **(C)** and *P. merdae* **(D)**. $N = 6$. Data are shown as the mean ± SE. $*p < 0.05$, $**p < 0.01$.

Since isomaltose is widely expected to be a prebiotic (30), we performed an *in vivo* experiment to test the effects of DSA administration in mice. No differences were seen in the Shannon index between the two groups. In addition, the β-diversity showed significantly different clusters. Although the DSA had little influence on the species diversity within a group, it modified the structure of the bacterial clusters in the DSA group when compared to the PBS group (Figure 3). Moreover, the abundances of three types of bacteria were found to have changed significantly due to the administration of DSA, namely, *A. lactatifermentans, M. intestinale*, and *P. merdae* (Figure 4).

M. intestinale is highly associated with dietary habits, and there are some studies showing that the consumption of a western diet and cafeteria foods may decrease its abundance in the gut (20, 21). *M. intestinale* is a glycan-degrading and butyrate-producing bacterium, and it has been reported that the abundance of *M. intestinale* increased or recovered with improvements of obesity-and intestinal inflammatory disease-related factors that were affected by high-fat diets and dextran sulfate sodium administration (22, 23). Thus, the consumption of DSA may be useful and effective for promoting a stable and healthy intestinal environment. The abundance of *M. intestinale* may have increased in the gut of mice that were administered DSA due to the fact that it is a glycan-degrading bacterium and DSA is considered to have more sugar chains because it contains more isomaltose than the commercial products. On the other hand, *P. merdae* has been isolated from human feces, and has been reported to be associated with several diseases, such as hypertension, polycystic ovary syndrome, cancer, and obesity (24). For example, while *P. merdae was found in increased abundance in people* with hypertension (25) and polycystic ovary syndrome (26), an increase in the abundance of *P. merdae* in the gut has been reported to contribute to the treatment of obesity-related cardiovascular disease. This effect was due to the ability of *P. merdae* to catabolism branched-chain amino acids, mediated by the porA gene (27). In other words, DSA intake may contribute to the prevention of lifestyle-related obesity and related metabolic diseases by increasing the abundances of *M. intestinale* and *P. merdae* in the gut. Interestingly, not only has *P. merdae* been shown to be more abundant in the gut of those in the 90-to 99-year-old group in East China (28), but *Muribaculaceae* species, including *M. intestinale*, have also been reported to be associated with longevity (29). Thus, while we believe that habitual DSA consumption would confer continual health benefits, a more detailed understanding of the function of the bacterial strain altered by DSA administration requires further investigation.

In the present study, we introduced a novel method of producing *koji amazake* that contains a high concentration of isomaltose. We focused on the rice *koji*-making process instead of the saccharification step, and prepared two types of rice *koji* that were obtained at different time points and were subsequently used in two saccharification steps; this method resulted in an increased concentration of isomaltose. Furthermore, the administration of DSA increased the abundances of the obesity-and longevity-related organisms *P. merdae and M. intestinale,* which can degrade glycan. These results suggest that DSA consumption may confer health benefits and help maintain a healthy intestinal environment.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

The animal study was approved by the Committee for Animal Experiments of Shinshu University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AM: Data curation, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. AS: Data curation, Investigation, Validation, Writing – original draft. FN: Data curation, Investigation, Writing – original draft. TF: Writing – review & editing. TT: Writing – review & editing. JT: Data curation, Investigation, Writing – review & editing. TS: Funding acquisition, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

Tadashi Fujii and Takumi Tochio are members of BIOSIS Lab. Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Enhancing iron and zinc bioavailability in maize (*Zea mays*) through phytate reduction: the impact of fermentation alone and in combination with soaking and germination

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Introduction: Phytates are nutrient-binding compounds found mainly in cereals and legumes, which may significantly contribute to micronutrient malnutrition in regions where phytate-rich cereals, such as maize, are staple food.

Objectives: This study investigated how maize fermentation, both alone and in combination with soaking and germination, can reduce phytate levels and enhance the estimated bioavailability of iron and zinc.

Methods: We evaluated various fermentation methods, including spontaneous fermentation; fermentation with starter cultures, either *Lactiplantibacillus plantarum* 299v® (Lp299) or yogurt containing viable *Lacticaseibacillus casei*; and fermentation with Lp299 of soaked and germinated maize. The outcome variables included changes in pH and lactic acid content during fermentation, and measurements of phytate levels (spectrophotometry), minerals (Atomic absorption) and protein (protein analyzer) in maize samples before and after treatments.

Results: Fermentation with Lp299 of soaked and germinated maize grains yielded a phytate reduction of up to 85.6% decreasing from $9.58 \pm 0.05\ g \cdot kg^{-1}$ in raw maize to $1.39 \pm 0.09\ g \cdot kg^{-1}$ after processing. Fermentation of raw maize flour using Lp299 or yogurt resulted in a similar phytate reduction of 65.3% ($3.35 \pm 0.26\ g \cdot kg^{-1}$) and 68.7% ($3.02 \pm 0.01\ g \cdot kg^{-1}$) respectively. Spontaneous fermentation yielded a phytate reduction of 51.8% ($4.65 \pm 0.40\ g \cdot kg^{-1}$). This reduction in phytate content enhanced the estimated bioavailability of iron and zinc, particularly in the soaking-germination-fermentation combination, where the Phytate:Zinc molar ratio (Phy:Zn) dropped from 40.76 to 7.77, representing 81% reduction from the raw maize. The Phytate:Iron molar ratio (Phy:Fe) dropped from 41.42 to 6.24 indicating an 85% reduction. Additionally, fermentation led to a significant increase ($p = 0.001$) in protein content in maize flour after fermentation, ranging from 7.3 to 10.3% after the various fermentation treatments. There was not significant difference in the protein increase when compared the fermentation types.

Conclusion: Lactic acid fermentation of soaked and germinated maize grains, emerged as the most promising process to enhance the bioavailability of essential minerals. This approach could help alleviate mineral deficiencies in populations dependent on maize-based diets. The findings underscore the

potential of fermentation to be applied at the household level, which may bring up an alternative for programs and policies focused on reducing micronutrient deficiencies and improving food security in developing regions.

KEYWORDS

maize, fermentation, soaking, germination, mineral bioavailability, phytates

1 Introduction

Globally, iron and zinc deficiencies pose a public health concern. The severity of this problem is particularly high in developing countries, while deficiencies are also rising among vegetarian and vegan populations in developed countries. These nutritional inadequacies contribute significantly to adverse health outcomes, such as iron deficiency anemia, which affects nearly 1.2 billion people worldwide (1). Such deficiencies result in productivity losses due to impaired cognition, stunted physical growth, and increased susceptibility to infections, morbidity and mortality (2). In Rwanda, anemia prevalence among children under five has been reported to range from 46.2 to 52.79% (3, 4).

Eastern and Western Africa have the highest per capita consumption of maize (*Zea mays*) as a staple crop. In these regions, the per capita consumption of maize ranges from 157–267 g per person per day. At these consumption levels, maize is expected to meet the dietary requirements for essential macro and micronutrients for children and women in these regions (5). However, the delivery of essential elements such as iron and zinc from the maize-based food products is compromised due to high levels of phytates. This situation poses a significant risk of micronutrients malnutrition and associated health disorders for people consuming maize as a staple food. Nearly 80% of the minerals in maize kernels concentrate in the germ portion that also serves as storage site for phytates and drastically affects minerals bioavailability (6). Additionally, phytates interact with available proteins and amino acids, forming insoluble complexes, and thereby making them unavailable for absorption in the gastrointestinal tract (55).

Nearly 1–3% (w/w) phytate appears in all plant seeds. Based on the cultivars and crop cultivation conditions, the concentration of phytates in maize ranges from 2.77 to 16.70 $mg \cdot g^{-1}$ of seeds (7). The estimated impact of phytates on minerals bioavailability is measured by calculating phytates to mineral ratios. Phytates:Zinc (Phy:Zn) molar ratio below 15 and Phytates:Iron (Phy:Fe) molar ratio below 1 are considered adequate for optimal absorption of listed minerals (8). However, maize exceeds these molar ratio thresholds, contributing to higher risks of iron and zinc deficiency among regular consumers of maize and maize-based products (9, 10). Considering their nutrients binding properties, several strategies have been evolved to mitigate the levels of phytates in maize. These strategies include mineral biofortification, as well as breading and genetic engineering approaches to reduce phytate levels in cereals. These methods are being explored as effective alternatives to increase mineral content and bioavailability in cereals (11). Depending on the specific objectives, the latest techniques may involve lengthy development times, and their widespread adoption can take time. In addition, consumer perception and acceptance of improved crops play a crucial role in the success of biofortification programs. Food processing methods, such as enzymatic treatment, heat treatments like cooking and baking are also used to reduced phytates. However, each method has its limitations. For example, enzymatic methods can be expensive and there is limited availability of phytase for human consumption. We should also consider that phytates are heat stable, so heat treatments like cooking and baking have limited efficacy reducing phytates. Dehulling has also been used to decrease phytates, but removing the outer layers of cereals can also remove valuable nutrients, including fiber, vitamins and minerals that are concentrated in the outer layers of many foods (12).

Simpler and more effective processing techniques, such as soaking, germination and fermentation, have proven effective at degrading phytates and improving mineral bioavailability in various crops (13–15). Fermentation an ancient food preservation technique, can enhance various nutritional and sensory properties depending on the starter culture and fermentation conditions (16, 17). Fermentation has been effective in reducing phytate content in cereals and pseudo cereals. During fermentation, either microbial phytase production or the activation of endogenous phytase can effectively hydrolyse phytates, thereby enhancing bioavailability of divalent minerals (18). Similar enzyme activation and phytate hydrolysis have been observed with soaking and germination techniques (19). Phytates reduction in maize through malting, germination and fermentation has been reported to range from 28 to 96% (13, 20, 21).

Given the dietary significance of maize in African countries and the risk factors that can undermine its nutritional value, designing culturally acceptable interventions to improve the nutrient delivery of this staple crop is essential for enhancing nutrition security in vulnerable populations. Fermentation has great potential to enhance the nutritional value of foods. However, despite its benefits, the widespread use of fermentation for nutritional improvement remains limited. This is partly due to a lack of standardized methods, variability in microbial cultures, and limited understanding of how fermentation conditions affect both nutrient retention and degradation. More research is needed to optimize fermentation processes and integrate them into contemporary food production, ensuring that both nutritional improvements and sensory qualities are preserved. By doing so, we can unlock the untapped potential of fermentation to combat nutrient deficiencies and improve food security, particularly in regions where malnutrition is common.

The focus of this study was to evaluate convenient, affordable, culturally acceptable and feasible processes to reduce phytates. We compared soaking, germination and fermentation (spontaneous, with lactic bacteria and yogurt containing viable bacteria), both alone

Abbreviations: FSp, Spontaneous fermentation; FLp, Fermentation of maize with *Lactiplantibacillus plantarum* 299v® as starter culture; FYLc, Fermentation of maize with yoghurt containing viable Lacticaseibacillus Casei; FLp-SG, Fermentation of soaked and germinated maize grains with *Lactiplantibacillus plantarum* 299v®; DM, Dry matter; Phy:Zn, molar ration phytate-to-zinc; Phy:Fe, molar ration phytate-to-iron.

and in combination, to reduce phytate levels in maize. We also assessed changes in the estimated bioavailability of iron and zinc following each processing method. Additionally, since protein is a key nutrient, we measured changes in protein content resulting from the various treatments. This study represents an opportunity to apply maize fermentation at household level, providing a sustainable process to improve iron and zinc bioavailability in vulnerable populations. Moreover, the findings could inform national programs and policies aimed at reducing malnutrition and food insecurity in African countries.

2 Materials and methods

2.1 Materials and sample preparation

We ordered the maize grains for this study from the Rwanda Agriculture Board (RAB), with the biological identification code ZM607 - MUTUTU-18A basic. We selected ZM607 - MUTUTU-18A for its high productivity in the lowlands commonly used for maize production. We purchased *Lactiplantibacillus plantarum 299v*® (Probi, Lund, Sweden) from a pharmacy in Lund, Sweden, and a commercial drinkable yogurt containing 20 billion *Lacticaseibacillus casei* starter culture (Actimel, Danone, France) from a supermarket (ICA, Lund, Sweden). We conducted all the experiments in Sweden.

We manually sorted the maize grains to remove damaged grains and other extraneous materials. We then divided the grains into two batches for further processing. For the first batch, we milled the grains into fine flour using a laboratory hammer mill fitted with a 0.5 mm sieve. The milled samples were packed in airtight plastic bags and stored at 4°C for further processing and analysis. This first batch was used for three different fermentation processes: spontaneous fermentation, fermentation with *L. plantarum 299v*® (Lp299), and fermentation with yogurt containing viable *L. casei*. For the second batch, we soaked and germinated the grains. After germination, grains were ground and sieved through a 0.5 mm sieve to obtain flour. This flour from soaked and germinated grains was then fermented with Lp299. We conducted the experiments in two independent runs. A detailed description of the processes is provided below, and Figure 1 presents a schematic representation of all steps and conditions used during the processes.

2.2 Soaking and germination

We soaked the maize grains in deionized water at a 1:3 (w/v) ratio at room temperature (18°C) for 24 h in jars, using static soaking without water changes, as described by Mihafu et al. (22). After soaking, we drained off the soaking water using an absorbent cloth and moved the soaked grains to the next step of germination. We took a sample of the soaked grains, wet ground it, and dried it at 105°C until it reached a constant weight, then stored it for further analysis.

We followed the method described by Mihafu et al. (22) for germination with some modifications. Instead of baskets, we used well-prepared plastic containers for germinating the soaked maize grains. We placed a moistened cotton cloth (0.25 m × 0.25 m) inside the container folding it to cover the base circumference. The soaked maize grains were spread on the cloth and were covered with another moistened cloth of the same length. To protect the grains from light and maintain warmth for sprouting, the container was covered with a black cloth. The maize grains were left to germinate for 80 h at room temperature (18°C) in Sweden, rather than 72 h, as the lower room temperature required a longer germination time compared to the original research conducted in Tanzania, where the room temperature was around 25°C. After germination, we spread the maize grains on baking paper and dried them in an oven (Termaks, Lund, Sweden) at 60°C for 5 h. We then ground the dried germinated maize grains using a laboratory hammer mill (Laboratory Mill 120, Lund, Sweden) and sieved the flour through a 0.5 mm sieve to obtain a fine consistency. The milled flour samples were packed in airtight plastic bags and stored at 4°C for further analysis and use in the fermentation process.

2.3 Fermentation, spontaneous and with starter culture

We followed the fermentation process described in our previous research (16, 18, 23) with some modifications. The modification in the current study included reducing the fermentation time to 24 h instead 48 h and using yogurt as starter culture. Briefly, a suspension of 500 g of maize flour was prepared in de-ionized water in plastic containers at the ratio of 1:2 (w/v). For each type of fermentation, we divided the slurry into two portions and placed them in separate hermetically sealed containers to ferment in replicates. Prior to incubation, we cultured the samples using either spontaneous or inoculated fermentation methods vis. Spontaneous fermentation of maize flour from raw grains (FSp); Lp299 led fermentation of maize flour developed from raw grains (FLp); lactic acid fermentation of maize flour with yogurt containing viable *L. casei* (FYLc); and fermentation with Lp299 of maize flour from soaked and germinated maize kernels (FLp-SG).

For FLp, we dissolved Lp299 (7.35 Log10 CFU·g^{-1}) at a proportion of 1% in distilled water before mixing it with maize flour and water. FYLc involved inoculating 5% yogurt (containing 20 billion *L. casei* cultures) by dissolving the inoculum in distilled water and mixing it with 500 g of maize flour at a 1:2 (w/v) ratio. For FLp-SG, we dissolved Lp299 in distilled water and mixed it with 500 g of maize flour obtained from soaked and germinated maize grains, maintaining the same 1:2 (w/v) ratio.

We incubated the fermentation containers at 30°C for 24 h, taking samples of the slurry every 4 h from 0 to 12 h, with a final sample taken at 24 h. We analyzed each sample for pH, titratable acidity (as lactic acid percentage), dry matter, and phytate content. At the end of the fermentation process, we decanted the fermentation water and transferred the samples to aluminum foil for drying in the oven (Termarks, Lund, Sweden) at 60°C for 5 h. The dried samples were then ground in a hammer mill and stored at 4°C for further analysis of phytate, protein, and mineral content.

2.4 Chemical analyses

2.4.1 Determination of moisture content

We determined the moisture content in maize flour in duplicates by following standardized procedures (18). Briefly, we transferred 5 g (±0.0001 g) of maize flour into dried and weighed dishes, then placed

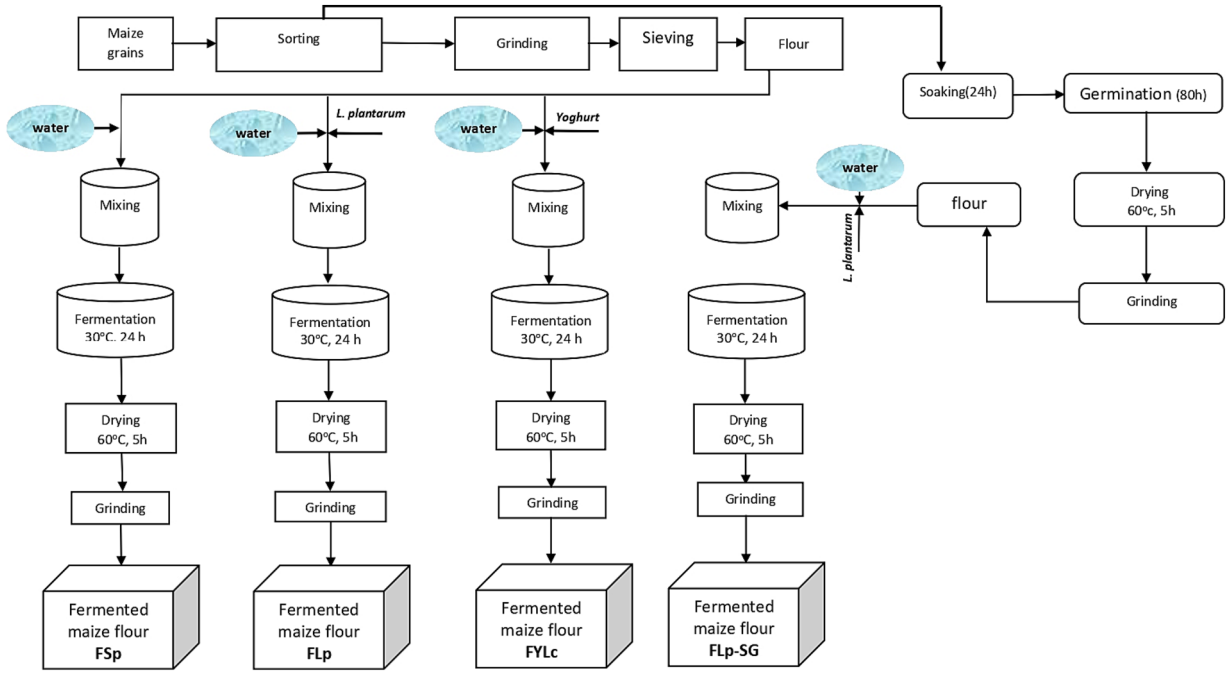

FIGURE 1
Flow chart of processing steps to obtain fermented maize flour under different conditions. FSp, Spontaneous fermentation of maize flour; FLp, Fermentation of maize flour with *L. plantarum* 299v®; FYLc, Fermentation of maize flour with yoghurt containing viable *L. casei*; FLp-SG, Fermentation of maize flour from soaked and germinated maize kernels with *L. plantarum* 299v as starter culture.

the samples in an oven at 105°C until they reached a constant weight. We computed the moisture content of the samples using a differential method.

2.4.2 pH and acidity determination

We determined the pH and acidity of fermented samples in duplicate, withdrawing samples every 4 h until the pH dropped below 4.6, following the method described in Castro-Alba et al. (18). Briefly, we suspended 10 g of each sample in 90 mL of de-ionized water and stirred the mixture for 10 min. We then filtered the suspension and measured the pH of the liquid by dipping the pH electrode (Metrohm 744 pH meter, Switzerland) into the homogenized mixture.

In addition to pH, we also determined the total acidity of the withdrawn samples in duplicate. We suspended 10 g of the fermented slurry sample in 90 mL of de-ionized water and stirred it for 10 min. Next, we titrated 75 mL of the homogenized sample against 0.1 N NaOH using a 1% phenolphthalein indicator. We recorded the volume of 0.1 N NaOH used for titration. We expressed the total acidity as $g{\cdot}kg^{-1}$ DM of lactic acid, where 1.0 mL of 0.1 N NaOH was equivalent to 9.0×10^{-3} g of lactic acid.

2.4.3 Determination of phytate content

We determined phytate following the method described by Makkar et al. (24) and the modifications presented by Ayub et al. (16). Briefly, we mixed 1.5 g of maize flour with 50 mL of extracting solution (3.5% HCl) in a 200 mL volumetric flask and stirred the mixture vigorously with a magnetic stirrer at 500 rpm for 60 min at room temperature. We then centrifuged the mixture at 3,000 g for 10 min at 20°C (Beckman Coulter Allegra x-15r, UK), and diluted 5 mL of the supernatant to 25 mL with distilled water.

To purify the phytate, we used an anion exchange column (200–400 mesh). We prepared duplicates of 0.7 cm × 15 cm columns, plugging them with a small quantity of cotton wool. We vertically fixed the columns and filled them with 0.5 g of AGI-X8 chloride anion-exchange resin (DOWEX®1×8 Chloride form, 100–200 mesh, Sigma) to separate inorganic phosphorus and other interfering compounds from inositol phosphates. We allowed 10 mL of diluted sample extracts to pass through the column. After that, we transferred 15 mL of 0.1 M NaCl into the column to elute the inorganic phosphorus and other interfering compounds, followed by 15 mL of 0.7 M NaCl to elute the phytate. We discarded the resin in the column after use.

Next, we added 1 mL of Wade reagent, made from 30 mg of $FeCl_3{\cdot}6H_2O$ and 357 mg of sulfosalicylic dihydrate, to 3 mL of the eluted sample for phytate analysis and vortexed the mixture for 5 s. We centrifuged the homogeneous mixture at 3,000 g for 10 min at 20°C (Beckman Coulter Allegra x-15r, UK). We recorded the absorbance values of the samples at 500 nm (Varian 50 Bio UV–Visible Spectrophotometer, Hamburg, Germany) against a reagent blank. We prepared a series of standard solutions containing 5, 10, 15, 30, and 50 $\mu g{\cdot}mL^{-1}$ of phytic acid in distilled water for quantification. We presented the results on a dry matter basis.

2.4.4 Determination of mineral content

We determined the mineral content in both unfermented and fermented maize flour following the procedure described by Lazarte et al. (10). We washed all glassware and materials used during the analysis with a 3% nitric acid solution to avoid contamination, then double-rinsed them with de-ionized water. Next, we placed approximately 0.5 g of each sample in Teflon vessels, mixing it with

2 mL of H_2O_2 (30% v/v) and 3 mL of HNO_3 (65% v/v). We tightly closed the vessels and performed acid digestion for one hour in a microwave reaction system (MRS5, Microwave Accelerated Reaction System, CEM, Matthews, NC, USA). After digestion, we diluted each sample to 25 mL with de-ionized water. We measured iron and zinc using Flame Atomic Absorption Spectrometry with an air-acetylene flame (Agilent Technologies 200 Series AA, Agilent, Santa Clara, CA, United States) at wavelengths of 213.9 and 248.3 nm, respectively. We prepared a 5-point calibration curve in the range of 100–2,000 mg·L^{-1} using certified Atomic Absorption Standard solutions for iron and zinc (Merck KGaA, Darmstadt, Germany).

2.4.5 Estimation of mineral bioavailability

We estimated iron and zinc bioavailability in both unfermented and treated samples using the molar ratios of phytate to mineral. We used a molar weight of 660 g·mol^{-1} for phytate. We then compared the Phytate:iron (Phy:Fe) and Phytate:zinc (Phy:Zn) of the samples to the recommended values for adequate iron and zinc bioavailability, where Phy:Fe should be less than 1 and Phy:Zn less than 15 (25).

2.4.6 Determination of protein contents in raw and processed maize flours

We determined the protein content of the unfermented and fermented samples using the Dumas (dynamic flash combustion) method (26) with a protein analyzer (Thermo Scientific™ FLASH™, EA 1112 series, United States). This analyzer detects the amount of nitrogen present in the sample after complete combustion. We then multiplied the nitrogen amount by a nitrogen-to-protein conversion factor of 6.25. We present the results of crude protein content in each sample on a dry matter basis.

2.5 Statistical analysis

We conducted all fermentation trials in duplicate, withdrawing two samples from each batch at each sampling time to analyze various parameters. We used the Kolmogorov–Smirnov test to evaluate the normality of the data, confirming that the datasets followed a normal distribution. As a result, we presented the data as mean values ± standard deviation and employed parametric tests for further statistical analysis. We performed a one-way ANOVA to assess the effects of fermentation type on pH, lactic acid content, iron, zinc, phytate, and phytate molar ratios. Following ANOVA, we used Tukey's *post-hoc* test for multiple comparisons to determine significant differences between the means of the four fermentation types, with the significance level set at $p < 0.05$. A two-way ANOVA was computed to investigate the effect of the interaction between fermentation type and fermentation time (for samples withdrawn at 0, 4, 8, 12, and 24 h fermentation) on the response variables phytate, pH, lactic acid and protein content. This was followed by multiple pairwise comparisons to identify significant effects at each sampling time.

Additionally, we conducted another round of one-way ANOVA, followed by Tukey's test, to assess the effects of soaking and germination on phytate, mineral content, and molar ratios. This analysis aimed to elucidate the changes due to the pre-treatments of soaking and germination. We used SPSS Statistics 24 (SPSS Inc., IBM Corporation, Armonk, United States) for all calculations.

3 Results

3.1 Moisture contents in raw and processed maize flours

We observed a moisture content of 10.6% in raw maize flour. After drying at 60°C for 5 h, the moisture content in spontaneously fermented flour (FSp) was 6.94%, in flour fermented with Lp299 (FLp) it was 6.13%, in flour fermented with yogurt as starter culture (FYLc) it was 6.49%, and in fermented flour from soaked and germinated maize grains (FLp-SG) it was 4.91%.

3.2 Effect of fermentation on pH and lactic acid

We found a decrease in pH and a relative increase in lactic acid content across all four fermentation types: FSp, FLp, FYLc, and FLp-SG, as shown in Figures 2A,B. The initial pH of the first two fermentations, FSp and FLp, was nearly identical at 6.60 and 6.61, respectively. Lactic acid levels in both samples were 9.0 g·kg^{-1}. A slight difference was observed in the FYLc sample at time 0, where the pH was 6.53 and lactic acid was 10.8 g·kg^{-1}. The fermented sample made from soaked and germinated maize kernels (FLp-SG) showed a lower initial pH of 6.31 and a higher titratable acidity expressed as 4.4 g·kg^{-1} of lactic acid. This indicates that soaking and germination lowered the pH and increased the lactic acid content even before fermentation began.

During the first 4 h of fermentation, the changes in pH across the four fermentation types were not significant ($p > 0.05$). From 4 to 12 h, we observed a more pronounced pH decrease in the maize slurry fermented with yoghurt (FYLc) and in the maize slurry from soaked and germinated kernels fermented with Lp299. Meanwhile the spontaneous fermentation group (FSp) showed no obvious pH change during this period, with a pH of 6.44 after 12 h. Between the 12 to 24 h of fermentation, all groups containing starter culture showed pH stabilization, with final pH values 3.66 for FLp, 3.97 for FYLc and 3.61 for FLp-SG, respectively. In contrast, the spontaneous fermentation showed a significant pH drop in the last 12 h, with the pH decreasing from 6.44 to 4.01 (Figure 2A).

During the fermentation period, lactic acid content steadily increased, with FLp-SG showing the most significant rise. By the end of fermentation, FLp-SG reached 80.1 g·kg^{-1} of lactic acid. Comparing the lactic acid content obtained from maize flour fermented with Lp299 and maize flour fermented with yogurt, FLp reached 62.1 g·kg^{-1}, while FYLc measured 42.3 g·kg^{-1} lactic acid. Between 0 and 8 h of fermentation, we observed a significant reduction in pH and a slower increase in lactic acid in the samples fermented with yogurt, compared to those fermented with Lp299. In samples that underwent spontaneous fermentation, lactic acid content increased from 9.0 to 18.9 g·kg^{-1} in the first 12 h. Whereas the rate of increase surged by approximately 129% over the next 12 h, resulting in lactic acid levels rising from 18.9 to 43.2 g·kg^{-1} (Figure 2B).

The results from the two-way ANOVA test showed that both pH and lactic acid content were significantly affected ($p = 0.000$) by fermentation time (4, 8, 12, and 24 h) and fermentation type (FSp, FLp, FYLc, and FLp-SG). The effect of fermentation time on pH and lactic acid was significant at $p = 0.000$, as was the effect of fermentation

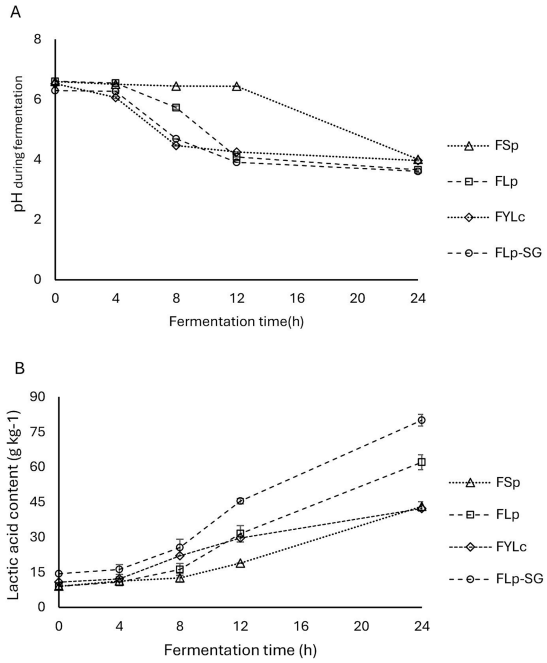

FIGURE 2
Changes in pH and lactic acid during fermentation. FSp, Spontaneous fermentation of maize flour; FLp, Fermentation of maize flour with *L. plantarum* 299v®; FYLc, Fermentation of maize flour with yoghurt containing viable *L. casei*; FLp-SG, Fermentation of maize flour from soaked and germinated maize kernels with *L. plantarum* 299v as starter culture.

type. Moreover, the interaction between these two factors-time and type of fermentation-had a statistically significant effect ($p = 0.000$) on the response variables. Figure 2A (pH) and Figure 2B (lactic acid) show the interaction plots of fermentation time and type, illustrating the impact of both factors on pH and lactic acid content.

A post-hoc analysis of multiple comparisons revealed similar trends for pH and lactic acid content. At time 0, there was no significant difference in pH and lactic acid content between the maize samples used in FSp, FLp, and FYLc ($p = 0.999$). However, these samples significantly differed from the FLp-SG sample ($p = 0.005$), the difference can be attributed to the soaking and germination steps prior to fermentation. At 4 h, lactic acid content in FSp was not significantly different from that in FLp ($p = 0.793$), FYLc ($p = 0.600$) but differed significantly from FLp-SG ($p = 0.005$). At 8 h fermentation, lactic acid content in FSp showed a slight but significant difference from that in FLp ($p = 0.046$), with a more pronounced significance level when comparing FSp to FYLc ($p = 0.002$) and FLp-SG ($p = 0.000$). At 12 and 24 h fermentation, lactic acid content was significantly different across all fermentation types ($p = 0.000$), except between FLp and FYLc where the p-value was 0.299 at 12 h and 0.600 at 24 h fermentation.

3.3 Effect of soaking, germination, and fermentation on phytate content

Our results indicate that 24 h soaking, and 80 h germination reduced phytate content in maize flour from 9.65 g·kg^{-1} to 8.44 g·kg^{-1} and 6.57 g·kg^{-1}, respectively. The rate of phytate reduction after soaking was 12.6%, and after germination, it was 31.9%, indicating that germination is a promising and simple technique for reducing phytate levels to some extend (Table 1).

This study found a significant ($p = 0.000$) reduction in phytate content after all types of fermentation, with notable differences between fermentation types due to variations in starter cultures and pretreatment methods like soaking and germination. The changes in phytate content are shown in Figure 3. After 24 h of fermentation, phytate reductions were as follows: 51.8 ± 4.10% for FSp, 65.3 ± 0.26% for FLp, 68.7 ± 0.13% for FYLc, and 85.6 ± 0.91% for FLp-SG. During FSp, the phytate reduction was slow in the first 12 h, followed by a rapid drop in final 24 h, as shown in Figure 3. The process that led to the highest phytate reduction was FLp-SG, with an 85.60% reduction at 24 h, achieved through fermentation of maize flour made from soaked and germinated kernels. These pre-treatments—soaking and germination—significantly enhanced phytate reduction. This phytate reduction was followed by that obtained in FYLc 24 h, where fermentation with yoghurt culture reduced phytate by 68.7%, followed by FLp 24 h with LP299 starter (65.3%), and FSp 24 h, which showed the lowest reduction at 51.8%.

The varying effectiveness of the fermentation processes in reducing phytate levels can be attributed to pretreatments like soaking (24 h) and germination (80 h) and the effectiveness of the starter cultures. No significant difference was observed between FLp and FYLc in phytate reduction, suggesting that Lp299 and *L. casei* are similarly effective in reducing phytate.

The results from the two-way ANOVA test showed that the phytate content in fermented maize samples was significantly affected ($p = 0.000$) by both time (4, 8, 12, and 24 h) and fermentation type (FSp, FLp, FYLc, and FLp-SG). Both factors had a statistically significant effect on phytate content, with $p = 0.000$ for fermentation time and $p = 0.000$ for fermentation type. Additionally, the interaction between the two factors also had a statistically significant effect ($p = 0.000$) on the response variable. Figure 3 shows the interaction plot of the two variables to better visualize how both factors affect phytate content.

Moreover, a post-hoc analysis of multiple comparisons (pairwise comparisons) revealed that at time 0, there was no significant difference in phytate content between the maize samples used in FSp, FLp, and FYLc ($p = 0.392$). However, these samples were significantly different ($p = 0.000$) from the FLp-SG sample at time 0 h, due to the soaking and germination of the FLp-SG sample prior to fermentation. At subsequent time points (4, 8, 12, and 24 h), there was a significant difference ($p = 0.000$) in phytate content based on the fermentation type.

3.4 Effect of soaking, germination, and fermentation on mineral content

Table 1 presents the mineral content of raw maize flour and fermented maize flour obtained after all processes (i.e., soaking, germination and fermentation). We observed a slight non-significant decrease in zinc content during FSp, FLp, and FYLc by 6, 13, and 7%, respectively. However, we found a significant lower zinc content after FLp-SG, where the concentration dropped from 21.10 to 17.73 mg·kg^{-1}, representing a16% decrease. This reduction can be attributed to the pre-treatment of soaking, where zinc may have

TABLE 1 Phytate, minerals and estimated mineral bioavailability before and after processing, results are presented as mean ± SD in dry weight.

Parameters	Unfermented maize	FSp	FLp	FYLc	Soaked maize	Germinated maize	FLp-SG
Moisture (%)	10.6 ± 0.11	6.94 ± 0.93	6.13 ± 0.15	6.49 ± 0.88	8.81 ± 0.15	10.15 ± 0.91	4.91 ± 0.21
Phytate ($g{\cdot}kg^{-1}$)	9.58 ± 0.05^{fD}	4.65 ± 0.40^{cC}	3.35 ± 0.26^{bB}	3.02 ± 0.01^{bB}	8.44 ± 0.06^{e}	6.35 ± 0.03^{d}	1.39 ± 0.09^{aA}
Zinc ($mg{\cdot}kg^{-1}$)	21.10 ± 0.89^{bB}	19.73 ± 0.50^{abAB}	18.33 ± 0.49^{abA}	19.57 ± 0.49^{abAB}	19.63 ± 2.01^{ab}	20.53 ± 0.45^{ab}	17.73 ± 0.68^{aA}
Iron ($mg{\cdot}kg^{-1}$)	19.57 ± 0.60^{aA}	19.67 ± 1.26^{aA}	18.07 ± 1.47^{aA}	17.70 ± 1.47^{aA}	18.43 ± 0.81^{a}	18.17 ± 1.14^{a}	18.87 ± 1.19^{aA}
Phy:Zn	40.76 ± 2.36^{eE}	23.42 ± 0.49^{cD}	18.14 ± 0.82^{bcC}	15.30 ± 0.32^{bB}	40.57 ± 1.29^{e}	30.65 ± 0.55^{d}	7.77 ± 0.24^{aA}
Phy:Fe	41.42 ± 1.27^{eD}	19.97 ± 1.16^{cC}	15.68 ± 0.32^{bB}	14.49 ± 1.27^{bB}	38.76 ± 1.75^{e}	29.63 ± 1.90^{d}	6.24 ± 0.39^{aA}
% of phytate reduction		51.8 ± 4.10	65.3 ± 0.26	68.7 ± 3.2	12.6 ± 0.6	31.9 ± 1.9	85.6 ± 0.91

For each parameter (each row), superscripts lowercase letters (a, b, etc.) indicate differences between untreated maize, and after the treatments; soaking, germination and all fermentations. Superscripts uppercase letters (A, B, etc.) indicate differences due to fermentation types against the untreated maize. Results are presented as mean ± SD and significant differences were computed at $p < 0.05$. FSp, Spontaneous fermentation of maize flour; FLp, Fermentation of maize flour with *L. plantarum* 299v®; FYLc, Fermentation of maize flour with yoghurt containing viable *L. casei*; FLp-SG, Fermentation of maize flour from soaked and germinated maize kernels with *L. plantarum* 299v as starter culture.

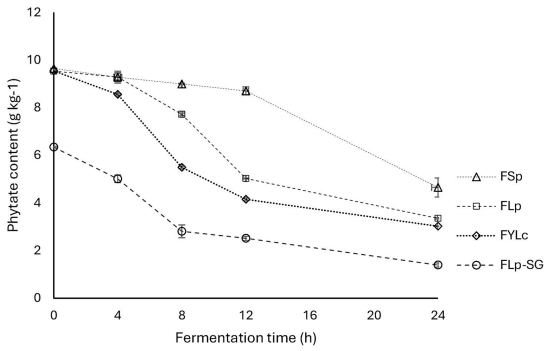

FIGURE 3
Changes in phytate content through various fermentation processes of maize flour. FSp, Spontaneous fermentation of maize flour; FLp, Fermentation of maize flour with *L. plantarum* 299v®; FYLc, Fermentation of maize flour with yoghurt containing viable *L. casei*; FLp-SG, Fermentation of maize flour from soaked and germinated maize kernels with *L. plantarum* 299v as starter culture.

leached into the discarded soaking water. There were no significant differences in the iron content before and after treatments.

3.5 Mineral bioavailability estimated by the phytate: mineral molar ratios

The recommended phytate to mineral molar ratios for adequate bioavailability of zinc and iron are Phytate:Zinc (Phy: Zn) <15 and Phytate:Iron (Phy: Fe) <1. In this study, the calculated molar ratios for and Phy:Fe and Phy:Zn, before and after treatments, are presented in Table 1. Before any treatment, the molar ratios in maize were significantly above the recommended values, with a Phy:Zn of 40.76 and a Phy:Fe of 41.42. All fermented maize flours showed improved phytate:mineral molar ratios, although most remain above the threshold values. The exception was the Phy:Zn of 7.77, found in the fermented maize flour developed from pre-soaked and germinated grains (FLp-SG). Compared to the Phy:Zn of raw maize, there was a substantial reduction from 40.76 to 7.77 (an 81% decrease), which fell well below the threshold level of 15. This result implies that zinc bioavailability in maize was no longer inhibited by phytates after the treatment FLp-SG. Phy:Zn ratio for spontaneously fermented flour also improved from 40.76 to 23.42, although it remained above the threshold of 15. Other fermentation treatments such as FLp and FYLc yielded comparable Phy:Zn ratio of 18.14 and 15.30, respectively, representing reductions of 55 and 62%, respectively, from the raw maize.

Phy:Zn ratios for only soaked or soaked-germinated maize were 40.57 and 30.65 respectively, indicating that these techniques alone were insufficient to significantly improve zinc bioavailability. Among all fermentation types, the FLp-SG process proved most effective at bringing Phy:Zn molar ratios below the recommended level 15, indicating that zinc bioavailability in this case was not longer impaired by phytate.

Phy:Fe molar ratio for untreated maize was 41,42. Soaked alone had minimal impact, reducing the ratio to 38.76, while germination brought it down to 29.63. In contrast, the FLp-SG treatment led to a substantial 85% reduction, lowering the Phy:Fe ratio to 6.24. This improvement was followed by FYLc (Phy:Fe ratio of 14.49), FLp (15.68) and FSp (19.97). Although none of the fermentation methods achieved the ideal phy:Fe molar ratio of below 1. Nevertheless, all the reductions were significant, and indicate potential for further enhancement of both fermentation methods and pre-treatments to improve iron bioavailability.

3.6 Effect of fermentation on protein content

Figure 4 shows the crude protein content of maize flour determined at 0, 12, and 24 h of fermentation for all the four fermentation types. During the study, protein content increased at varying rates as fermentation time progressed for all the four fermentation types. Maize flour that underwent spontaneous fermentation for 24 h (FSp) showed a slight protein content increase of 8.2% from the baseline. Compared to the control or raw maize flour, protein content significantly increased ($p < 0.05$) by 9.3% in FLp, 10.3% in FYLc and 7.3% [in] FLp-SG.

The results from the two-way ANOVA test showed that protein content was significantly affected by fermentation time ($p = 0.000$) at intervals of 0, 12 and 24 h, but was not significantly affected by the fermentation type ($p = 0.459$) among FSp, FLp, FYLc, and FLp-SG. The interaction between the two factors – fermentation time and type-was also not statistically significant ($p = 0.756$) for

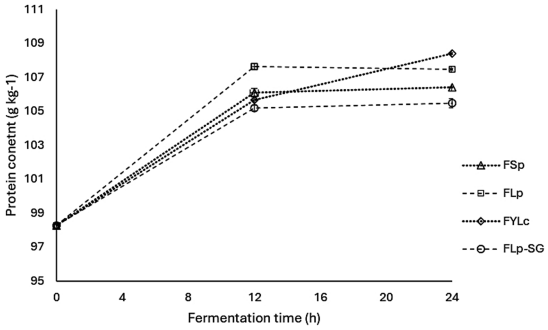

FIGURE 4
Changes in protein content during maize flour fermentation. FSp, Spontaneous fermentation of maize flour; FLp, Fermentation of maize flour with *L. plantarum* 299v®; FYLc, Fermentation of maize flour with yoghurt containing viable *L. casei*; FLp-SG, Fermentation of maize flour from soaked and germinated maize kernels with *L. plantarum* 299v as starter culture.

protein content. Figure 4 presents the interaction plot of the two variables, providing visual representation of how both factors impacted protein content. Furthermore, the post-hoc analysis of multiple comparisons indicated that at time 0, 12 and 24 h, protein content was not significantly different across samples FSp, FLp, FYLc and FLp-SG with *p*-values of 0.000 at 0 h, ranging from 0.163 to 0.801 at 12 h, and from 0.097 to 0.569 at 24 h. However, protein content showed significant differences due to fermentation time; at 0 h, protein content was significantly lower ($p = 0.000$) than that recorded at 12 h, after which it did not significantly change ($p = 0.595$) until 24 h fermentation. This trend was consistent across all fermentation types.

4 Discussion

4.1 Effect of fermentation on pH and lactic acid

The reduction of pH during fermentation can be attributed to the lactic acid bacteria added as Lp299 or *L. casei* in yoghurt, these lactic bacterium converts available carbohydrates to organic acids that are responsible for the pH reduction and lactic acid increase. It is reported that in spontaneous fermentation, endogenous microbial activity reduces the substrate's pH by using carbohydrates and producing organic acids such as lactic, citric, and acetic acids (27). In our study, relatively lower rate of reduction in pH and corresponding slight increase in lactic acid contents during first 12 h of spontaneous fermentation resulted from the time needed for endogenous microbes to activate and adapt to the fermentation conditions. The higher lactic acid content in FYLc during the first half of the fermentation period likely resulted from the lactic acid already present in the yogurt. After 8 h, the faster increase in lactic acid production in samples fermented with Lp299 strains can be due to the depletion of available carbohydrates, caused by the higher metabolic activity of the microflora during the initial phase of fermentation.

The results on the effect of fermentation on pH and the lactic acid contents of the substrate align with previous research. For instance, earlier studies reported that maize flour fermentation anticipated considerable pH reduction from 6.67 to 3.85 and total acidity increased from 37.97 to 71.59 $g \cdot kg^{-1}$ (28). Our study recorded a slightly higher pH of 4.01. Unlike the 129-h fermentation reported by Beugre et al. (28), we achieved this pH reduction in just 24 h, which highlights the potential for reducing fermentation time in cereals and other complex plant foods. Similarly, Ejigui et al. (29) found a 37% decrease in pH after 96 h of maize fermentation. While we observed a 39% reduction in 24 h. These differences can be attributed to the crop cultivar, grain size and fermentation conditions such as time, temperature and starter cultures. Improved microbiological activity to support lactic acid fermentation, decrease pH and increase corresponding lactic acid contents has also been endorsed by Wedad et al. (30) during 16 h sorghum fermentation.

Our study recorded a rapid 79% increase in lactic acid content from the baseline for 24 h fermentation, which closely aligns with the findings of Beugre G. et al. (28), where titratable acidity increased by 87% over 48 h fermentation. We observed a significant pH decrease ($p < 0.001$) and a corresponding significant increase in lactic acid content ($p < 0.001$) (Figure 2B). Among the fermentation processes, maize flour fermented with Lp199 after soaking and germination showed the greatest pH reduction (from 6.61 to 3.61) and lactic acid content increase (from 9.0 to 80.1 $g \cdot kg^{-1}$) (Figures 2A,B). This improved performance can be attributed to the early activation of endogenous microflora during soaking and germination of the maize kernels. Soaking and germination resulted in a slight drop in pH and an increase in lactic acid content. Differences between spontaneous fermentation and those using Lp299 or yogurt starter cultures can be linked to the predefined inoculation of the starter cultures, which supports the rapid activation of fermentation (31, 32).

The extended shelf life of fermented foods is largely linked to higher lactic acid contents and significant drop in pH, which inhibit spoilage bacteria and other pathogens. This also enhance the quality and consumer acceptability of fermented products. Achieving high lactic acid concentrations or a rapid pH decline to around 4.0 is highly desirable for preventing microbial spoilage and extending the shelf stability properties of maize flour (33). This quality is especially important when preparing maize-based weaning formulas for infants and young children, as it reduces the risk of spoilage or microbial contamination (31).

4.2 Effect of soaking and germination on phytate content

The results indicate that the phytate content in raw maize was consistent with previous studies, which reported levels ranging 7–14 $mg \cdot g^{-1}$ (27, 34). Phytate reduction in cereal products during soaking is attributed to the solubility of phytate in water. Since phytate is water soluble, soaking facilitates its reduction. In our study, phytate reduction during soaking was modest (12.6%), similar to findings by Kruger et al. (34), who reported a 14% reduction in whole white maize grains after soaking for 12 h at 25°C. Other authors have found that soaking removed up to 21% of phytate from maize grains (35). Previous studies have shown that the impact of soaking on phytate content depends not only on soaking conditions – such as pH, time, volume of soaking water and temperature – but also to the grain variety and state of the soaked grain. Hotz and Gibson (27) observed

a 43% reduction of phytate after 12 h soaking of unrefined white maize flour at 25°C and a higher reduction (49%) when excess water was decanted. In their study, the ratio of maize to water was 1:4, and the soaking temperature was higher than in our present research. Duhan et al. (36) observed a 29% reduction in phytate from whole millet after 24 h of soaking, compared to 39 and 52% for once-dehulled millet and millet flour, respectively. These results indicate a greater potential for reducing phytate content by processing grains into flours rather than soaking intact grains. This demonstrates that increasing the surface area of seeds or grains enhances exposure to the soaking medium, improving the removal of soluble phytates.

It is important to note that soaking is a common pre-treatment method used to enhance the nutritional quality of pulses, legumes, and grains. While soaking can reduce anti-nutritional factors like phytates, it can also lead to the loss of some water-soluble nutrients. Nutrients such as B-complex vitamins (thiamine, riboflavin, niacin, and folate), vitamin C, minerals (potassium and magnesium), and soluble proteins can leach into the soaking water (37). Therefore, it is important to assess nutrient loss when evaluating soaking as a processing method.

The results of 80 h germination of maize grains showed a 31.9% reduction in phytate content. This result agrees with findings by Mihafu et al. (22) in Tanzania, where phytate reduction ranged from 8.3 to 34.1% after 72 h of maize germination. Additionally, Coulibaly et al. (38) reported a 23.9% phytate reduction in millet after 72 h of germination, with 96 h of malting leading to a 45.3% reduction. The reduction in phytate during germination is primarily attributed to phytate hydrolysis triggered by the activation of phytase enzymes, which increase during germination (39). These enzymes break down phytate, releasing phosphorus, myo-inositol, and minerals that serve as nutrients for the growing seedling. Other factors contributing to phytate hydrolysis include the species and varieties of the grains, temperature, moisture content, pH, duration of germination, phytate solubility, and the presence of inhibitors like tannins in sorghum (40).

4.3 Effect of fermentation on phytate content

Our statistical comparison using one-way ANOVA for different fermentation techniques revealed significant differences ($p = 0.000$) in their effectiveness at reducing phytate content in maize after 24 h fermentation (Figure 3). Furthermore, the two-way ANOVA test confirmed that, in addition to fermentation type, fermentation time also plays a significant role in reducing phytate content. While reduction of phytate was slower in the first 4 h, it was intensified between 4 and 12 h followed by a slower decreased in the last 12 h of fermentation. More specifically for FSp the reduction of phytate content during the first 12 h reached only 8% from the baseline, and during the following 12 h the reduction reached 51.8%. This reduction goes in line with the pH that remained above 6 during the first 12 h, a level that does not support optimal phytase activity. The pH dropped to 4.01 during the last 12 h explaining why most of the phytate reduction occurred during the later half of the 24-h fermentation. A similar pattern of phytate reduction was observed in a study where maize was spontaneously fermented for four days at 30°C, achieving a 61.5% reduction in phytates (29). Minor differences in rate of phytates reduction between our and theirs can be attributed to variations in temperature and the longer fermentation time. This slower reduction in the early stages of spontaneous fermentation can also be attributed to the slow decrease in the substrate's pH, as discussed in the earlier section. Endogenous phytases of plant origin are most effective at degrading phytates in a pH range of 4.0–5.6. During fermentation the pH reduction and lactic acid production create the optimal environment for these phytases to become active and hydrolase the phytate molecules (20).

In our study, fermentations with starter cultures showed higher phytate reduction compared to spontaneous process, with FLp achieving a 65.3% reduction and FYLc reaching 68.7% in 24-h fermentation. For these two fermentations the fastest phytate reduction was detected between 4 and 12 h of fermentation, 47% out of the 65.3% for FLp and 57.5 out of the 68.7% for FYLc. Previous research has demonstrated that using specific starter cultures leads to greater phytate reduction than spontaneous fermentation. Hotz and Gibson (27) found a 49% phytate reduction when starter culture from a fermented beverage was added during maize fermentation at 25°C for 15 h, this was four times higher than the 12% reduction observed in spontaneous fermentation. Moreover, specific starter cultures such as *Lactobacillus amylovorus* and *Lentilactobacillus buchneri* have been shown to be effective phytase producers, achieving up to a 95.5% phytate reduction after 72 h of fermentation at 30°C (41).

Spontaneously fermented maize flour (FSp) showed the lowest phytate reduction, achieving only a 51.8% decrease from the baseline which was unfermented maize flour. This result may be due to the low specificity of microbial phytase from the endogenous microflora, as reported by Shimelis and Rakshit (32). In contrast, the highest phytate reduction occurred with the combined FLp-SG process, where fermentation with Lp299-inoculated pre-soaked and germinated maize led to an 85.6% reduction. The pre-treatments of soaking and germination enhanced phytate reduction by 20.3% (from 65.3% in FLp to 85.6% in FLp-SG). These findings highlight the effectiveness of pre-treatments in increasing phytate reduction before fermentation. Hotz and Gibson (27) also studied germination prior to fermentation, finding a 17% increase in phytate reduction when germination was used before natural fermentation of maize at 25°C for 48 h. Their work showed phytate reduction of 29% for flour from germinated maize grains, compared to 12% for unprocessed maize flour fermented for 96 h. However, their overall lower phytate reduction rates may have been influenced by the lower fermentation temperature (25°C). Our results align more closely with those reported by Khetarpaul and Chauhan (42) who reported an 88.3% reduction in phytate content by fermenting pre-germinated pearl millet flour (germinated at 30°C for 24 h) with *Saccharomyces cerevisiae* var. *diasticus*, *Saccharomyces cerevisiae*, *Levilactobacillus brevis*, and *Limosilactobacillus fermentum* at 30°C for 72 h. Among the factors which can accelerate or decelerate phytate reduction during fermentation of plant-based food, phytase activity is the most important factor that depends on the pH of substrate. Other factors include the microbial species, temperature and presence of anti-nutrients (43). Reducing phytate content, whether to a small or large extent, is beneficial for human nutrition as it decreases the risk of forming insoluble mineral complexes and improves mineral absorption in the gut.

Moreover, we found no significant difference in the effectiveness of FLp and FYLc fermentations for phytate reduction, indicating

that yogurt containing viable lactic bacteria is as effective as the probiotic strain Lp299 as a starter culture. Overall, higher phytate reduction was achieved by incorporating pre-fermentation treatments such as soaking and germination before yogurt fermentation. This opens the possibility of applying this process at the household level. However, several challenges must be addressed before this can be fully realized, which we discuss extensively at the end of the discussion section.

4.4 Effect of soaking, germination fermentation on mineral content and estimated mineral bioavailability

The estimated bioavailability of minerals such as iron, zinc, and calcium are often calculated using the molar ratios of phytate to mineral because the presence of phytates significantly affects the absorption and utilization of these minerals in the human body. The molar ratio of phytate to minerals reflects the degree to which phytates can bind and prevent the absorption of essential minerals. The mechanism behind the molar ratios is funded by the fact that phytates are negatively charged molecules that strongly bind to positively charged minerals like iron, zinc, and calcium. These bonds form insoluble complexes, which prevent the minerals from being absorbed in the small intestine (44). The more phytate there is in relation to the mineral, the greater the likelihood of mineral-phytate complex formation, reducing the mineral's availability for absorption. Thus, lower molar ratios generally indicate better potential for mineral absorption, making this calculation important for dietary planning and food processing aimed at improving the nutritional value of plant-based foods.

In this study, we found slight to non-significant changes in minerals contents of the fermented, soaked and germinated maize flours, results that agree with the findings of Castro-Alba et al. (18, 23) and Ayub et al. (16). In those studies, fermentation of pseudocereals did not have a significant effect modifying iron, zinc and calcium content of the raw and fermented pseudocereals. In our study a significant reduction of zinc was only found in FLp-SG and attributed to the soaking process used prior to this fermentation. This loss may be linked to the leaching effect of soluble mineral fractions in soaking water that was removed. Referring to the effect of lactic acid fermentation in improving minerals bioavailability of maize flours, we proposed Lp299 fermentation in continuum of maize kernel soaking and germination as the alternative that brings down the Phy:Zn ratio to a threshold level of 7.77 which is below 15 (desirable value). This result is similar with the results reported in a study in cassava fermentation, where the Phy:Zn ratio was improved from 16.31 to 1.71 after 48 h spontaneous fermentation at 25°C (45). Improvement of estimated bioavailability of zinc and iron in maize flour proves the efficiency of fermentation in phytate reduction and adds up to nutritional advantages.

Since application of plant—based fermented flours in food product development and food value addition is increasing, fermented maize flour can help improve essential minerals intake and absorption. Considering the context of developing countries where maize is a staple cereal, practicing soaking, germination and lactic acid fermentation in combination can alleviate the burden of micronutrient deficiencies and malnutrition.

4.5 Effect of fermentation on protein contents

Our results on protein contents of the fermented flours are nearly consistent with the results of Ogodo et al. (46) where 48 h fermentation of maize flour with lactic acid bacterial consortium improved protein contents from 9.44 to 12.9%. In another study, protein contents of maize flour were increased from 29.7 to 43.5% during various fermentations maize (47). Protein increases during fermentation have been attributed to several biochemical and microbiological processes that occur during fermentation. Microorganisms such as bacteria and yeast carbohydrates and other nutrients present in the substrate, they also consume nitrogen sources (aminoacids and peptides) to synthesize their own proteins, leading to an increase in bacterium biomass (48). Proteolytic activity has also been reported during fermentation, as this process involves the action of various enzymes, including proteases produced by the microorganism and the cereal itself. Proteolytic enzymes break down complex proteins into simpler peptides and free aminoacids, which may lead to an increase in protein content (49). Cell wall degradation led by the enzymes produced during fermentation has been reported (50). Some proteolytic enzymes can break down the aleurone layer's cell wall structure, thus facilitating the release of proteins that are trapped within the bran of cereals. Thus, the synthesized proteolytic enzymes improve protein digestibility, and the changes in amino acid profile have positive effects increasing the overall nutritional quality of cereals. In this study, a slight increase in protein contents of during FYLc may be linked to the protein contents of the yoghurt used as inoculum for flour fermentation. Fermentation led improvement in protein content and quality of the maize flour is nutritionally advantageous and can further help to improve the palatability and texture of maize-based food products.

Along with protein, other nutrients in the substrate, such as starch and sugar, may also undergo significant changes during fermentation, warranting further research attention. It was reported that fermentation led to the breakdown of starch into simpler, more digestible sugars, and the reduction of sugars as microorganisms use them for energy. Besides proteolytic enzymes, amylolytic enzymes are also produced during fermentation. These amylases break down complex carbohydrates (starch) into simpler sugars (for example, maltose, and glucose), this process is called saccharification (51). Thus, fermented foods will usually result in lower glycemic index than their unfermented counterparts, which may have significant nutritional and health benefits. However, the specific changes depend on the type of microorganisms involved and the fermentation conditions.

We have noted in our previous studies of quinoa fermentation (23, 52) that fermentation can significantly affect the sensory characteristics, altering their taste, texture, aroma, and appearance. During fermentation, microbial activity breaks down complex carbohydrates and proteins, leading to the production of various compounds that influence sensory attributes such as taste. Overall, while fermentation enhances the nutritional value of cereals, it can also introduce complex sensory challenges that may either enhance or detract from consumer acceptability, depending on cultural preferences and intended use of the product. In our previous studies, we optimized the time of fermentation and concluded that the longest the fermentation time the more undesirable sensory characteristics

are developed. For quinoa fermentation, we reduced the fermentation time from 48 to 9 h and added roasting after fermentation. This significantly improved the sensory characteristics of the fermented quinoa flour (23). We acknowledge that sensory is very important for consumer acceptability, while in the present study we reduced the fermentation time from 48 to 24 h, and we (4 people) in the research group evaluated basic characteristics such as aroma and taste, which seem to be acceptable. Further comprehensive studies should explore how different fermentation conditions - such as time, temperature, and microbial strains - affect these sensory properties. In the study conducted by Annan et al. (53) was highlighted the importance of the effect of different starter cultures in traditional African maize fermentation. It was shown that fermented maize dough in Ghana called "Koko" was preferred after spontaneous fermentation than after the use of a combination of starter cultures. Conversely, the sensory characteristics of the Nigerian "Ogi" prepared from fermented maize slurry were improved when starter cultures of *Levilactobacillus brevis* and *Saccharomyces cerevisiae* were used during fermentation (54). Understanding these changes will help optimize fermentation processes for improved consumer satisfaction and product development, especially in diverse cultural contexts where sensory preferences may vary.

By promoting local food processing techniques such as soaking, germination, and fermentation, families can improve the nutritional value of staple foods like maize without requiring expensive or complex technology. The extend by which this adoption could improve nutritional situation should be explored. Countries like Rwanda, where fermented foods are already part of their dietary habits and traditions are an important niche to start fermentation programs, where lactic bacteria found in commercial yogurt can be used as a starter culture. Fermentation is a traditional practice in many cultures, which makes its adoption more likely when compared to more modern, unfamiliar technologies. While modern food processing technologies such as ultrasound, pulsed electric fields, and enzymatic treatments offer promising alternatives for reducing phytates, fermentation remains a highly effective, affordable, and culturally adaptable method. It should not be dismissed as a "primitive" technology, as its potential to combat micronutrient deficiencies is considerable.

However, it is important to acknowledge the challenged associated with the widespread adoption of fermentation in household settings. Locations-specific fermentation conditions need to be validated, these regarding factors such as temperature, humidity and time, which can vary from one country to another. Further research and policy support and national programs are fundamental to develop detailed guidelines for home use of fermentation. The guidelines would offer households easy-to-follow instructions that ensure optimal nutrient enhancement while maintaining food safety. Widespread adoption of home fermentation can have profound social and economic impacts in developing countries, contributing to economic empowerment and improved public health. Furthermore, promoting home fermentation supports food sovereignty by empowering communities to rely on their traditional food systems rather than external technologies.

4.6 Limitations of the study

This study primarily focused on the nutritional effects of fermentation without considering the potential impact on sensory characteristics such as taste, texture, and aroma. Sensory properties such as aroma and taste were evaluated only by the research group. As these sensory attributes significantly influence consumer acceptability, the findings may not fully represent the practical implications of fermented products in real-world food systems. Future studies should incorporate sensory evaluation to provide a more comprehensive understanding of the acceptability of fermented foods. Another limitation of the study is the number of replicates used, two replicates of processing and two replicates of analysis, was relatively low, which may limit the statistical power and generalizability of the results. A larger sample size would enhance the reliability of the findings and allow for more robust conclusions. Further research on the field of fermentation should also include a throughout investigation on the impact of fermentation on the content and bioavailability of other important nutrients (starch, sugars) and bioactive compounds such as polyphenols.

5 Conclusion

This study demonstrates that simple processing techniques such as soaking, germination and fermentation effectively reduced phytates in maize flour. These methods have the potential to be applied at household level. For instance, FYLc, which uses yoghurt as starter culture, provided comparable results to the fermentation with Lp299. We also found that pre-treatments like soaking and germination used prior fermentation helped to further reduce the phytate content in maize. Reducing phytate reduction in this staple crop significantly improves the estimated bioavailability of essential minerals like iron and zinc which are crucial for addressing nutritional deficiencies in vulnerable populations. Additionally, the fermentation processes also increased the protein content in maize flour, which is particularly relevant in the current shift toward more protein-rich plant-based alternatives.

These findings underscored the potential of applying fermentation at the household level as a cost-effective dietary strategy to enhance protein content and improve mineral absorption, especially in populations vulnerable to protein and micronutrient deficiencies. The widespread adoption of household fermentation could improve nutrition security in developing regions. Moreover, well-document fermentation methods hold the potential to inform and shape national nutrition policies and programs aimed at combating malnutrition and micronutrient deficiencies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SN: Conceptualization, Data curation, Formal analysis, Investigation, Validation, Writing – original draft. TI: Formal analysis, Validation, Visualization, Writing – review & editing. CL: Conceptualization, Data curation, Formal analysis, Investigation,

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Antioxidant capacities and non-volatile metabolites changes after solid-state fermentation of soybean using oyster mushroom (*Pleurotus ostreatus*) mycelium

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Given the abundance of beneficial properties and enzymes secreted by edible oyster mushrooms, their mycelium could serve as a starter for fermented foods to enhance their nutritional and bioactive quality. This study aimed to investigate the effects on the nutritional ingredients, antioxidant activity, and non-volatile metabolites during solid-state fermentation (SSF) of soybeans by *Pleurotus ostreatus* mycelium. The results indicated that the contents of dietary fiber and starch in fermented soybeans decreased, while the amounts of protein and lipid increased after SSF ($P < 0.05$). Analysis of the total phenolic content (TPC) and antioxidant activities of the fermented soybeans revealed that the methanolic extracts significantly increased TPC and antioxidant activities against intracellular reactive oxygen species (ROS) in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages, as well as against DPPH and ABTS radicals *in vitro*. A total 154 differential metabolites were identified after SSF, and a Spearman correlation study revealed a direct relationship between antioxidant activities and certain metabolites including phenolic compounds, oligopeptides, and free fatty acids etc. Among these metabolites, phenolic compounds produced by the shikimic acid pathway were diverse in variety and had the greatest multiple differences. The study discovered that a potential mechanism involving SSF with *P. ostreatus* mycelium increased the antioxidant activity of soybeans.

KEYWORDS

***Pleurotus ostreatus*, solid-state fermentation, soybean, antioxidant activity, metabolome**

1 Introduction

Soybeans (*Glycine max* L.) are the main crop grown among legumes and rank the second-largest food crop in the world after cereals. After years of expansion, soybeans have become a mainstay of Southeast Asian cooking, and their particular characteristics have attracted the attention of the food industry (1, 2). Because of their nutritional qualities and useful components, soybeans are regarded as one of the best meat alternatives (3–5). Furthermore, numerous epidemiological studies have demonstrated the advantages of soybeans in lowering the prevalence of heart problems, menopausal symptoms, and cancers such as prostate, breast, and colon cancers (5–9).

A great option for meeting individual increasing needs for healthy, tasty, and sustainably produced food is fermented soybean (10–12). Multiple complex processes, including microbial metabolism, enzyme catalysis, lipid oxidation, amino acid breakdown, and Maillard browning, are involved in the fermentation of soybeans (13–15). Trypsin

inhibitors, which prevent the absorption of nutrients from food, are efficiently eliminated from soybeans during fermentation, reducing their antinutritional effects (16, 17). Certain vitamin contents, such as vitamin K_2 and vitamin B_{12}, noticeably increase in fermented soybean products (12, 18). Fermentation breaks down phytates that bind with minerals, making iron and copper more bioavailable (19). Through fermentation, isoflavone' glycoside forms are changed into aglycone forms, increasing its bioavailability (20).

The oxidative stress caused by reactive oxygen species (ROS) has been linked to the development of multiple chronic diseases, such as rheumatoid arthritis, diabetes mellitus, neurological disorders, and cataract formation (21). It has been discovered that the soybean fermentation produces significant antioxidant activity in both primary and secondary metabolites including, free amino acids, small peptides, flavonoids, phenolic acids and phytosterols, etc. (3, 10). Furthermore, by increasing the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), soybeans through fungal solid-state fermentation demonstrated a preventive effect on lipid peroxidation in the liver of cholesterol-fed rats (22). Stronger antioxidant activity was found in fermented soybean foods made with *B. subtilis* GD1, *B. subtilis* N4, *B. velezensis* GZ1, *L. delbrueckii* subsp. bulgaricus, and *Hansenula anomala* (23). Various types of microbes, such as filamentous fungi including *Aspergillus* spp., *Mucor* spp. and *Rhizopus* spp., bacteria including *Bacillus* spp., and lactic acid bacteria are involved in soybeans fermentation (3, 10). Mushroom mycelium grows quickly and is also used as a starter for fermenting soybeans. After 20 days of fermentation with *Irpex lacteus* mycelia, soybeans exhibited ~4–5 times more total phenolic, flavonoid, isoflavone, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging (24). The fermentation process changed the physical characteristics of soybean and enhanced their antioxidant qualities, which could be affected by the choice of microbial species, temperature, duration, and concentration of salt and environmental factors (3, 14, 25). Phenolic compounds were found to be the major contributor to the antioxidant effects in fermented soybeans (3). β-glucosidase convert isoflavone glucosides to isoflavone aglycones and other metabolites during the mold-fermented soybean process. It has been proposed that isoflavone aglycones have a greater antioxidant capability than their glycoside forms (3, 26). However, due to the decreased β-glucosidase activity, isoflavone aglycones had very minor effects on the antioxidant activity of the bacterially fermented soybean. Small peptides and free amino acids particularly those made of hydrophobic, aromatic, acidic, and basic amino acids accumulated as a result of the high activity of proteases in bacterial fermented soybean, which were strongly correlated with the antioxidant activity (27). Microorganisms convert substances into bioactive compounds that are associated with antioxidant activity through enzymatic processes (13). Thus, it is crucial to use novel microorganisms with special enzyme-producing capabilities to create new functional fermented foods.

Pleurotus ostreatus, also known as oyster mushroom, is a white-rot macrofungus that secretes a variety of enzymes, particularly lignocellulolytic enzymes, throughout its growth (28). Its chemical composition consists of polysaccharides (β-glucans), dietary fibers, unsaturated fatty acids, peptides, terpenoids, sterols, and physiologically active proteins such as lectins and enzymes (29). Numerous studies have documented the bioactive effects of compounds derived from *P. ostreatus*. Two novel sesterterpenes and two new triterpenes that demonstrated antiprotozoal action against *Trypanosoma cruzi* and *Plasmodium falciparum* were identified by Annang et al. (30). From the methanolic extracts of *P. ostreatus*, the coumarins 5-methoxy-(E)-subodiene and toddaculin were isolated, as well as the steroid ergosterol, which showed notable antifungal activity against *Penicillium digitatum* (31). Three novel amino acid compounds with antifungal activity against *Candida albicans* were isolated from the ethyl acetate extracts of *P. ostreatus*' solid culture (32). Furthermore, 3-(2-aminophenylthio)-3-hydroxypropanoic acid from *P. ostreatus* aqueous extracts showed potent antibacterial and antifungal properties (33).

While some research has been done on the use of oyster mushrooms to increase the nutritional and health-promoting properties of food products (29, 34, 35), there have been no studies conducted on the use of *P. ostreatus* mycelium to improve the quality of soybean products. To enhance the antioxidant properties of soybeans, our research focused on the potential effects of novel fermented soybeans using oyster mushroom with strong antioxidant activity. The objective of these investigations was to ascertain the alterations in the non-volatile metabolites and antioxidant capacity during the fermentation of soybeans with *P. ostreatus*. The findings of this study could provide a theoretical foundation for further research and commercial development, such as fermenting soybeans with edible mushrooms.

2 Materials and methods

2.1 Materials

Soybeans (*Glycine max* L.) were sourced from Mudanjiang City, Heilongjiang Province, China. *Pleurotus ostreatus* (CGMCC 5.784) was purchased from the China General Microbiological Culture Collection Center (CGMCC) and stored in potato dextrose agar (PDA) slant medium at 4°C.

DPPH was purchased from Aladdin Biochemical Technology Co., Ltd (Shanghai, China). Gallic acid, Folin-Ciocalteu's phenol reagent and 2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Yuanye Bio-Technology Co., Ltd (Shanghai, China). RAW 264.7 macrophages were purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd (Beijing, China). All other chemicals and solvents were of analytical reagent grade and acquired from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2 Methods

2.2.1 Preparation of fermented soybeans by P. ostreatus mycelium

The soybeans were subjected to solid state fermentation (SSF) according to the study by Zhai et al. (36) with slight modifications.

The slant mycelia of *P. ostreatus* were transferred onto petri plates containing a broth made of 0.4% PDA for cultivation at 25°C for ~10 days. Soybeans were soaked in deionized water (1:5, w/v) for 8 h at room temperature, and the moisture content of soybeans was about 60%. Tissue culture bottles (80 × 90 mm) were filled with 120 g of soaked soybeans and sterilized by autoclaving at 121°C for 90 min. Three tiny mycelia of 1 cm × 1 cm from a fully colonized petri dish cultured on PDA were added to the surface of the cooled soybeans as an inoculant. The bottles were then incubated at 25°C and 70% humidity in the dark until the fungal mycelia covered the entire bottle (33 days). The soybeans used as the control underwent the same procedure without being inoculated.

2.2.2 Assay of general composition of soybeans

Both the unfermented soybeans (USB) and fermented soybeans (FSB) were heat-dried at 60°C for 24 h until they achieved a constant weight after the culture. After that, the material was ground using a grinder with a Ø40 mm diameter for further analysis. The unfermented and fermented soybeans were analyzed for their chemical composition. The content of moisture, crude protein, reducing sugars (37), starch (38), total dietary fiber, soluble dietary fiber, and insoluble dietary fiber (39) were determined according to standard methods described by the Association of Official Analytical Chemists (AOAC). Lipid content was determined using the method reported by Idamokoro et al. (40). All determinations were performed in triplicate, and the mean values with standard deviations were calculated and reported.

2.2.3 Assay of the total phenolic content

Determining the total phenolic content (TPC) was conducted by the method described by Donlao and Ogawa (41). Dried and crushed samples after fermentation were homogenized with 80% (v/v) methanol (10 g sample/200 mL 80% MeOH w/v). Afterward, the extract was filtered and collected in a dark glass bottle. Briefly, 20 μL of the diluted extract was mixed with 20 μL of 50% Folin-Ciocalteau reagent in 96-well plates and kept in the dark. After a 30 min interval, 40 μL of 7.5% Na_2CO_3 and 120 μL of deionized water were added. After letting the mixture sit in the dark for 2 h, the SYNERGY H1 microplate reader (Biotek Instruments, Inc. USA) was used to measure the absorbance at 765 nm. The results were expressed as milligrams of gallic acid equivalent per g (mg GAE/g).

2.2.4 Determination of antioxidant activity

The supernatant of methanolic extracts was concentrated and lyophilized, then prepared into different concentrations (ranging from 0.5 to 16 mg/mL) with 80% (v/v) methanol to determine antioxidant activity. DPPH radical scavenging activity was measured according to the method reported by Sanjukta et al. (42). Briefly, 100 μL of the supernatant of methanolic extracts, Trolox and diluted methanolic extracts (0.5, 1, 2, 4, 8 and 16 mg/mL) were mixed with DPPH-methanol (0.2 mM, 100 μL) in a 96-well plate. The mixture was kept in the dark for 30 min at room temperature and the absorbance at 517 nm was read. 80% (v/v) methanol was used instead of the samples for the blank control. DPPH scavenging activity was determined as follows: DPPH scavenging activity = $(1\text{-}A_s/A_b) \times 100\%$, where A_b is the absorbance of the 80% (v/v) methanol reacted with DPPH, and A_s is the absorbance of the different samples reacted with DPPH. The calibration curve was drawn with different concentrations of Trolox as the horizontal coordinate (x) and DPPH scavenging activity as the vertical coordinate (y) ($y = 0.0131x + 0.0067$; $R^2 = 0.9988$). The Trolox calibration curve (0 ~ 70 μmol/L) was used to quantify antioxidant activity allowing for the expression of the ability to radical scavenging activity of unfermented and fermented soybeans as μmol Trolox equivalents per gram.

The scavenging activities of samples against ABTS were determined according to the method reported by Ketnawa and Ogawa (43). ABTS solution (7.00 mmol/L) and $K_2S_2O_8$ solution (4.90 mmol/L) were mixed in a 2:1 (v: v) ratio and kept in the dark for 16 h. Subsequently, deionized water was added to the mixture until the absorbance at 734 nm was 0.7 ± 0.02. The mixture was used as the ABTS working solution. 40 μL of the supernatant of methanolic extracts, Trolox and diluted methanolic extracts (0.5, 1, 2, 4, 8 and 16 mg/mL) was added to 200 μL of ABTS working solution. The mixture was incubated in the dark at room temperature for 5 min and the absorbance at 734 nm was read. 80% (v/v) methanol was used instead of the samples for the blank control. ABTS scavenging activity was determined as follows: ABTS scavenging activity = $(1\text{-}A_s/A_b) \times 100\%$, where A_b is the absorbance of the 80% (v/v) methanol reacted with ABTS, and As is the absorbance of the different samples reacted with ABTS. The calibration curve was drawn with different concentrations of Trolox as the horizontal coordinate (x) and ABTS scavenging activity as the vertical coordinate (y) ($y = 0.0056x + 0.0058$; $R^2 = 0.9995$). The Trolox calibration curve (0 ~ 160 μmol/L) was used to quantify antioxidant activity allowing for the expression of the ability to radical scavenging activity of unfermented and fermented soybeans as μmol Trolox equivalents per gram.

Intracellular reactive oxygen species (ROS) scavenging activity was assayed according to Xu et al. (44) in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages with modifications. RAW 264.7 macrophages were inoculated (25,000 cells/well) in 96-well plates and allowed to develop for 24 h. Cells were then exposed to 1.5 μL of unfermented and fermented soybeans (ranging from 0 to 40 μg/mL) and 200 μL of culture media including 1 μL/mL LPS for 24 h. As a negative control, cell culture media without soybeans was added. After incubation, each well received 200 μL of 2′,7′-dichlorofluorescein diacetate DCFH-DA solution (10 μM), which was then incubated for 30 min at 37°C in an incubator with 5% CO_2. Fluorescence intensities of each well were measured at 485 and 528 nm using a Synergy H_1 microplate reader (BioTek, USA). The percentage generation of ROS in comparison to the negative control was calculated based on the value of F485/528. ROS% = (The value of sample)/(The value of negative control) × 100%. After fluorescence intensity evaluation, the dye was disposed of and the cells were rinsed twice with ice-cold PBS. Subsequently, fluorescence images were rapidly captured using a green fluorescent protein (GFP) channel at a 20 × magnification on an EVOS M7000 system (Thermo Fisher Scientific, Waltham, MA).

2.2.5 Analysis of metabolites

To extract metabolites, 50 mg of sample and 400 μL of 80% (v/v) methanol containing 0.02 mg/mL of an internal standard (L-2-chlorophenylalanine) were added to a 1.5 mL centrifuge tube (45). After 30 s of vortex mixing, the samples were sonicated for 30 min at 5°C. Subsequently, the samples were kept at −20°C for 30 min to precipitate the proteins. The materials were then centrifuged for 15 min at 4°C and 13,000 g and the supernatant was collected for non-targeted metabolome analysis (46). A quality control (QC) sample was prepared by mixing all samples to be a pooled sample.

The LC-MS/MS analysis was conducted using the SCIEX UPLC-Triple TOF 5600 system with an ACQUITY HSS T3 column (100 mm × 2.1 mm i.d., 1.8 μm; Waters, USA) at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The injection volume was 10 μL. The mobile phases comprised 0.1% formic acid in water: acetonitrile (95:5, v/v) (solvent A) and 0.1% formic acid in acetonitrile: isopropanol: water (47.5:47.5:5, v/v/v) (solvent B). The flow rate was 0.40 mL/min, and the column temperature was maintained at 40°C. The Ultra Performance Liquid Chromatography (UPLC) system was coupled to a quadrupole-time-of-flight mass spectrometer (Triple TOFTM 5600+, Sciex, USA) equipped with an electrospray ionization (ESI) source operating in both positive and negative modes. The optimal conditions were set as follows: source temperature at 550°C; curtain gas (CUR) at 30 psi; Ion Source Gas1 and Gas2 at 50 psi each; ion-spray voltage floating (ISVF) at −4,000V in negative mode and 5,000 V in positive mode; declustering potential at 80 V; collision energy (CE) set to 20–60 eV rolling for MS/MS. Data acquisition was performed using the Information Dependent Acquisition (IDA) mode, and detection was carried out over a mass range of 50–1,000 m/z.

2.2.6 Statistical analysis

All results of experiments conducted in triplicate were presented as mean ± standard deviation (SD). Data were analyzed using SPSS software (Inc., Chicago, IL, USA) employing one-way ANOVA and T-test with a confidence interval of 95 % ($P < 0.05$) for means. Regular graphs and tables were made in excel. The graphs in metabolomics were made through the free online platform of majorbio choud platform (www.cloud.majorbio.com).

Principal component analysis (PCA) was used to determine the degree of metabolite difference between different samples and the degree of difference within groups. The difference of specific metabolites before and after fermentation was observed through the analysis of metabolite cluster heat map. Orthogonal partial least squares discriminant analysis (OPLS-DA) was carried out to determine the differences in non-volatile metabolites between unfermented and fermented soybeans. Variable importance in the projection (VIP) ≥ 1, fold change (FC) ≥ 2 or ≤ 0.5 and $P < 0.001$ were used as screening conditions to identify differential metabolites. Spearman correlation analysis was performed for the difference metabolites and antioxidant activity after SSF.

TABLE 1 General composition indicators in unfermented soybeans (USB) and fermented soybeans (FSB)[1].

Item (%)	USB	FSB
Protein	35.47 ± 0.14^{b}	45.33 ± 0.11^{a}
Lipid	19.38 ± 0.31^{b}	20.34 ± 0.28^{a}
Soluble dietary fiber	5.64 ± 0.05^{a}	2.82 ± 0.09^{b}
Insoluble dietary fiber	13.87 ± 0.15^{a}	9.98 ± 0.11^{b}
Reducing sugar	0.94 ± 0.00^{b}	3.97 ± 0.07^{a}
Starch	16.62 ± 0.15^{a}	11.23 ± 0.16^{b}

[1]Different lowercase letters on the same line were significantly different ($P < 0.05$).

3 Results and discussion

3.1 Basic characteristic of fermented soybeans by *P. ostreatus*

Pleurotus species have the ability to extensively degrade a wide variety of substrates to release small molecules from carbohydrates and other macromolecular nutrients of soybeans. This is achieved by secreting the requisite extracellular oxidative (ligninolytic) and hydrolytic (cellulases and hemicellulases) enzymes to liberate low-molecular-weight molecules that can be absorbed for their nourishment (47). The soybeans were inoculated with mycelia of *P. ostreatus,* and cultured at 25 ± 0.1°C. The mycelium grew slowly and filled the entire bottle within 33 days. The general composition of fermented soybeans by *P. ostreatus* is shown in Table 1. Compared to the unfermented soybeans, the starch and dietary fiber (DF, including soluble and insoluble dietary fiber) in fermented soybeans were consumed during the growth of *P. ostreatus* mycelia, reducing the starch and DF content from 16.62% to 11.23% and from 19.51% to 12.8%, respectively. Consequently, the reduced sugar content increased from 0.94% to 3.97%. Oyster mushrooms are capable of enzymatically breaking down a variety of substrates that comprise dietary fiber and starch into low molecular weight soluble chemicals. Amylase is the enzyme that breaks down starch, while cellulase, xylanase, and laccase are the three main groups that break down dietary fiber. Lim et al. investigated the activities of amylase (EC 3.2.1.1), cellulase (EC 3.2.1.4), laccase (EC 1.10.3.2), and xylanase (EC 3.2.1.8) in discarded *P. ostreatus* mushroom compost. The results showed that amylase, cellulase, xylanase, and laccases activity were 2.97 U/g, 1.67 U/g, 91.56 U/g, and 2.97 U/g, respectively (48). Since the biomass of *P. ostreatus* increased throughout growth, the fermented soybeans' crude protein and lipid contents increased by 27.80% and 5.0%, respectively, compared to the unfermented soybeans, reaching 45.33% and 20.34% after fermentation (Table 1).

Certain solid fermented products showed comparable tendencies. Zhai et al. (36) found that adding *Agaricus blazei* to wheat, rice, oats, maize, millet, broomcorn millet, and sorghum during fermentation increased the amount of reducing sugar by 100.77, 7.12, 12.35, 10.85, 57.55, and 58.68 times, respectively, compared to the control. The solid-state fermentation of tempeh resulted in a 21.7% increase in protein content as well as an

increase in essential amino acids content, particularly amino acids containing sulfur (49).

Microbial enzymes cause the free phenolic compounds to break free from their bound form (50). Phenolic compounds, which serve as metal chelators, hydrogen donors, reducing agents (free radical terminators), and singlet oxygen quenchers, constitute the majority of antioxidants. The antioxidative activity of food is highly associated with the total phenolic compounds (3, 51). Phenolic compounds found in soybean consist of phenolic acids (including ferulic acid, p-coumaric acid, vanillic acid, chlorogenic acid, caffeic acid, syringic acid, salicylic acid, protocatechuic acid, etc.), isoflavones (daidzein, glycitein, genistein and their glucosides) and anthocyanins (52). Phenolic compounds such as phenolic acids (gallic, caffeic, and p-coumaric acids, benzoic acid and cinnamic acid derivative), flavonoids (myricetin, rutin, naringenin, quercetin, morin, and hesperetin), coumarins, quinones, and styrylpyrones also exist in oyster mushrooms (53). Additionally, during fermentation, legumes produce more beneficial phenolic compounds because the microorganisms secreting ligninolytic and carbohydrate-metabolizing enzymes hydrolyze the phenolic glycosides to release free aglycones (51, 54). Therefore, it is assumed that the application of oyster mushroom (*P. ostreatus*) mycelium fermentation can greatly increase the content of phenolic compounds in soybean. In this study, the TPC of the unfermented soybeans was 4.62 mg/g. After SSF, the TPC of the fermented soybeans increased to 20.72 mg/g, marking a 4.47-fold increase compared to the control ($P < 0.05$) (Figure 1A). The fermentation of oyster mushroom mycelium led to a significant increase in total phenolic compounds, surpassing the rise observed in other fermented soybean products. For instance, when black beans were solid-state fermented using various Generally Recognized as Safe (GRAS) filamentous fungi, particularly *Aspergillus awamori*, the TPC of fermented soybeans increased from approximately 16 to 27.2 mg gallic acid equivalent/g (55). Similarly, employing *B. subtilis* BCRC 14715 during solid state fermentation resulted in an increase in the TPC of black soybean methanolic extract from 17.75 to 22.66 mg gallic acid equivalent/g (56). Furthermore, the TPC of Douchi was found to be 75.5% higher than that of unfermented soybean (57). TPC levels in commercially fermented soybean products from China with the highest TPC concentration being 12.29 ± 1.21 (mg GAE/g) were lower than the TPC level in this study (57). Nevertheless, Shukla et al. observed TPC values (22.2 30.6 mg GAE/g) in Korean fermented soybean paste, which were comparatively higher than the results of our analysis (58).

3.2 Assay of antioxidant activity of fermented soybeans by *P. ostreatus*

Different antioxidant methods have been introduced to measure the antioxidant capacity of antioxidants in order to meet the assays for both hydrophilic and lipophilic antioxidants as well as the use of different radical sources. These methods can be separated into two categories: single electron transfer (SET) and hydrogen atom transfer (HAT) methods based on the reaction processes (59). The 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay is a widely utilized technique that provides an initial method for measuring antioxidant activity (60). It is now widely accepted that the direct HAT mechanism mediates the interaction between DPPH and phenols (59). The ABTS scavenging assay can be used with hydrophilic or lipophilic compounds. The reactions with ABTS radicals involve both HAT and SET mechanisms, in contrast to the HAT-only reactions with DPPH radicals (59, 61). The use of cell models to investigate a compound's antioxidant activity has been expanding in prominence since they allow for a more realistic biological environment (62). Using a combination of DPPH scavenging assay, ABTS scavenging assay, and cellular ROS assays in this study, the antioxidant activity of the fermented soybean methanolic extracts were assessed in comparison to the unfermented soybeans. The results are displayed in Figures 1–3. In terms of scavenging DPPH radicals, the fermented soybeans exhibited a value of 20.03 μmol TE/g. It was 3.92 times greater than that of the unfermented soybeans, which had a value of 5.11 μmol TE/g (Figure 1B). The DPPH scavenging activity of fermented soybeans increased gradually at concentrations of 0.5∼4 mg/mL, and at each dose, the efficiency of scavenging radicals was higher than that of the unfermented soybeans (Figure 1C). The DPPH value in this study was similar to the highest values (20.58 ± 0.60 μmol TE/g) that have been documented for Chinese commercially fermented soybeans (57). Consistent with the finding of this study, DPPH radical scavenging activities of soybeans fermented by basidiomycetes (*Ganoderma lucidum*, *Hericium ramosum* and *Hericium erinaceus*) were significantly higher than those of unfermented soybeans (63). After fermentation using a single culture of *Rhizopus oligosporus*, the DPPH scavenging activity of soybeans increased dramatically, with antioxidants soluble in water and ethanol increasing by 4.3 and 3.7 times, respectively, compared to unfermented soybeans (64). In the ABTS assays, unfermented soybeans exhibited a scavenging activity of 15.98 μmol TE/g for ABTS radicals, while following SSF, the scavenging activity of fermented soybeans for ABTS radicals increased by 2.13-fold to 34.09 μmol TE/g (Figure 1D). The level of ABTs in the Douchi fermented soybean product with edible mushroom for 30 days, however, was less than the amount in this study, at around 28 μmol TE/g (65). The effectiveness of scavenging ABTS radicals was higher for fermented soybeans at each dose compared to unfermented soybeans, and the ABTS scavenging activity of the fermented soybeans increased progressively at concentrations of 2∼8 mg/mL (Figure 1E). Soybeans fermented by *Cordyceps militaris* also demonstrated a significant scavenging effect against DPPH and ABTS radicals in a dose-dependent manner at each concentration level (66). Similarly, the scavenging activities of DPPH and ABTS increased in CKJ (*cheonggukjang*) (67). The elevated levels of DPPH and ABTS corroborated the findings of a prior study (65), which reported that after 30 days of fermentation, fermented soybeans exhibited a greater capacity for antioxidants than raw soybeans. Through metabolism and modification, phenolic chemicals become more bioavailable, which also helps to boost antioxidant activity during fermentation (3). It is widely acknowledged that significant antioxidant activities of food products are related to TPC (68). However, fungal fermentation is a complex metabolic process that can yield various antioxidant compounds, including phenolic compounds, antioxidant peptides

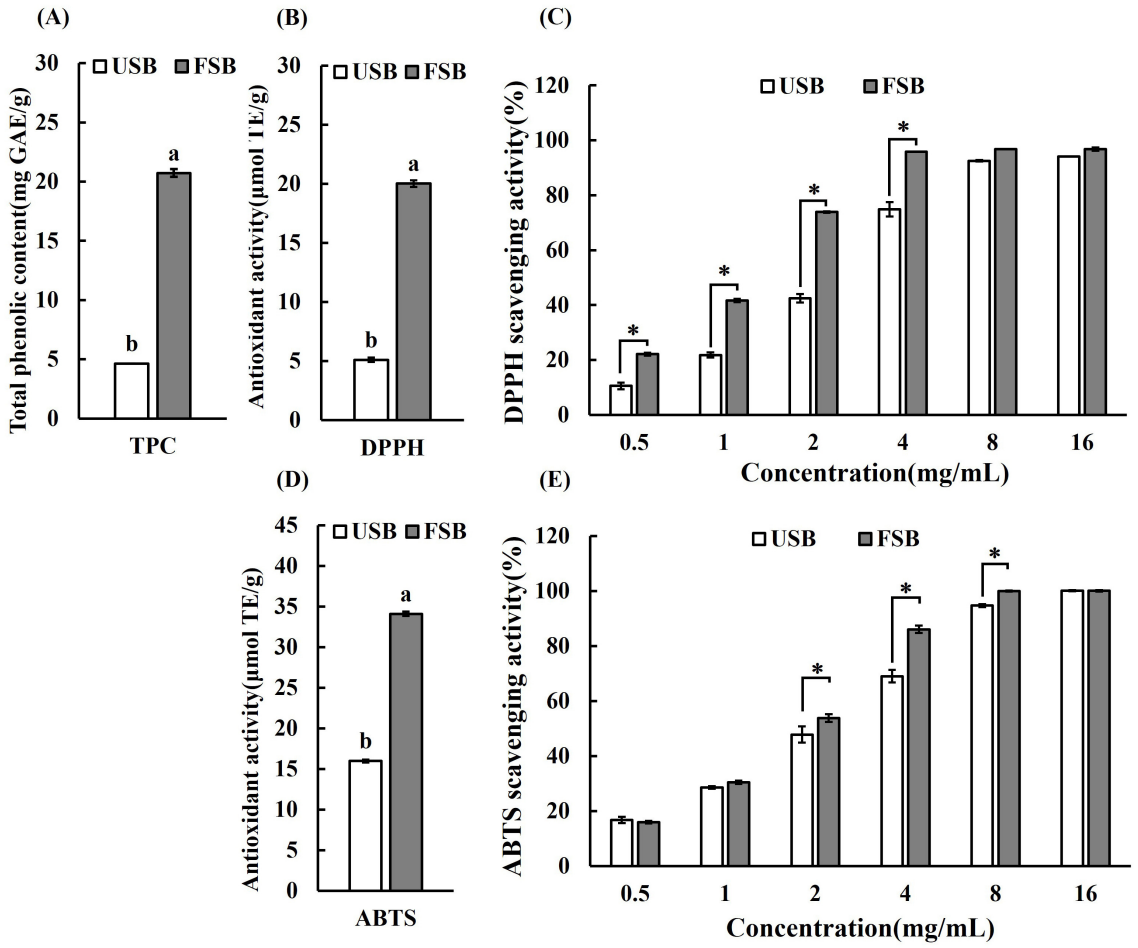

FIGURE 1
The total phenolic contents and the antioxidant capacity of unfermented soybeans (USB) and fermented soybeans (FSB) by P. ostreatus mycelium. **(A)** The total phenolic contents (TPC), **(B)** The results of DPPH (expressed in μmol TE/g), **(C)** DPPH radical scavenging activity, **(D)** The results of ABTS (expressed in μmol TE/g), **(E)** ABTS radical scavenging activity. Values are expressed as mean ± SD ($n = 3$). Bars with different lowercase letters represent significant difference ($P < 0.05$). Bars with * represent significant difference between USB and FSB under the same concentration ($P < 0.05$).

and exopolysaccharides (EPS) (69). Furthermore, 80% methanol extracts may contain a variety of materials with antioxidant activity that function in different ways. The results further demonstrated that fermented soybeans possessed greater antioxidant capacities than unprocessed soybeans (57).

ROS are generated when macrophages are attacked by LPS. ROS play a role in controlling gene expression and activating cell signaling when macrophages participate in host cell defense mechanisms (70). In this study, intracellular ROS levels were measured using DCFH-DA, and the results are shown in Figure 2. The cell viability of RAW 264.7 macrophages did not significantly differ between the 0–40 μg/mL fermented soybeans under LPS induction (Data not shown). The ROS fluorescence intensity significantly decreased with the addition of 5 μg/mL fermented soybeans compared to unfermented soybeans indicating that fermented soybeans could inhibit the generation of ROS (Figure 2). Fluorescence images of fermented soybeans also confirmed this result (Figure 3). However, higher concentrations of unfermented soybean and fermented soybean extracts did not differ significantly in the production of ROS. This finding is consistent with previous reports (70), which found that black bean steamed liquid lyophilized product reduced ROS levels in LPS-induced RAW 264.7 macrophages. Therefore, fermented soybeans within a certain concentration can prevent macrophages from expressing ROS to mitigate intracellular oxidative stress.

Despite differing views among the scientific community, the methods used to evaluate antioxidant activity *in vitro* are inexpensive, simple to use, don't require extremely sensitive equipment, and aid in the planning of upcoming bioactive chemical studies (71). Considering the results of this study cannot be instantly applied to the "prevention, treatment, or cure" of non-communicable diseases in humans, the mechanisms of action behind the apparent antioxidant activity of fermented soybean *in vivo* must be elucidated.

3.3 Analysis of metabolite differences of fermented soybeans by *P. ostreatus*

The primary and secondary metabolites produced by microorganisms during fermentation change the properties

of fermented foods. Therefore, monitoring small molecule metabolites in fermented foods using metabolomics is crucial for evaluating their functional properties (72). PCA, based on multivariate statistics, revealed the overall differences in metabolites of unfermented and fermented soybeans. The PCA scores of samples (including QC samples) are shown in Figure 4A. In the cationic mode, the first and second principal components explained 80.1% and 12.6% of the total variation, respectively. All QC samples were tightly clustered together, indicating good analytical stability and experimental repeatability (73). The results indicated a relative aggregation among the three parallel samples of the same treatment, with no significant difference, while two distinct clusters were observed in unfermented soybeans and fermented soybeans. This clear distinction between soybeans before and after fermentation suggests that their metabolism undergoes significant changes. This finding aligns with a previous study (74), indicating that fermentation has a significant effect on metabolites. The highlighted metabolites that contributed to the metabolic changes were further investigated using the OPLS-DA approach (Figure 4B).

OPLS-DA analyses were carried out to determine the differences in non-volatile metabolites between unfermented and fermented soybeans. The VIP (Variable Importance in Projection), fold change (FC) and *P*-value were utilized to screen for differential metabolites. The VIP value generated from the OPLS-DA model serves as a crucial indicator, filtering out the variables that contribute significantly to the model. It is generally believed that metabolites with a VIP value of $\geq$ 1 are deemed significantly different. FC represents the extent of difference in substance expression between two groups of samples; a FC of $\geq$ 2 indicates up-regulation, and a FC of $\leq$ 0.5 indicates down-regulation. A $P < 0.001$ indicates a significant difference. Therefore, metabolites with VIP values $\geq$ 1, FC values $\geq$ 2 or $\leq$ 0.5, and $P < 0.001$ were considered significantly different (75). Previous research suggests that carbohydrates, amino acids, organic acids, nucleotides, lipids, flavonoids, phenolic acids, alkaloids, terpenoids, tannins, coumarins and lignin are among the main differential metabolites following SSF (65, 74, 76). In this study, a total of 154 different metabolites were identified after fermentation, consisting of 122 up-regulated metabolites, and 32 down-regulated metabolites (Figure 4C). These metabolites included 40 amino acids, peptides, and analogs; 32 others; 24 lipids; 21 phenolic compounds; 13 carbohydrates and carbohydrate conjugates; 11 terpenoids; 6 nucleosides, nucleotides, and analogs; 4 organic acids and 3 alkaloids (Figure 4D). After SSF, amino acids, carbohydrates and their derivatives, and phenolic compounds emerged as the main differential metabolites, which are related to the whole complex growth metabolism during fungal fermentation, including the synthesis of amino acids, the release of active peptides, the degradation of cellulose, the hydrolysis of bound phenols, and the generation of new active ingredients (77). *P. ostreatus* may grow in almost any organic substrate generated by our food production system (78), So *P. ostreatus* also grew in soybean and degraded protein, carbohydrate, lipid, and other components

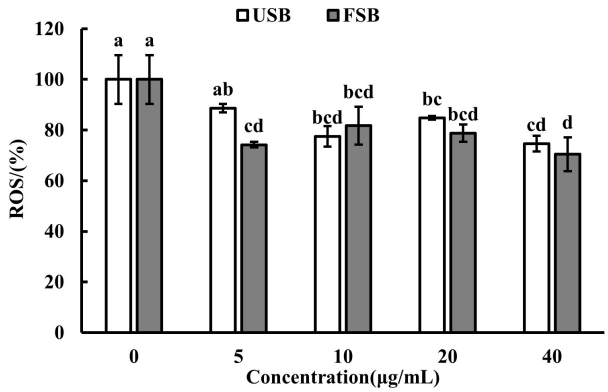

FIGURE 2
Reactive oxygen species (ROS) levels of LPS-induced RAW 264.7 macrophages treated by unfermented soybeans (USB) and fermented soybeans (FSB). Values are expressed as mean ± SD (n = 3). Bars with different lowercase letters represent significant difference ($P < 0.05$).

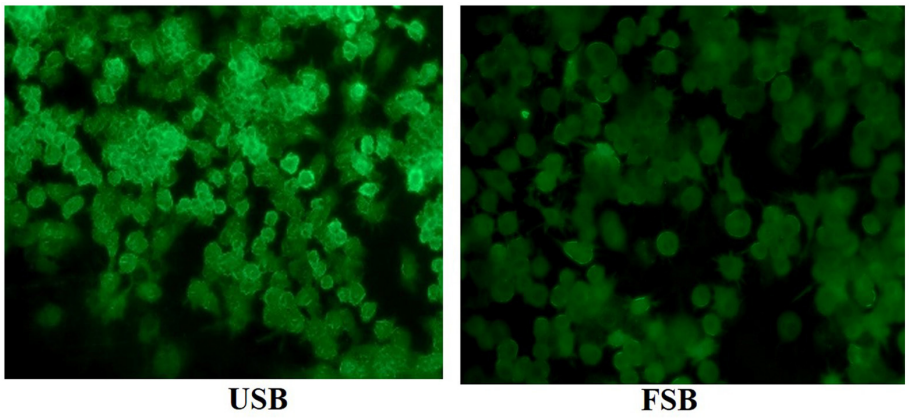

FIGURE 3
Fluorescence images of LPS-induced RAW 264.7 macrophages treated with methanol extracts (5 μg/mL) of unfermented soybeans (USB) and fermented soybeans (FSB).

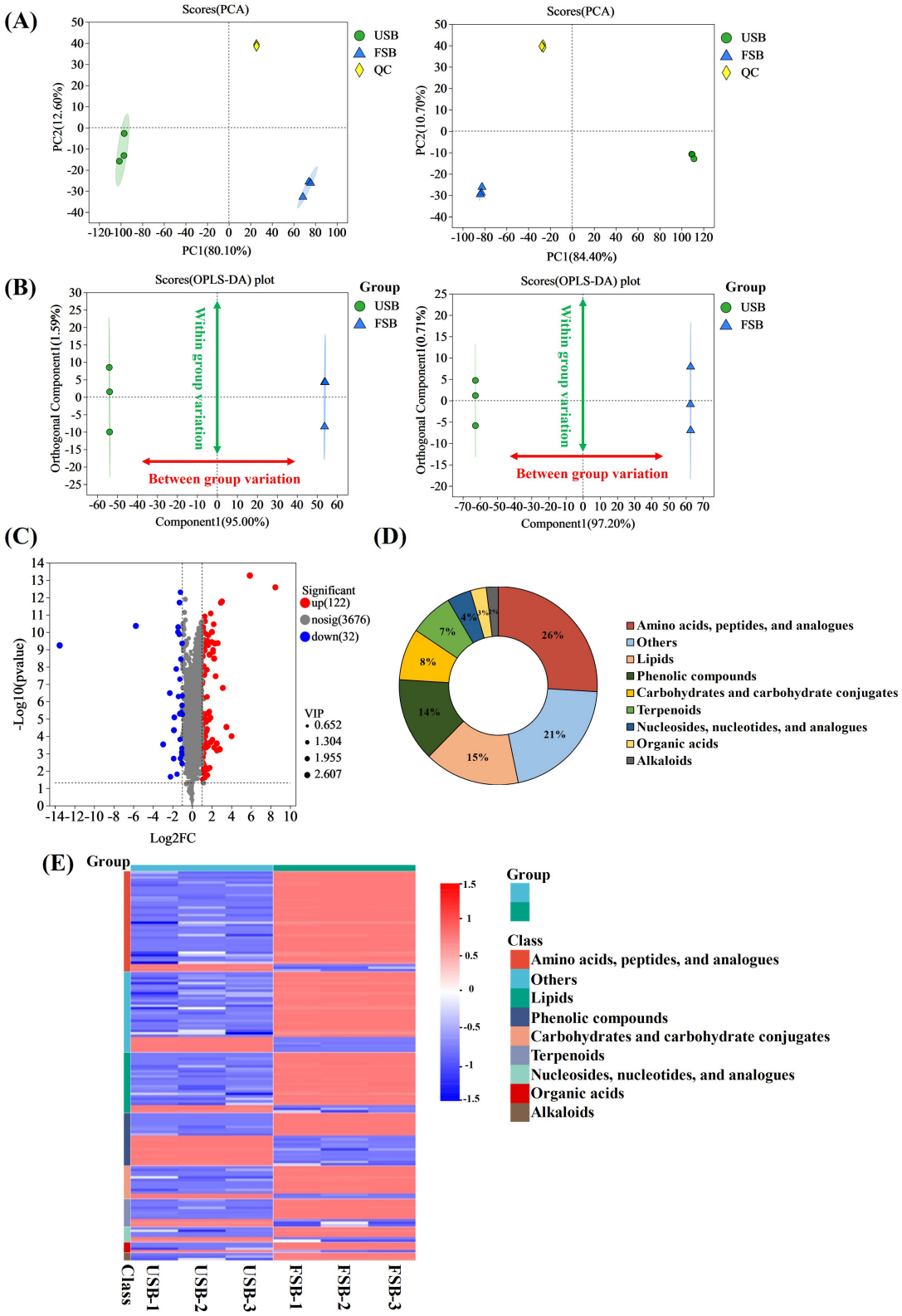

FIGURE 4
Analysis of different metabolites between unfermented soybeans (USB) and fermented soybeans (FSB). **(A)** Principal component analysis, **(B)** Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA), **(C)** Volcano plots of different metabolites, **(D)** Classification of different metabolites, **(E)** Clustering heatmaps of different metabolites were carried out to determine the differences in non-volatile metabolites between unfermented and fermented soybean samples. By VIP $\geq$ 1, FC $\geq$ 2 or $\leq$ 0.5 and $P < 0.001$ were used as screening conditions to identify differential metabolites.

of soybean into small molecular compounds including amino acids, peptides, carbohydrates and free fatty acids to survive. On the other hand, *P. ostreatus* is an outstanding microbial cell manufacturer that produces secondary metabolites. The mycelium of six species of the genus Pleurotus were discovered to contain a wide variety of secondary metabolites including phenolic compounds, cinnamic acid, phenylalanine, indole derivatives, sterols, terpenoids, lovastatin, and ergothioneine (79, 80). To better understand the non-volatile metabolites of soybeans during fermentation, a heatmap (Figure 4E) was employed as the basis for hierarchical cluster analysis (HCA). Consistent with the PCA results, the results indicated that the three biological replicates were clustered together because of the relative contents of non-volatile metabolites.

Notably, we discovered that N-Jasmonoylisoleucine (JA-Ile) was 2.49 times more abundant in fermented soybeans than in unfermented soybeans (Supplementary Table S1). The jasmonate signal molecule JA-Ile is generated from polyunsaturated fatty acids (81), and plays primary roles in regulating numerous ecological interactions with biotic and abiotic environments. JA-Ile could induce the biosynthesis of diverse secondary metabolites such as terpenoids, phenylpropanoids, phenols and alkaloids (82, 83). It was reported that the defense hormone jasmonate regulation in *P. ostreatus* triggered the mycelial production of terpenoids and steroids in response to fungivore attacks (84). In this study, JA-Ile-mediated core signaling may also play a key role in regulating the biosynthesis of a wide variety of secondary metabolites by solid-state fermentation of soybeans using *P. ostreatus*.

3.4 Analysis of different metabolites and correlation with antioxidant capacities

The Correlation between metabolites and antioxidant capacity are presented in Figure 5. As shown in Figure 5, 58 metabolites showed positive correlations with the antioxidant activity of the fermented soybean, while 23 metabolites had negative associations.

3.4.1 Amino acids, peptides, and analogs

In the OPLS-DA analysis comparing fermented and unfermented soybeans, 40 differential metabolites of amino acids, peptides, and analogs were identified. Among them, amino acids, peptides and analogs are the most varied metabolites (Supplementary Table S1). During soybean fermentation, several oligopeptides were released through proteolytic processes involving the breakdown by proteolytic enzymes generated from *P. ostreatus* mycelium. It had been reported that the hydrophobic amino acid especially phenylalanine (Phe) was exposed to hydroxyl radicals and converted to tyrosine to scavenge hydroxyl radicals or reduce metal cations (85). Higher antioxidant activity was also attributed to the aromatic amino acids tyrosine (Tyr), tryptophan (Trp), cysteine (Cys) and methionine (Met), which could supply electrons or hydrogen atoms (86). The hydrophobic amino acids may help reduce peroxidation by making peptides more soluble in lipids and improving their ability to interact with radical species (87). Tyr is produced when Phe is exposed to hydroxyl radicals, and Tyr exhibits the hydroxyl radical's scavenging ability (88). Pro increased antioxidant enzyme activities such as ascorbate peroxidase and peroxidase (89). Pro-based peptides are widely recognized for their essential roles in signal transduction pathways and their antibacterial, immunomodulatory, and antioxidant characteristics, among other health-promoting activities (90, 91). Correlation analysis thus revealed the increased antioxidant activities of dipeptides and other oligopeptides that comprised hydrophobic amino acids, aromatic amino acids, and Pro (Figure 5A). Dipeptides could be fully absorbed from the intestinal lumen into the bloodstream, which resulted in the expression of biological actions at the tissue levels (92). Dipeptides through fermentation exhibited antioxidant activity and angiotensin I-converting enzyme suppressive behavior (93, 94). Dipeptides associated with antioxidant activity including phenylalanine-phenylalanine (Phe-Phe), phenylalanine-isoleucine (Phe-Ile), tryptophan-aspartate (Try-Asp), hydroxyprolyl-tryptophan, γ-Glutamylhydroxyproline and γ-Glutamylproline were among the metabolites contributing mainly to the observed discrimination in this study (Figure 5A). The amounts of dipeptides containing Phe, Phe-Phe, and Phe-Ile in the fermented group increased significantly ($P < 0.001$) in comparison to the unfermented group, reaching values 6.09 times and 2.86 times higher, respectively (Table 2). Additionally, the results of the correlation assay indicated that the other oligopeptides containing hydrophobic and aromatic amino acids might have antioxidant qualities (Figure 5A). On the contrary, the contents of cyclosquamosin G and Tyr-Pro-Thr-Val-Asn declined after fermentation, and there was negative correlation between their antioxidant activity and content (Table 2, Figure 5A). The results made in this study require additional verification as there are currently no studies on the relationship between these two chemicals and antioxidant activities.

3.4.2 Lipids

Lipids are essential components for cells and have been demonstrated to play a vital role in foods undergoing microbial fermentation (95). In this study, the lipid content in the fermented soybeans increased from 19.38% to 20.34% (Table 1), indicating the generation of more lipids during SSF. The variations in phospholipids during fermentation could lead to modifications in the structure of cellular membranes, influencing various biological functions associated with phospholipids (96). In this study, the levels of CDP-DG[a-13:0/20:4(6E,8Z,11Z,13E)-2OH(5S,15S)] and PC(2:0/PGJ2) significantly increased after soybean fermentation compared to the unfermented soybeans, while the levels of CDP-DG(16:0/6 keto-PGF1α) and PC[20:5(5Z,8Z,10E,14Z,17Z)-OH(12)/2:0] decreased ($P < 0.001$). Enzymes are known to hydrolyze phospholipids into LPC, LPE and fatty acids (65, 75). In addition, the content of Lyso PE [0:0/16:1(9Z)] increased significantly, reaching a 2.57- fold increase compared to unfermented soybeans after fermentation (Supplementary Table S1). According to the results, fermentation significantly impacted soybean free fatty acid such as 3-Hydroxysebacic acid, 3-Methyl-5-pentyl-2-furantridecanoic acid and dihydrowyerone acid ($P < 0.001$) (Table 2). Correlation analysis showed that the contents of these three fatty acids were

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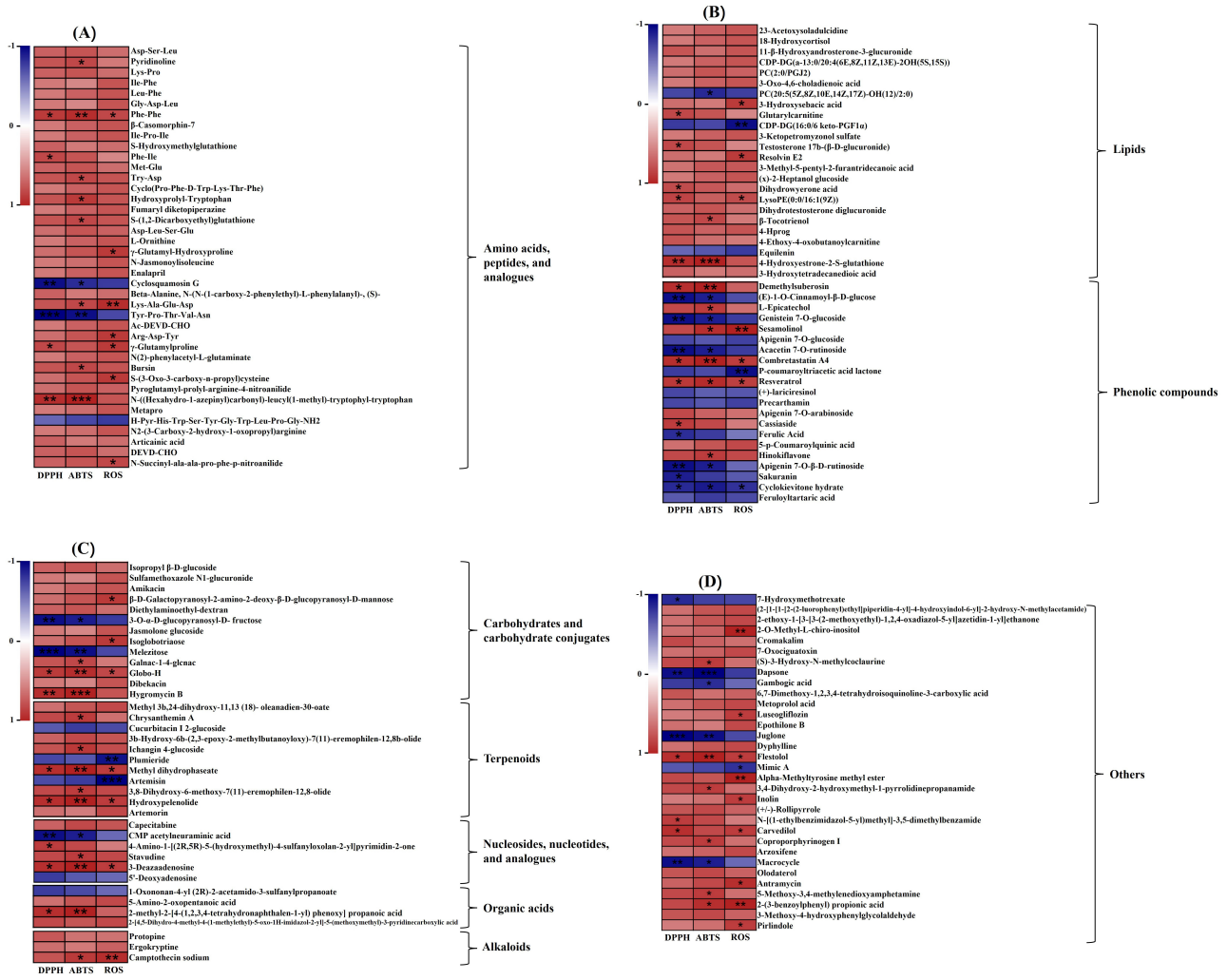

FIGURE 5
The heatmaps of the correlation analysis between 154 differential metabolites and antioxidant activity. **(A)** Amino acids, peptides, and analogs, **(B)** lipids and phenolic compounds, **(C)** carbohydrates and carbohydrate conjugates, terpenoids, nucleosides, nucleotides, and analogs, organic acids and alkaloids, **(D)** others (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

positively correlated with antioxidant activity (Figure 5B). The unsaturated fatty acids showed a high sensitivity to oxidation and were easily peroxidized by OH-attack. Polyunsaturated fatty acid peroxidation produces isoprostanes (97), and their concentrations are thought to be a good indicator of oxidative stress (98). It remains to be confirmed whether the three fatty acids found in this study have the aforementioned effects. Rong et al. (65) discovered a relationship between free fatty acids and douchi's antioxidant activity, which is in agreement with the results of this investigation.

Notably, upregulated sterol derivatives contained 18-Hydroxycortisol, 11-β-Hydroxyandrosterone-3-glucuronide, 3-Oxo-4,6-choladienoic acid, 3-Ketopetromyzonol sulfate and testosterone 17b-(β-D-glucuronide), dihydrotestosterone diglucuronide and 23-Acetoxysoladulcidine in the metabolites of lipids (Supplementary Table S1). Ergosterol abundant in oyster mushrooms (99), may serve as the precursor for the synthesis of these sterol derivatives during soybean fermentation by *P. ostreatus* mycelium. This could potentially explain the high lipid content observed in fermented soybeans. Among these sterol derivatives, testosterone 17b-(β-D-glucuronide) was thought to be associated with antioxidant activity of fermented soybean in this study (Figure 5B). Thus far, there has been no related research showing a connection between this compound and antioxidant activity; more experiments are necessary to validate this result. In this study, the results of correlation analysis showed that β-Tocotrienol and 4-Hydroxyestrone-2-S-glutathione were positively correlated with antioxidant activity in the fermented soybean (Figure 5B). Vitamins E and glutathione were considered that the powerful antioxidants for their ability to combat reactive oxygen and nitrogen species (100). β-Tocotrienol and 4-Hydroxyestrone-2-S-glutathione are the derivatives of vitamins E and glutathione, which could explain the antioxidant activity of these two compounds.

3.4.3 Phenolic compounds

The phenolic compounds including phenolic acids, coumarins, lignans, chalcones, avonoids, lignins, and stilbenes are the secondary metabolism of most fungus and plants, which are

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TABLE 2 58 differential metabolites related to antioxidant activity of fermented soybeans.

Category	Metabolite	VIP	*P* value	Abundance		FC	M/Z
				USB[a]	FSB[b]		
Amino acids, peptides, and analogs	Pyridinoline	2.36	2.01E-12	0.82 ± 0.01	6.28 ± 0	7.62	427.18
	Phe-Phe	2.25	4.32E-10	0.98 ± 0.02	5.95 ± 0.01	6.09	311.14
	Phe-Ile	2.15	3.94E-05	2.17 ± 0.35	6.2 ± 0.01	2.86	279.17
	Try-Asp	2.11	8.37E-12	1.61 ± 0.01	5.96 ± 0	3.71	318.11
	Hydroxyprolyl-Tryptophan	2.08	6.76E-04	0.92 ± 0.79	5.27 ± 0.02	5.75	298.12
	S-(1,2-Dicarboxyethyl) glutathione	2.03	9.48E-11	3.22 ± 0.01	7.27 ± 0.01	2.26	422.09
	γ-Glutamyl-Hydroxyproline	2.02	1.23E-09	2.65 ± 0.01	6.21 ± 0.02	2.35	584.22
	Cyclosquamosin G	2.01	3.61E-09	6.4 ± 0.02	2.88 ± 0.02	0.45	846.42
	Lys-Ala-Glu-Asp	1.99	6.57E-07	3.09 ± 0.12	6.97 ± 0	2.25	460.21
	Tyr-Pro-Thr-Val-Asn	1.97	1.17E-03	6.72 ± 0.03	3.23 ± 0.73	0.48	593.29
	Arg-Asp-Tyr	1.95	3.56E-03	2.97 ± 0.98	6.45 ± 0.03	2.17	453.21
	γ-Glutamylproline	1.94	4.67E-06	3.65 ± 0.19	7.36 ± 0	2.02	243.10
	Bursin	1.89	6.34E-11	2.97 ± 0.01	6.47 ± 0.01	2.18	374.17
	S-(3-Oxo-3-carboxy-n-propyl) cysteine	1.88	2.92E-08	2.98 ± 0.05	6.46 ± 0.01	2.17	220.03
	N-((Hexahydro-1-azepinyl)carbonyl)-leucyl(1-methyl)-tryptophyl-tryptophan	1.87	7.60E-10	2.71 ± 0.01	6.15 ± 0.02	2.27	656.29
	N-Succinyl-ala-ala-pro-phe-p-nitroanilide	1.75	3.10E-02	2.91 ± 1.66	6.03 ± 0.01	2.07	642.29
Lipids	PC[20:5(5Z,8Z,10E,14Z,17Z)-OH(12)/2:0]	2.04	9.93E-11	6.44 ± 0.01	2.35 ± 0	0.37	644.33
	3-Hydroxysebacic acid	2.04	6.46E-05	3.12 ± 0.36	6.76 ± 0.02	2.17	260.15
	Glutarylcarnitine	2.01	1.01E-04	2.26 ± 0.39	5.78 ± 0.01	2.56	276.14
	CDP-DG(16:0/6 keto-PGF1α)	1.97	8.32E-04	6.6 ± 0.05	3.14 ± 0.66	0.48	534.76
	Testosterone 17b-(β-D-glucuronide)	1.95	6.83E-05	2.44 ± 0.38	6.17 ± 0.01	2.53	499.21
	Resolvin E2	1.93	5.11E-06	1.91 ± 0.17	5.16 ± 0.04	2.70	357.20
	Dihydrowyerone acid	1.86	1.62E-04	2.94 ± 0.43	6.35 ± 0.01	2.16	291.09
	LysoPE(0:0/16:1(9Z))	1.85	3.59E-04	2.17 ± 0.52	5.57 ± 0.02	2.57	472.24
	β-Tocotrienol	1.82	1.42E-02	2.31 ± 1.34	5.51 ± 0.05	2.39	393.31
	4-Hydroxyestrone-2-S-glutathione	1.75	9.58E-10	2.94 ± 0.01	5.95 ± 0.02	2.02	590.21
Phenolic compounds	Demethylsuberosin	2.61	5.48E-14	0.11 ± 0	6.78 ± 0	59.13	267.04
	(E)-1-O-Cinnamoyl-β-D-glucose	2.58	5.83E-10	5.79 ± 0.03	0 ± 0	0.00	621.22
	L-Epicatechol	2.36	2.62E-13	0.02 ± 0	5.5 ± 0	358.11	289.07
	Genistein 7-O-glucoside	2.34	4.52E-05	6.61 ± 0.04	1.82 ± 0.44	0.28	433.11
	Sesamolinol	2.22	3.75E-10	1.42 ± 0.01	5.72 ± 0.02	4.02	414.15
	Acacetin 7-O-rutinoside	2.17	5.09E-06	7.02 ± 0.02	2.93 ± 0.21	0.42	606.19
	Combretastatin A4	2.16	1.41E-09	1.37 ± 0.01	5.96 ± 0.03	4.36	677.26
	P-coumaroyltriacetic acid lactone	2.16	3.29E-07	5.76 ± 0.02	1.18 ± 0.12	0.21	252.04
	Resveratrol	2.12	3.52E-11	1.24 ± 0.01	5.66 ± 0.01	4.58	273.08
	Cassiaside	2.01	5.13E-06	1.81 ± 0.21	5.8 ± 0.01	3.21	385.09
	Ferulic acid	1.96	1.68E-06	6.53 ± 0.01	3.19 ± 0.13	0.49	177.05
	Hinokiflavone	1.90	4.33E-04	3.02 ± 0.58	6.61 ± 0.02	2.19	537.08
	Apigenin 7-O-β-D-rutinoside	1.85	5.18E-08	5.8 ± 0.06	2.43 ± 0	0.42	577.16
	Sakuranin	1.82	5.18E-07	5.2 ± 0.03	1.96 ± 0.09	0.38	447.13

(Continued)

TABLE 2 (Continued)

Category	Metabolite	VIP	*P* value	Abundance		FC	M/Z
				USB[a]	FSB[b]		
	Cyclokievitone hydrate	1.77	5.05E-04	6.02 ± 0.04	2.89 ± 0.53	0.48	803.26
Carbohydrates and carbohydrate conjugates	β-D-Galactopyranosyl-2-amino-2-deoxy-β-D-glucopyranosyl-D-mannose	2.28	4.64E-10	2.28 ± 0.01	6.78 ± 0.02	2.97	545.22
	3-O-α-D-glucopyranosyl-D- fructose	2.05	5.45E-06	7.3 ± 0.01	3.64 ± 0.2	0.50	365.11
	Isoglobotriaose	1.96	2.48E-09	2.9 ± 0.01	6.23 ± 0.02	2.15	520.23
	Melezitose	1.96	4.48E-10	6.48 ± 0.02	3.16 ± 0.01	0.49	527.16
	Galnac-1-4-glcnac	1.92	5.04E-11	3.04 ± 0.01	6.68 ± 0.01	2.19	405.15
	Globo-H	1.82	9.39E-09	3.18 ± 0.01	6.42 ± 0.03	2.02	574.20
	Hygromycin B	1.80	7.02E-10	3.02 ± 0.01	6.19 ± 0.02	2.05	572.23
Terpenoids	Chrysanthemin A	2.35	1.71E-12	0.75 ± 0.01	6.15 ± 0	8.24	615.25
	Ichangin 4-glucoside	2.08	4.56E-04	2.3 ± 0.63	6.12 ± 0.04	2.66	692.29
	Plumieride	2.05	2.19E-02	6.01 ± 0.02	1.3 ± 2.24	0.22	451.12
	Methyl dihydrophaseate	2.00	3.08E-10	2.04 ± 0.01	5.95 ± 0.02	2.92	317.14
	Artemisin	1.95	1.92E-03	6.09 ± 0.04	2.66 ± 0.82	0.44	301.09
	3,8-Dihydroxy-6-methoxy-7(11)-eremophilen-12,8-olide	1.93	5.40E-07	1.95 ± 0.11	5.59 ± 0.01	2.87	317.14
	Hydroxypelenolide	1.86	2.69E-08	2.42 ± 0.05	5.81 ± 0	2.40	289.12
Nucleosides, nucleotides, and analogs	Cmp-nana	2.13	1.97E-12	7.5 ± 0	3.06 ± 0	0.41	595.13
	4-Amino-1-[(2R,5R)-5-(hydroxymethyl)-4-sulfanyloxolan-2-yl]pyrimidin-2-one	2.01	8.16E-03	2.04 ± 1.35	5.83 ± 0.01	2.87	276.10
	Stavudine	1.97	1.24E-11	2.63 ± 0.01	6.44 ± 0	2.45	447.15
	3-Deazaadenosine	1.91	8.96E-09	2.78 ± 0.04	6.35 ± 0.01	2.28	301.07
Organic acids	2-methyl-2-[4-(1,2,3,4-tetrahydronaphthalen-1-yl) phenoxy] propanoic acid	1.96	2.37E-11	2.5 ± 0.01	6.29 ± 0.01	2.51	679.33
Alkaloids	Camptothecin sodium	2.01	6.63E-03	1.72 ± 1.42	5.95 ± 0.01	3.47	401.09
Others	7-Hydroxymethotrexate	2.38	4.41E-11	5.65 ± 0.02	0.11 ± 0	0.02	491.14
	2-O-Methyl-L-chiro-inositol	2.17	4.49E-04	1.26 ± 0.77	5.98 ± 0.01	4.73	387.15
	(S)-3-Hydroxy-N-methylcoclaurine	2.03	1.49E-10	1.87 ± 0.01	5.93 ± 0.01	3.17	336.12
	Dapsone	2.03	1.34E-08	5.29 ± 0.04	1.71 ± 0.02	0.32	519.11
	Gambogic acid	2.02	4.38E-06	6.57 ± 0.02	3.02 ± 0.18	0.46	646.34
	Luseogliflozin	2.00	8.17E-03	3.01 ± 1.33	6.76 ± 0.02	2.24	467.21
	Juglone	1.99	4.75E-07	6.76 ± 0.01	3.33 ± 0.1	0.49	207.07
	Flestolol	1.94	5.32E-06	2.81 ± 0.2	6.52 ± 0.01	2.32	372.16
	Mimic A	1.93	5.02E-13	6.46 ± 0	2.81 ± 0	0.44	565.21
	Alpha-Methyltyrosine methyl ester	1.93	2.75E-04	2.57 ± 0.53	6.26 ± 0.01	2.43	254.10
	3,4-Dihydroxy-2-hydroxymethyl-1-pyrrolidinepropanamide	1.92	1.62E-03	2.84 ± 0.76	6.16 ± 0.02	2.17	431.21
	Inolin	1.91	1.75E-02	2.05 ± 1.78	6.06 ± 0.01	2.96	366.13
	N-[(1-ethylbenzimidazol-5-yl)methyl]-3,5-dimethylbenzamide	1.89	7.92E-05	2.66 ± 0.33	5.79 ± 0.03	2.18	308.18
	Carvedilol	1.88	8.05E-09	3.44 ± 0.03	6.9 ± 0.01	2.00	451.18
	Coproporphyrinogen I	1.85	9.21E-10	2.7 ± 0.01	6.04 ± 0.02	2.24	705.31
	Macrocycle	1.78	4.37E-10	6.18 ± 0.02	3.07 ± 0	0.50	551.15

(Continued)

TABLE 2 (Continued)

Category	Metabolite	VIP	*P* value	Abundance		FC	M/Z
				USB[a]	FSB[b]		
	Antramycin	1.72	8.74E-06	2.9 ± 0.18	5.83 ± 0	2.01	360.12
	5-Methoxy-3,4-methylenedioxyamphetamine	1.71	9.90E-10	2.07 ± 0.01	4.95 ± 0.02	2.39	254.10
	2-(3-benzoylphenyl) propionic acid	1.69	7.39E-03	1.94 ± 1.05	4.97 ± 0.02	2.56	299.09
	Pirlindole	1.62	6.27E-06	2.52 ± 0.14	5.09 ± 0.02	2.02	271.15

[a]USB represents unfermented soybeans.
[b]FSB represents fermented soybeans.

important phytochemicals with a broad range of antioxidant capacities (101). By supplying hydrogen atoms and generating antioxidant radicals, pheonlic compounds could react with free radicals and transform them into more stable products. These products were stabilized by the delocalization of the unpaired electron along the phenolic ring, which resulted in stable resonance hybrids (102). The majority of phenolic compounds found in unfermented soybeans are anthocyanins, phenolic acids (ferulic acid, *p*-coumaric acid, chlorogenic acid, caffeic acid, syringic acid, vanillic acid, salicylic acid, protocatechuic acid, etc.), and isoflavones (daidzein, glycitein, genistein, and their glucosides) (52). However, in this study, it was observed that coumarin, flavonoids, lignan and stilbene were the phenolic compounds that underwent the most significant changed during the solid-state fermentation of soybeans by *P. ostreatus* mycelium, rather than phenolic acids (Supplementary Table S1). A significant positive correlation was observed between antioxidant activity and the phenolic compounds including demethylsuberosin, L-Epicatechol, sesamolinol, combretastatin A4, resveratrol, hinokiflavone and cassiasside (Figure 5B). Coumarins exhibit a wide range of functions as antibacterial, anticancer, and anticoagulant agents (103). Demethylsuberosin, a coumarin, was found in fermented soybeans that had undergone fermentation at a level 59.12 times higher (Table 2). The presence of demethylsuberosin in *Angelica decursiva* and *Citrus aurantium* L. var. *amara* Engl was reported to be related to the ability to suppress lipid accumulation and lower blood pressure (104, 105). L-Epicatechol is the flavonoid compound with the largest FC among all the differential metabolites, and its content is 358.1 times that of unfermented soybeans. L-Epicatechol identified as a potent antioxidant compound in wild soybeans, with levels 1,750 times higher than those in cultivated soybeans (76). Sesamolinol, a lignan, significantly increased in concentration during fermentation, reaching levels 4.02 times higher than in unfermented soybeans. Sesamolinol has been reported to reduce oxidative stress and affect lipid metabolism in liver cells (106). In this study, the contents of combretastatin A4 and resveratrol in fermented soybeans were 4.36 times and 4.58 times higher than unfermented soybeans, respectively (Table 2). Combretastatin A4 and resveratrol are examples of stilbene substances. A powerful inhibitor of tubulin polymerization, combretastatin A4 has a significant suppressive effect on the development of tumor cells (107). Resveratrol possesses numerous biological properties and health benefits, including cardioprotective, neuroprotective, anti-inflammatory, anti-cancer, anti-diabetic, and antioxidant qualities (108, 109). Cassiaside is a phenolic glycoside analogous, and it was discovered that cassiaside exhibited hepatoprotective benefits and that the reduction in ROS generation in tert-butyl hydroperoxide induced HepG2 cells (110).

In the OPLS-DA analysis of the entire fermentation process, nine other flavonoids were among the differential phenolic compounds, including genistein 7-O-glucoside, apigenin 7-O-glucoside, acacetin 7-O-rutinoside, apigenin 7-O-arabinoside, hinokiflavone, apigenin 7-O-β-D-rutinoside, sakuranin, cyclokievitone hydrate and precarthamin (Supplementary Table S1). Among them, the contents of six flavonoid o-glycosides including genistein 7-O-glucoside, apigenin 7-O-glucoside, acacetin 7-O-rutinoside, apigenin 7-O-β-D-rutinoside, sakuranin and precarthamin, cyclokievitone hydrate (isoflavonoid) were significantly reduced, consistent with a previous study showing a decrease in flavonoid glycosides dropped after soybean fermentation (65). During fermentation, the avonoid glucosides including genistein 7-O-glucoside, acacetin 7-O-rutinoside, apigenin 7-O-β-D-rutinoside, sakuranin and cyclokievitone hydrate were negatively correlated with antioxidant activity, respectively (Figure 5B). Fermentation could facilitate the conversion of glycoside molecules, thereby enhancing the antioxidant capacity of soybeans (65). In addition, the health-promoting biflavone hinokiflavone (111) was found to be significantly elevated after SSF, increasing 2.19 times more than those in unfermented soybeans, and correlation analysis revealed a positive association between this component and the fermented soybean's antioxidant properties (Figure 5B).

3.4.4 Carbohydrates and carbohydrate conjugates

The genus *Pleurotus* is among the most exploitable xylotrophic fungi, capable of producing ligninolytic enzymes such as MnP and laccase, in addition to complex enzymes such as endo-glucanase, exo-glucanase, β-glucosidase, endo-xylanase, xylosidase, endo-mannanase and mannosidase (112). The carbohydrates in fermented soybean include starch, cellulose, hemicellulose, and pectin. Under the action of the carbohydrate–active enzymes, the amounts of starch and dietary fiber decrease, while most monosaccharides and oligosaccharides increased gradually during SSF (113). As fermentation progresses, these sugars transformed into organic acids, sugar alcohols, or other sugar conjugates that participate in various metabolic processes (113, 114). The carbohydrate contents of fermented soybeans, particularly the concentration of monosaccharides or oligosaccharides, have

shown conflicting findings due to the use of different fermentation strains, techniques, and durations (74, 114, 115). In this study, 13 differential metabolites of carbohydrates and carbohydrate conjugates were identified by the OPLS-DA analysis, and the metabolites were reported in Supplementary Table S1. Correlation heatmaps were generated to 7 potential carbohydrates and carbohydrate conjugates associations with the antioxidant activity of the soybeans fermented by *P. ostreatus* mycelium (Figure 5C). It was reported that oligosaccharides had the potential to enhance the activity of key antioxidant enzymes that scavenge reactive oxygen species (ROS) including peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD) (116).

3.4.5 Terpenoids

In this study, it was observed that sesquiterpenoids, triterpenoids and terpenoid glycosides changed most significantly in soybean after SSF. 7 sesquiterpenoids compounds were identified, including chrysanthemin A, 3b-Hydroxy-6b-(2,3-epoxy-2-methylbutanoyloxy)-7(11)- eremophilen- 12,8b-olide, methyl dihydrophaseate, artemisin, 3,8-Dihydroxy-6-methoxy-7(11)-eremophilen−12,8-olide, hydroxypelenolide and artemorin. Except for artemisin, the concentration of all sesquiterpenoids increased after SSF in this study (Supplementary Table S1). It was reported that sesquiterpenes constitute approximately 58% of the newly identified bioactive terpenes from endophytic fungi in the past decade, demonstrating various activities such as anti-viral, an-ti-microbial, antioxidant, anti-cancer, anti-acetylcholinesterase (AChE) properties (117). Our findings support this information. Sesterterpene synthase catalyzes the initial scaffold-generating step, which is primarily responsible for the structural variety of sesterterpenes. The sesterterpene synthases that have been found in fungi are bifunctional, having two domains: the terpene synthase domain and the trans-prenyltransferase domain, thus it is conducive to synthesize the product in a head-to-tail manner (80, 118). Among the triterpenoids, the level of methyl 3b,24-dihydroxy-11,13(18)-oleanadien−30-oate significantly increased, while the content of cucurbitacin I 2-glucoside decreased significantly after soybean fermentation. Triterpenoids, primarily soyasaponins, were found to be significant components of terpenoid differential metabolites in the soybean fermentation process (65). By eliminating the extra energy in singlet oxygen and releasing it as heat, the terpenoids serve as singlet oxygen quenchers, bringing the oxygen back to its unexcited state and allowing the terpenoids to be recycled as antioxidants (102). The results showed that antioxidant activity and five distinct terpenoids correlated positively, while antioxidant activity and two terpenoids correlated negatively (Figure 5C). There have been no reports of these terpenoids having any connection to antioxidant activity thus far. Fermentation might enhance the potential antioxidant activity and health benefits of soybean by up-regulation of terpene-active compounds.

3.4.6 Other compounds

From the results of Figure 5D, it could be seen that other non-volatile metabolites also had a high contribution to antioxidant activity, such as alkaloids, organic acids, nucleosides, ketones, and aldehydes (119–122). Camptothecin as an indole alkaloid, a modified monoterpene found in some plants (angiosperms) and endophytic fungi, is the source of the major anticancer medicine (123). This study is the first to demonstrate camptothecin can also be created by SSF of soybean with *P. ostreatus* and have a potential antioxidant activity (Figure 5D). Juglone was negatively correlated with antioxidant activity in this study (Figure 5D). It was reported that juglone (5-hydroxyl-1,4-naphthoquinone) showed the antioxidant capacities, which could serve to combat oxidative stress (124). Due to the function of the ligninolytic enzymes from *P. ostreatus*, Juglone was downregulated after SSF (Table 2). Therefore, it was negatively correlated with antioxidant activity (Figure 5D). The methylated inositol 2-O-Methyl-L-chiro-inositol exhibited anti-diabetic, anti-platelet aggregation, and free radical scavenging activities (125). In this study's correlation analysis, we also discovered 2-O-Methyl-L-chiro-inositol's possible antioxidant properties (Figure 5D). Carvedilol was positively correlated with antioxidant activity in this study (Figure 5D). Though carvedilol is a non-selective β-blocker that is frequently used to treat ischemic heart disease and hypertension, it demonstrated strong antioxidant properties unrelated to its adrenergic receptor-blocking ability (126).

3.4.7 Integration of metabolic pathway of the metabolites associated with antioxidant activity

Based on the KEGG pathway analysis and metabolic data, a biological networking pathway map was created to show the dynamic and explain the non-volatile metabolite related to antioxidant activity of soybean after SSF with *P. ostreatus* (Figure 6). Soybean raw materials as well as macromolecules like proteins, carbohydrates, and lipids in this study could be broken down by *P. ostreatus* during fermentation through a variety of enzymatic activities (47). This generated substrates such as sugars, fatty acids, and amino acids that may be utilized in other cellular processes to form the characteristic metabolites that follow SSF. We only found small amounts of fatty acids in the differential metabolites after 33 days of fermentation, and neither monosaccharides nor amino acids were found (Figure 6). The aromatic amino acids phenylalanine, tyrosine, and tryptophan were synthesized through shikimate pathway, which are the precursors to the synthesis of phenolic compounds (127). Flavonoids, stilbenoids, coumarins, lignans and aromatic alkaloids were among the chemical substances whose overexpression in this study suggested that SSF could significantly increase the metabolism of the shikimic acid pathway and its downstream pathways (Figure 6). Camptothecin as an indole alkaloid, a modified monoterpene found in some plants (angiosperms) and endophytic fungi, is the source of the major anticancer medicine (123). This study is the first to demonstrate camptothecin can also be created by SSF of soybean with *P. ostreatus* (Figure 6). Through the EMP pathway, microorganisms convert glucose to pyruvate, which can then be transformed to a variety of carbohydrates and carbohydrate conjugates. Broad spectrum antibacterial agents were made from naturally occurring aminoglycoside antibiotics, which were mostly derived from bacteria (*Streptomyces* sp. and *Micromonospora* sp.) (128). An aminoglycoside antibiotic

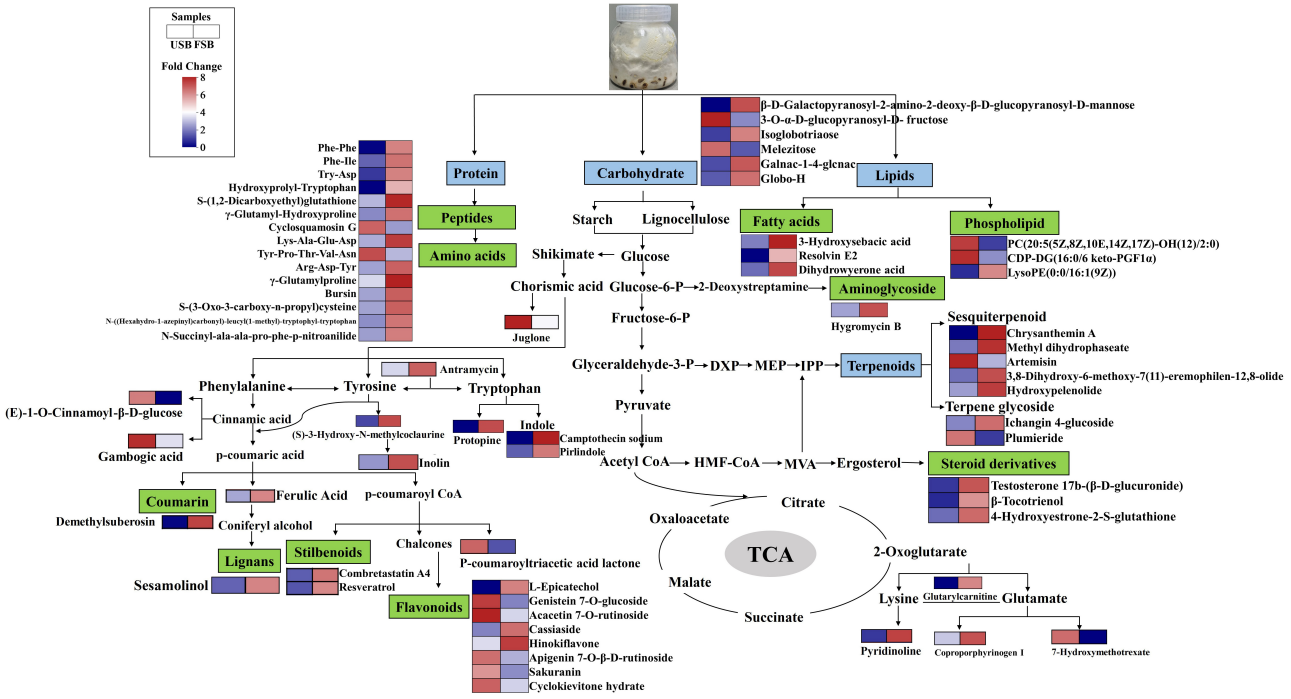

FIGURE 6
Biosynthetic pathway map of differential metabolites associated with antioxidant activity.

hygromycin B was identified in this study, demonstrating that this kind of compound can be synthesized and metabolized by mushroom mycelium (Figure 6). Terpenoids are the diverse family of natural products, and all terpenoids are derived from two 5-carbon precursors, dimethylallylpyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP), which are produced by the 2-C-methylerythritol phosphate (MEP) and mevalonate (MVA) pathways (129) (Figure 6). Among the components of fungi, steroids play a significant role, which are mostly metabolites of ergosterol. Numerous new metabolites of sterol and sterol derivatives from ergosterol were identified from fungal sources in recent years (130). Ergosterol conversion may be the source of the steroid derivatives in this investigation (Figure 6).

4 Conclusion

In this study, the utilization of *P. ostreatus* mycelium to enhance the quality of soybean products was explored for the first time. The investigation focused on nutritional ingredients, antioxidant activity and non-volatile metabolites during the SSF process. The results indicated a decrease in dietary fiber and starch content, while protein and lipid levels increased after SSF ($P < 0.05$). A significant increase in total phenolic content (from 4.62 to 20.72 mg GAE/g) was observed, along with increased antioxidant activities against intracellular reactive oxygen species (ROS), DPPH, and ABTS in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Untargeted metabolomic analysis using UPLC-MS/MS revealed drastic differences in the metabolite profile of soybeans processed by SSF compared to the unfermented soybeans. A total of 154 differential metabolites covering 10 subclasses were detected after SSF, with amino acid and derivatives, carbohydrates, lipids, phenolic compounds, and terpenoids being the major classes of differential metabolites. The antioxidant activity of fermented soybeans might be associated with specific phenolic compounds, oligopeptides and free fatty acids, etc. SSF has been recognized as an effective bioprocess for enhancing the antioxidant activities of soybeans. The most abundant of these metabolites were phenolic compounds generated through the shikimic acid pathway. In the future, metabolomics could be used to explore the contribution of bioactive components to functional properties, thereby optimizing the production of bioactive components in fermented soy foods. Consequently, we suggest two applications for solid fermented soybean using mycelium from *P. ostreatus*: first, to improve the soybean's properties for the creation of meat analog, and second, as a bioreactor to produce bioactive compounds and medicines.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

MH: Conceptualization, Data curation, Methodology, Software, Writing – original draft. QP: Methodology, Resources,

Visualization, Writing – original draft. XX: Data curation, Formal analysis, Investigation, Validation, Writing – original draft. BS: Conceptualization, Supervision, Writing – review & editing. YQ: Funding acquisition, Project administration, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1509341/full#supplementary-material

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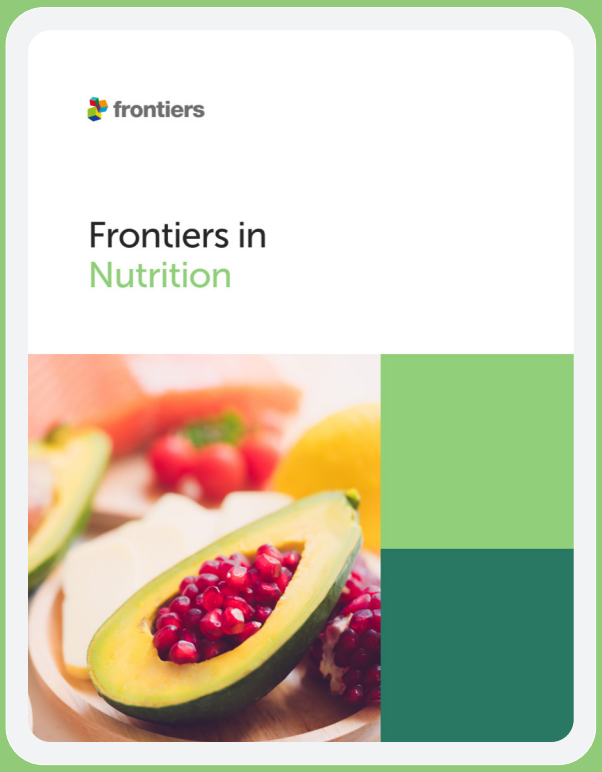

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