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Original Research

Untargeted Metabolomics of Milk Co-Fermentation by *Streptococcus salivarius* and *Lactobacillus delbrueckii*

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Abstract

Background: Co-fermentation with synergistic lactic acid bacteria may improve fermented milk quality and functionality, but the metabolomic changes during simultaneous cultivation of *Streptococcus salivarius* and *Lactobacillus delbrueckii* remain insufficiently characterized. **Methods:** Milk standardized to 3.5% fat and 12% total solids was inoculated with the two strains at a 1:1 ratio and fermented at 42 °C until pH 4.5. Fermentation kinetics were monitored by pH measurement. Untargeted metabolomics was performed using ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS) for non-volatile metabolites and headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME-GC-MS) for volatile organic compounds. Data were analyzed by principal component analysis (PCA), orthogonal partial least squares discriminant analysis (OPLS-DA), Student's *t*-test, VIP screening, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment. **Results:** pH reached 4.5 within 16 h. UHPLC-MS retained 69,015 features (563 standard; 2601 database annotations); PCA/OPLS-DA clearly separated A4/A8/A16/A24 by fermentation time. Short-chain fatty acids (SCFAs), free fatty acids (FFAs), di-/tripeptides and free amino acids increased, whereas lactose decreased with elevated glucose, galactose and lactic acid; KEGG highlighted pyruvate, tricarboxylic acid (TCA) cycle, butanoate, amino-sugar/nucleotide-sugar and purine/pyrimidine metabolism. GC-MS annotated 181 volatiles; acetaldehyde, diacetyl/acetoin and some alcohols/esters rose but remained non-significant. **Conclusions:** *Streptococcus salivarius–Lactobacillus delbrueckii* co-fermentation efficiently acidified milk and substantially remodeled the non-volatile metabolome, whereas volatile changes were suggestive but remained inconclusive, possibly due to the limited sample size and other factors. The identified biomarkers hold potential value for fermentation monitoring, biopreservation, and functional dairy product development, warranting further *in vivo* validation and process optimization.

Keywords: dairy fermentation; metabolomics; *Streptococcus salivarius*; *Lactobacillus delbrueckii*

1. Introduction

Fermented dairy products have been consumed for centuries and are widely recognized for their nutritional value, sensory properties, and potential health benefits. The metabolic activities of starter cultures during fermentation fundamentally transform the chemical composition of milk, generating a diverse array of metabolites including organic acids, peptides, fatty acids, and volatile compounds that contribute to product flavor, texture, and functionality [1,2,3]. Traditional fermentation often relies on single-strain or defined mixed cultures; however, co-fermentation using synergistic bacterial combinations has gained increasing attention as a strategy to enhance product quality and develop novel functional attributes [4,5].

Streptococcus salivarius and *Lactobacillus delbrueckii* possess distinct metabolic characteristics during dairy fermentation. *Lactobacillus delbrueckii* is a well-characterized lactic acid bacterium (LAB) with high lactose utilization and acid-producing capacity, serving as an essential starter strain for yogurt and fermented milk production [6]. *Streptococcus salivarius* is generally recognized as a commensal bacterium colonizing the human oral cavity and gastrointestinal tract and also functions as a core starter culture for dairy fermentation. It has received growing research interest owing to its probiotic properties and capacity to synthesize bacteriocins and other bioactive metabolites. Nevertheless, *Streptococcus salivarius* exhibits weak proteolytic activity and depends on proteases secreted by *Lactobacillus delbrueckii* to degrade casein and acquire sufficient peptides and free amino acids for growth [7]. Co-cultivation of the two strains induces metabolic synergy, which improves fermentation efficiency and shapes unique metabolomic profiles compared with single-strain fermentation.

Non-targeted metabolomics has emerged as a powerful analytical approach for comprehensively characterizing the complex metabolic changes occurring during food fermentation [8,9]. By combining ultra-performance liquid chromatography (UPLC) with mass spectrometry for non-volatile metabolites and gas chromatography-mass spec-

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trometry (GC-MS) for volatile organic compounds, researchers can obtain a holistic view of the fermentation metabolome [10]. This dual-platform approach enables the identification of differentially abundant metabolites that may serve as biomarkers for fermentation quality, product functionality, and shelf stability.

Despite the potential advantages of co-fermentation, the metabolomic changes occurring during the simultaneous cultivation of *S. salivarius* and *L. delbrueckii* in milk remain poorly characterized. Therefore, the objective of this study was to apply an untargeted metabolomics approach to investigate the molecular changes in milk during co-fermentation with these two bacterial species. Specifically, we aimed to (i) evaluate the fermentation kinetics and acidification profile, (ii) characterize the alterations in non-volatile and volatile metabolomes, and (iii) identify potential biomarkers associated with fermentation efficacy and functional properties.

2. Materials and Methods

2.1 Bacterial Strains and Culture Conditions

Streptococcus salivarius and *Lactobacillus delbrueckii* were purchased from Dali Laisi'er Dairy Co., Ltd., with catalog numbers LSE-G and LSE-L, respectively, and subsequently stored in the laboratory. Stock cultures were maintained at –80 °C in 20% (v/v) glycerol. Prior to experiments, strains were activated by two successive transfers in sterile reconstituted skim milk (10% w/v) supplemented with 1% (w/v) yeast extract and 1% (w/v) glucose, followed by incubation at 37 °C for 12 h (*S. salivarius*) and 42 °C for 12 h (*L. delbrueckii*) [11], respectively.

2.2 Fermentation Protocol

Fresh bovine milk was standardized to 3.5% fat and 12% total solids, pasteurized at 85 °C for 15 min, and cooled to inoculation temperature. The co-fermentation was initiated by inoculating activated cultures of *S. salivarius* and *L. delbrueckii* at a 1:1 ratio (each at 10^6 CFU/mL) into the prepared milk base. Fermentation was carried out at 42 °C [12], and pH was monitored at 2 h intervals using a calibrated pH meter until the fermentation endpoint (pH 4.5) was reached. Fermented milk samples to be analyzed were collected, immediately snap-frozen in liquid nitrogen, and stored at –80 °C until metabolomic analysis (the fermentation time points for the analyzed samples were 4 h, 8 h, 16 h, and 24 h).

2.3 Non-Volatile Metabolite Extraction and UHPLC-MS Analysis

Non-volatile metabolites were extracted using a modified Bligh–Dyer method [13]. Briefly, 200 mg frozen sample was homogenized in 1 mL of chilled methanol–water (4:1, v/v) containing an internal standard. After vortexing, the mixture was centrifuged at 12,000 ×g for 10 min at 4 °C. The supernatant was collected and filtered through a 0.22 μm membrane filter. For polar metabolite separation, a Vanquish ultra-high-performance liquid chromatograph (UHPLC, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Waters ACQUITY UPLC BEH Amide column (2.1 mm × 50 mm, 1.7 μm) was used in this study. The mobile phase consisted of mobile phase A (aqueous solution containing 25 mmol/L ammonium acetate and 25 mmol/L ammonia solution) and mobile phase B (acetonitrile). The sample tray temperature was set at 4 °C, and the injection volume was 2 μL. An Orbitrap Exploris 120 mass spectrometer controlled by Xcalibur software (version 4.4, Thermo Fisher Scientific, Waltham, MA, USA) was used to acquire full-scan MS and MS/MS data. The detailed mass spectrometric parameters were as follows: sheath gas flow rate, 50 Arb; auxiliary gas flow rate, 15 Arb; capillary temperature, 320 °C; full MS resolution, 60,000; MS/MS resolution, 15,000; collision energy, SNCE 20/30/40; spray voltage, 3.8 kV (positive mode) or –3.4 kV (negative mode).

2.4 Volatile Compound Analysis by GC-MS

Volatile organic compounds (VOCs) were analyzed using headspace solid-phase microextraction (HS-SPME) combined with GC-MS [14]. Samples (5 mL) were placed in 20 mL headspace vials containing 1.5 g NaCl and equilibrated at 50 °C for 15 min. A 50/30 μm DVB/CAR/PDMS fiber (Supelco, Bellefonte, PA, USA) was exposed to the headspace for 30 min at 50 °C. Desorption was performed in the GC injector at 250 °C for 5 min in splitless mode. GC separation was conducted on a DB-WAXetr column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies, Santa Clara, CA, USA) using an Agilent 7890B GC system coupled to a 5977B mass selective detector. Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The oven temperature was programmed from 40 °C (held 3 min) to 230 °C at 5 °C/min.

2.5 Data Processing and Statistical Analysis

For metabolomic analysis, each group consisted of four biological replicate samples (parallel samples), and each sample was independently subjected to metabolomic data acquisition and analysis. For raw data preprocessing, features with a relative standard deviation (RSD, also known as the coefficient of variation, CV) exceeding 50% were first removed to eliminate noise. Peak area data were retained only when no single group contained more than 50% missing values *and* the total missing values across all groups did not exceed 50%. Missing values were imputed using half of the minimum value for each feature, and the data were normalized to the total ion current (TIC) of each sample prior to subsequent analysis. Metabolites were annotated against the Human Metabolome Database (HMDB), the Kyoto Encyclopedia of Genes and Genomes (KEGG), and an in-house library based on exact mass (±5

ppm), isotope patterns, and MS/MS fragmentation information. Compounds in the GC-MS data were identified using AMDIS software (National Institute of Standards and Technology, Gaithersburg, Maryland, USA) and the NIST 20 mass spectral library.

Multivariate statistical analysis, including principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA), was performed using SIMCA-P+ 14.0 (Umetrics, Umeå, Sweden) after Pareto scaling. Variable importance in projection (VIP) scores >1.0 and p-values < 0.05 (Student's t-test) were used to identify significantly differential metabolites. Metabolic pathway enrichment was analyzed using MetaboAnalyst 5.0 (Professor David Wishart's team at the University of Alberta, Edmonton, Alberta, Canada.).

3. Results and Discussion

3.1 Fermentation Kinetics and Acidification Profile

The co-fermentation of milk with *S. salivarius* and *L. delbrueckii* proceeded efficiently, with the pH decreasing from an initial value of approximately 6.5 to the fermentation endpoint of 4.5 within 16 h [15]. This acidification rate is consistent with typical yogurt fermentation profiles and indicates effective metabolic synergy between the two strains. The progressive acidification reflects the combined proteolytic and glycolytic activities of the starter cultures, wherein *L. delbrueckii* likely contributed significantly to lactose utilization and lactic acid production, while *S. salivarius* may have complemented the fermentation through the production of bacteriocins and other metabolites that modulate the microbial environment [16,17,18]. The attainment of pH 4.5 within 16 h suggests that this co-culture combination is technically viable for industrial fermented milk production.

3.2 Nonvolatile Metabolites

3.2.1 Multivariate Statistical Analysis

In untargeted UHPLC-MS analysis performed in both positive and negative ion modes, 69,015 features were retained after data preprocessing. Among these features, 563 were annotated by matching retention time, MS1, and MS/MS information with reference standards, while 2601 were annotated based on MS1 and MS/MS spectra obtained from public databases. The 3D PCA score plot indicated that the four sample groups (A4, A8, A16, and A24) exhibited an obvious temporal gradient distribution along the PC1 axis (Fig. 1). The early fermentation groups (A4 and A8) and late fermentation groups (A16 and A24) clustered on the negative and positive sides of the PC1 axis, respectively, demonstrating that the fermentation process induced systematic remodeling of the milk metabolome.

To improve group separation and screen key differential metabolites, orthogonal partial least squares discriminant analysis (OPLS-DA) was further adopted for pairwise comparison. Using SIMCA software (V18.0.1, Sartorius

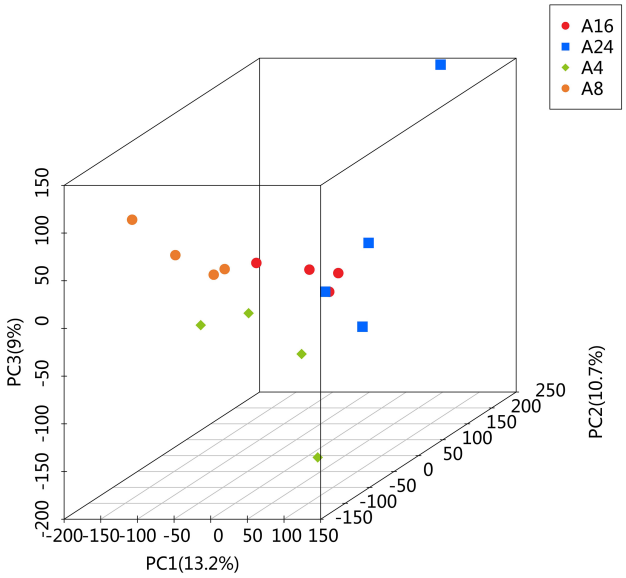


Fig. 1. Score scatter plot 3D of principal component analysis (PCA) model for Group A4 vs A8 vs A16 vs A24.

Stedim Data Analytics AB, Umeå, Sweden), all data were logarithmically (LOG) transformed and unit variance scaling (UV)-normalized prior to OPLS-DA modeling based on the first principal component. The results revealed significant separation between every two groups (Figs. 2,3,4,5), and the most distinct separation was observed between the A4 and A16 groups (Fig. 3). Biological replicates within each group clustered closely, indicating that fermentation time significantly altered the metabolite profiles of fermented milk and verifying the excellent reproducibility of the present experiment.

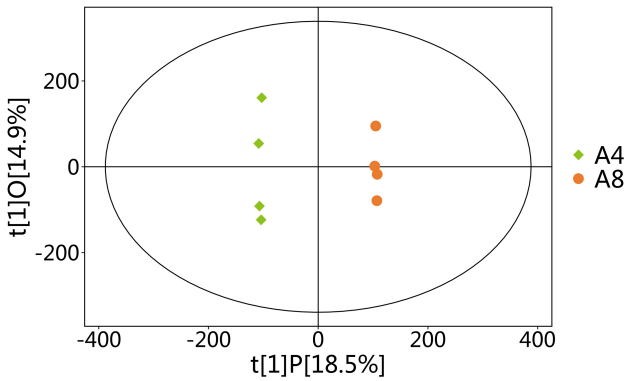


Fig. 2. Scatter plot of the orthogonal partial least squares discriminant analysis (OPLS-DA) model for Group A4 versus Group A8.

3.2.2 Analysis of Differentially Expressed Metabolites

Volcano plots were used to visualize the global distribution of metabolic differences between groups. The re-

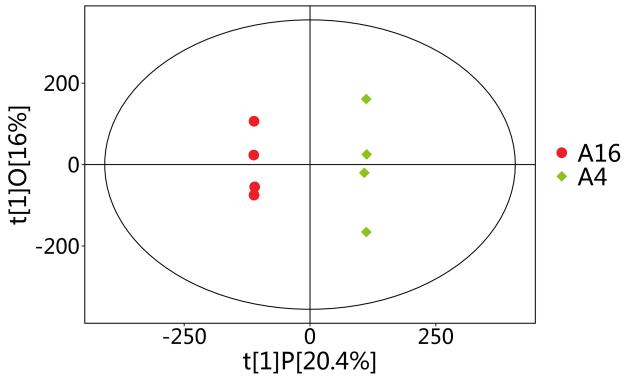


Fig. 3. Scatter plot of the OPLS-DA model for Group A4 versus Group A16.

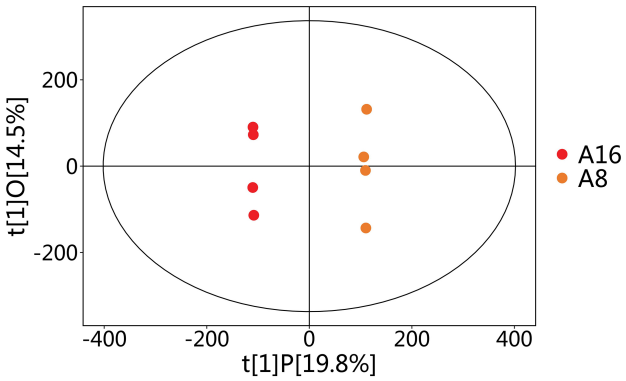


Fig. 4. Scatter plot of the OPLS-DA model for Group A8 versus Group A16.

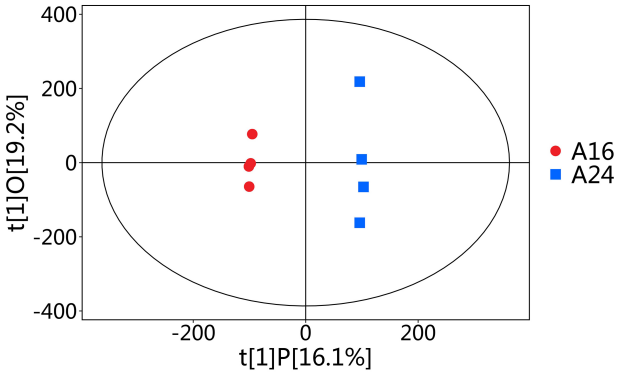


Fig. 5. Scatter plot of the OPLS-DA model for Group A16 versus Group A24.

sults of differential metabolite screening were visualized via volcano plots, taking the comparison between Group A4 and Group A16 as an example. Each point in the volcano plot corresponded to one metabolite. The x-axis represented the fold change of each metabolite for pairwise comparison ($\log_2$ scale); the y-axis represented the *p*-value obtained from Student's *t*-test ($-\log_{10}$ scale), and the size of each point indicated the VIP value derived from the OPLS-DA model; a larger point indicated a higher VIP value. Significantly upregulated metabolites were marked in red, significantly downregulated metabolites in blue, and metabolites with no significant differences in gray. The top 10 significantly upregulated and top 10 significantly downregulated metabolites were labeled on the plot, respectively (Fig. 6).

As shown in Fig. 7, the downregulated metabolites were mainly organic acids (e.g., pyruvate), vitamin derivatives (e.g., 1,25-Dihydroxyvitamin D3-26,23-lactone and Menadione bisulfite), glycosides (e.g., skimmin), and saponins (e.g., senegin II). Conversely, the up-regulated metabolites were primarily peptides (e.g., Tyr-Val), amino acid derivatives (e.g., 5-hydroxytryptophan), lignans (e.g., epiyangambin), and cardiac glycosides (e.g., corchoroside A).

It should be noted that these compounds (lignans and cardiac glycosides) are typical plant secondary metabolites rather than canonical bacterial fermentation products. Therefore, their presence in fermented milk is more reasonably attributed to carryover within the dairy matrix—particularly feed-derived phytochemicals transferred from bovine feed into milk via the rumen–blood–mammary gland pathway—or to the release or modification of plant-associated precursors during fermentation through acidification, enzymatic hydrolysis, or microbial deglycosylation. However, because the 0 h unfermented milk samples were not subjected to quantitative and qualitative instrumental analysis, the baseline identity and abundance of these compounds cannot be determined. Thus, the present data support the possibility that these substances are present in milk, whereas their exact origin—pre-existing milk-associated compounds, feed-derived transfer [19,20] or fermentation-induced transformation [21]—still requires experimental confirmation.

3.2.3 KEGG Enrichment Analysis of Differentially Expressed Metabolites

KEGG pathway enrichment analysis was performed to systematically characterize the functional categories of differential metabolites. The horizontal bar plot of KEGG classification categorized these differential metabolites into eight primary classes, including amino acid metabolism, carbohydrate metabolism, energy metabolism, global and overview maps, metabolism of cofactors and vitamins, metabolism of other amino acids, nucleotide metabolism, and xenobiotics biodegradation and metabolism (Fig. 8). The global and overview maps was the dominant category, in which metabolic pathways occupied the largest proportion (83.75%). Other representative subpathways within this category contained biosynthesis of amino acids, biosynthesis of cofactors, and 2-oxocarboxylic acid metabolism. Carbohydrate metabolism contained glyoxylate and dicarboxylate metabolism, tricarboxylic acidcycle (TCA cycle), and amino sugar and nucleotide sugar

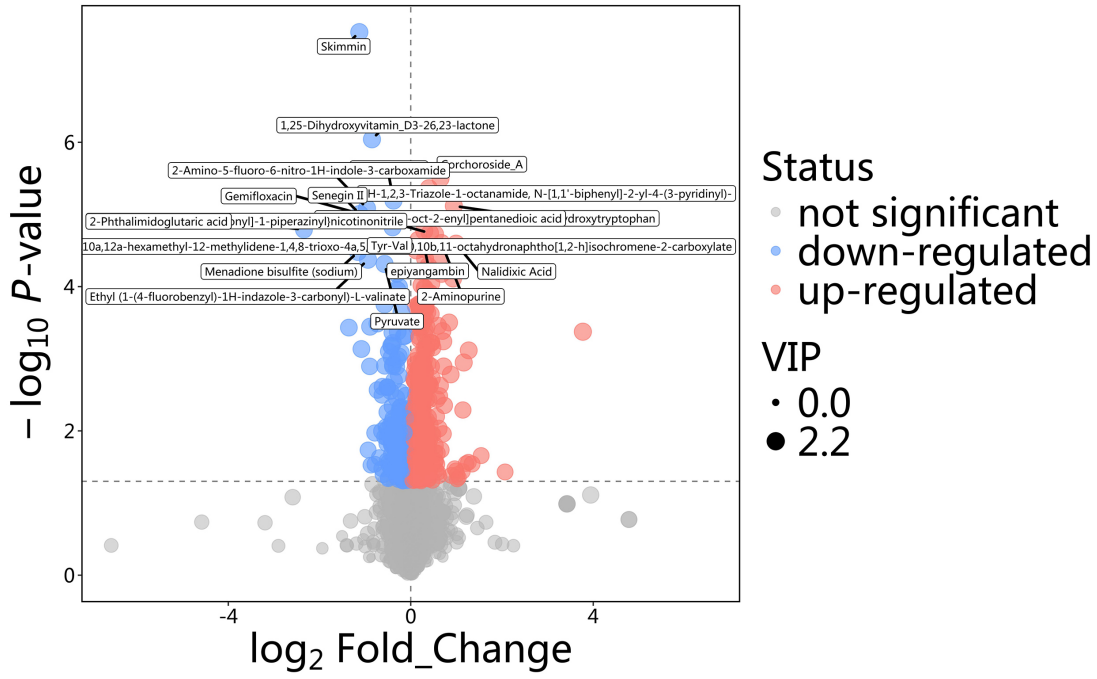


Fig. 6. Volcano plot of metabolites identified as differentially expressed between Group A4 and Group A16.

metabolism. Energy metabolism included other carbon fixation pathways, while pantothenate and coenzyme A (CoA) biosynthesis belonged to the metabolism of cofactors and vitamins. Nucleotide metabolism consisted of purine metabolism (13.75%) and pyrimidine metabolism (8.75%). In addition, alanine, aspartate and glutamate metabolism was assigned to amino acid metabolism, D-amino acid metabolism to metabolism of other amino acids, and benzoate degradation to xenobiotics biodegradation and metabolism.

The results of the metabolic pathway analysis are presented as a rectangular tree diagram. In this diagram (Fig. 9), each rectangle represents a metabolic pathway; the size of the rectangle indicates the magnitude of the pathway's influence factor in the topological analysis—the larger the rectangle, the greater the influence factor. The color of the rectangle represents the *p*-value from the enrichment analysis (the negative natural logarithm, i.e., -ln(p)); the darker the color, the smaller the *p*-value, and the more significant the enrichment. The treemap highlighted that multiple central carbon and nitrogen metabolic pathways were markedly perturbed, such as alanine, aspartate and glutamate metabolism, pyruvate metabolism, butanoate metabolism, citrate cycle (TCA cycle), and amino sugar and nucleotide sugar metabolism. Several secondary metabolic pathways were also affected, including arginine and proline metabolism, fructose and mannose metabolism, peptidoglycan biosynthesis, pentose phosphate pathway, purine metabolism and pyrimidine metabolism.

The enrichment bubble plot visualized the enrichment factor, metabolite count and *p*-value of each enriched KEGG pathway. The bubble size indicated the number of enriched differential metabolites, and bubble color represented the statistical significance (Fig. 10). Metabolic pathways possessed the highest metabolite count. Nucleotide metabolism, alanine, aspartate and glutamate metabolism, and purine metabolism showed relatively high rich factors and low *p*-values, indicating strong enrichment. By contrast, other carbon fixation pathways and benzoate degradation exhibited weaker enrichment effects. Collectively, the multi-dimensional KEGG enrichment results demonstrated that the co-fermentation process profoundly reshapes the core metabolic networks of the fermenting strains.

3.2.4 Effects of Co-fermentation on Milk Quality

Nucleotides and Derivatives: Elevated levels of nucleosides and nucleotide derivatives were observed, which may reflect bacterial biomass turnover and autolysis. These compounds can serve as flavor enhancers and may also participate in immunomodulatory functions [22].

Fatty Acids and Lipids: Free fatty acids (FFAs), including short-chain fatty acids (SCFAs) such as butyric acid, propionic acid, and caproic acid, were markedly increased following fermentation. These compounds arise from the lipolytic activity of bacterial esterases and phospholipases acting on milk triglycerides and phospholipids [23]. The accumulation of FFAs not only contributes to the characteristic flavor of fermented dairy products but also exhibits antimicrobial properties that may inhibit spoilage organisms, thereby potentially extending product shelf life [24].

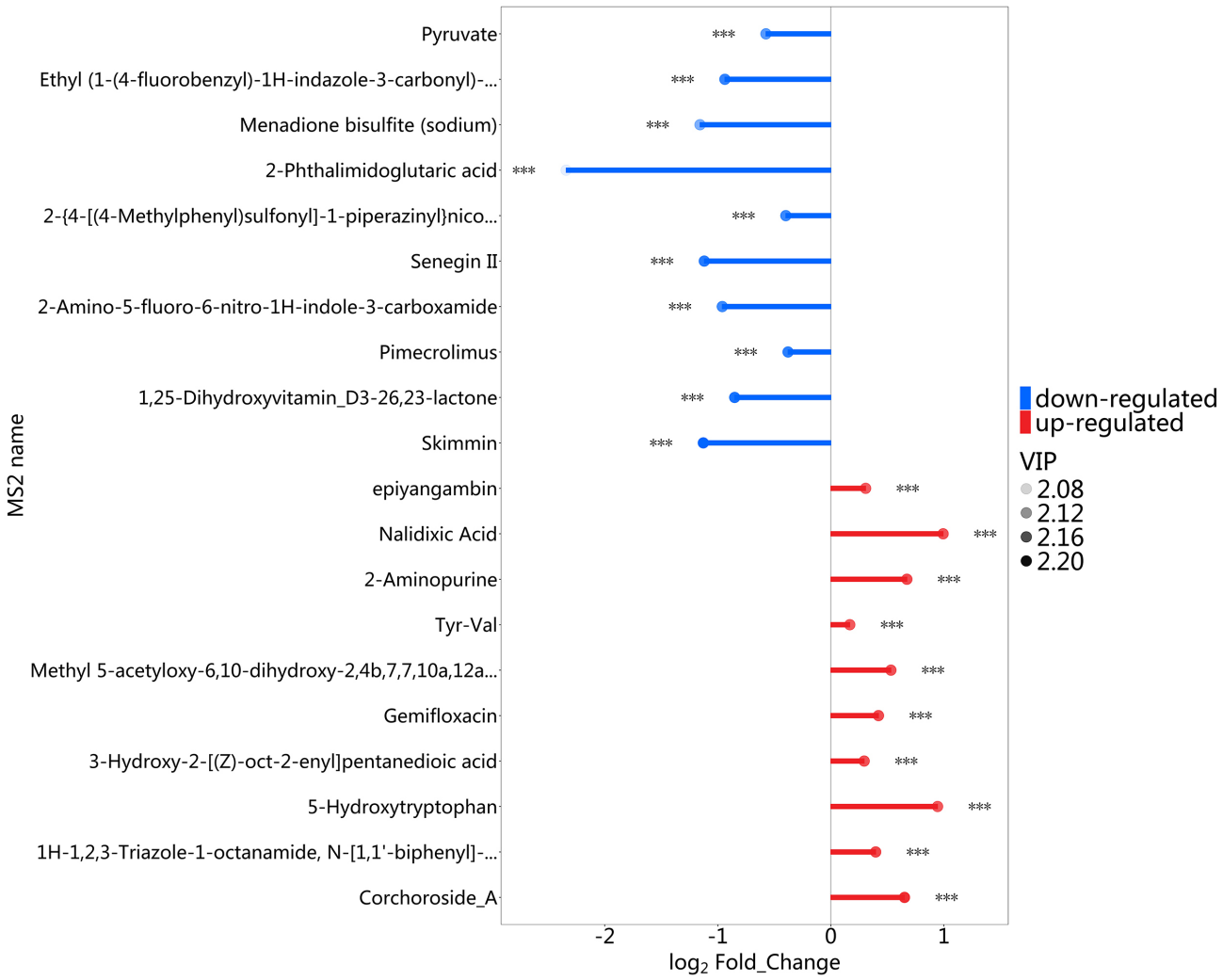


Fig. 7. Matchstick drawing of Group A4 versus Group A16.

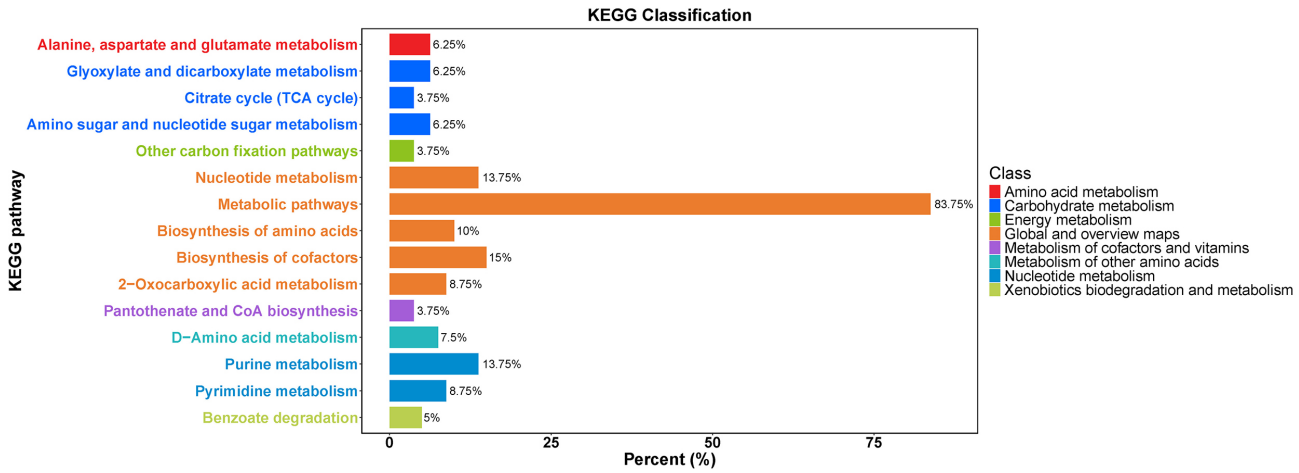


Fig. 8. Kyoto Encyclopedia of Genes and Genomes (KEGG) classification for Group A4 vs A8 vs A16 vs A24.

Peptides and Amino Acids: A substantial increase in dipeptides, tripeptides, and free amino acids was observed, reflecting the proteolytic activity of both bacterial strains. *L. delbrueckii* is known to possess a complex proteolytic system comprising cell envelope proteinases and intracellular peptidases that hydrolyze caseins into bioactive peptides

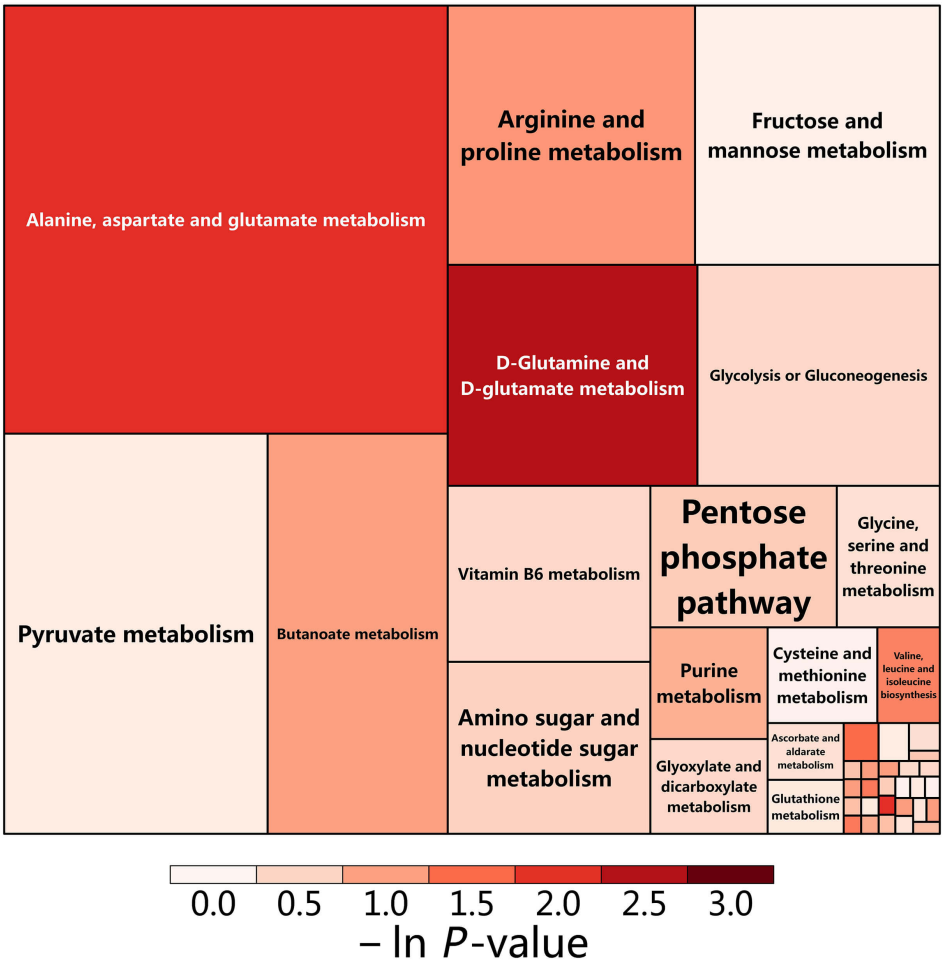


Fig. 9. Pathway analysis.

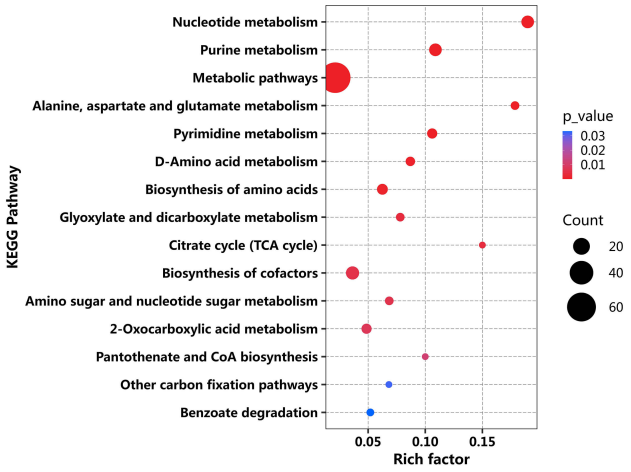


Fig. 10. KEGG enrichment.

[25]. Several of the identified peptides mapped to known sequences with angiotensin-converting enzyme (ACE) inhibitory, antioxidant, or antimicrobial activities, suggesting that the co-fermentation may enhance the functional value of the final product [26].

Carbohydrates and Organic Acids: As expected, lactose levels decreased significantly, concomitant with increased concentrations of glucose, galactose, and lactic acid. The depletion of lactose and accumulation of organic acids are hallmarks of LAB fermentation and contribute to both the preservation effect and the tangy flavor profile of fermented milk [27]. Additionally, trace amounts of acetic acid and formic acid were detected, likely arising from heterofermentative pathways or the metabolic activity of *S. salivarius*.

3.3 Volatile Metabolites

After preprocessing of raw GC-MS data, 225 features were retained, among which 181 compounds were successfully annotated (Fig. 11). The volatile profile was dominated by organic acids, alcohols, aldehydes, ketones and esters. Several flavor-related compounds exhibited distinct changing trends, yet none of them reached statistical significance. Statistically, the overall volatile flavor profile was not substantially remodeled, which might be attributed to the limited sample size.

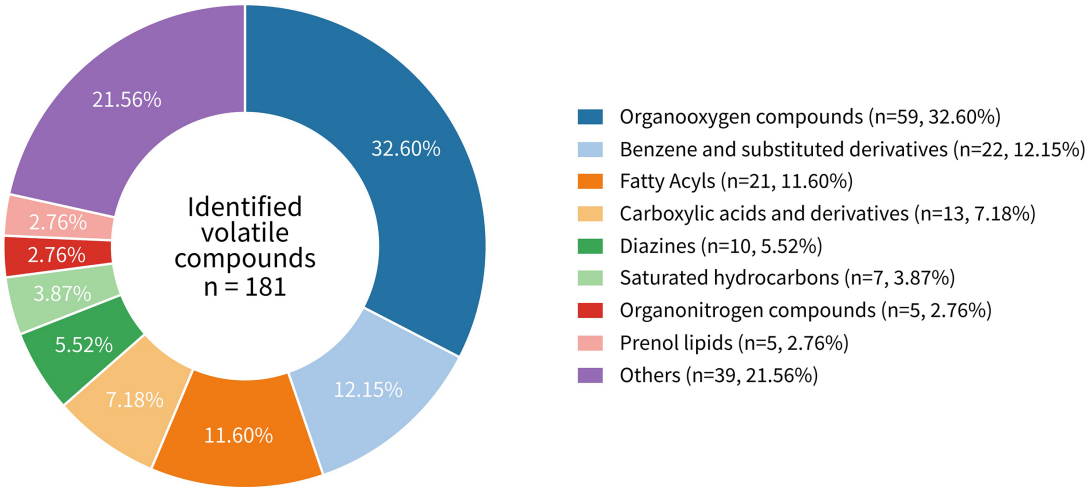


Fig. 11. Volatile compounds by subclass.

Organic Acids and Carbonyl Compounds Acetaldehyde, the principal flavor compound in yogurt, showed a increase, consistent with the threonine aldolase activity of *L. delbrueckii* [28]. Diacetyl and acetoin, key aroma compounds contributing to buttery notes, were also elevated. These volatile carbonyl compounds are critical determinants of consumer acceptability in fermented dairy products [29].

Alcohols and Esters: Ethanol, 2-methyl-1-butanol, and 3-methyl-1-butanol increased during fermentation, likely derived from amino acid catabolism (Ehrlich pathway) and carbohydrate metabolism, in which lactic acid bacteria degrade corresponding amino-acid precursors, and they contribute mild alcoholic notes to fermented-milk aroma [30]. Several ethyl esters, including ethyl acetate and ethyl hexanoate, were detected at higher levels in the fermented samples, contributing fruity and floral notes to the aroma profile. However, none of these substances reached statistical significance.

Sulfur Compounds: Trace amounts of dimethyl sulfide and methanethiol were identified, arising from methionine catabolism. While present at low concentrations, these sulfur-containing volatiles significantly influence the overall aroma perception of fermented milk [31,32].

3.4 Identification of Key Biomarkers and Functional Implications

OPLS-DA modeling identified several metabolites with high VIP scores that could serve as potential biomarkers for monitoring the co-fermentation process. These included lactic acid, acetaldehyde, specific bioactive peptides (e.g., β-casein derived fragments), and certain medium-chain fatty acids. The presence of these biomarkers not only indicates successful fermentation but also points to the potential functional benefits of the co-culture system.

The accumulation of bacteriocin-like peptides and organic acids suggests that this co-fermentation strategy may confer biopreservative properties, thereby extending the shelf life of the final product by suppressing the growth of pathogenic and spoilage microorganisms [33]. Furthermore, the observed changes in the metabolome, particularly the enrichment of bioactive peptides and SCFAs, indicate potential benefits for gut microecological balance. SCFAs are known to serve as energy substrates for colonocytes, modulate intestinal pH, and influence the composition of the gut microbiota [34]. The co-fermentation approach may therefore yield a product with enhanced functional properties beyond basic nutrition.

3.5 Study Limitations

While this study provides a comprehensive metabolomic characterization of milk co-fermentation by *Streptococcus salivarius* and *Lactobacillus delbrueckii*, several limitations should be acknowledged:

3.5.1 Absence of Single-Strain Fermentation Controls

Only the co-fermentation system was investigated; parallel monocultures of *S. salivarius* and *L. delbrueckii* were not included. Consequently, the individual contributions of each strain to the observed metabolic changes cannot be unambiguously resolved, and the proposed metabolic synergy remains inferential rather than experimentally demonstrated.

3.5.2 Lack of Authentic Standard Validation for Metabolite Identification

Metabolite annotation relied primarily on accurate mass (±5 ppm), isotopic patterns, and MS/MS fragmentation matched against HMDB, KEGG, and an in-house library. The majority of identifications were not confirmed with authentic standards by retention time and fragmentation spectra, leaving some annotations—particularly for isobaric/isomeric compounds—tentative.

3.5.3 No Bioactivity Assays to Support Functional Claims

The proposed functionalities of identified metabolites, such as ACE-inhibitory, antioxidant, and antimicrobial activities of bioactive peptides and short-chain fatty acids, were inferred from database annotation and literature. These claims remain speculative in the absence of *in vitro* or *in vivo* validation (e.g., ACE inhibition assays, antioxidant assays, antimicrobial testing, and animal or human intervention studies).

4. Conclusion

This study provides the first comprehensive metabolomic characterization of milk co-fermented with *Streptococcus salivarius* and *Lactobacillus delbrueckii*. The co-culture effectively acidified milk to pH 4.5 within 16 h, demonstrating technical feasibility for dairy applications. Untargeted UHPLC-MS and GC-MS analyses revealed significant alterations in the milk metabolome, with notable changes in fatty acids, peptides, carbohydrates, and volatile compounds. The identification of key biomarkers associated with fermentation efficacy, biopreservation, and potential microecological modulation highlights the promise of this co-fermentation strategy for the development of novel functional fermented dairy products. Future research should focus on validating the bioactivities of the identified metabolites *in vivo* and optimizing the co-culture ratios and fermentation conditions for industrial scale-up.

Availability of Data and Materials

All data reported in this paper are available from the corresponding author upon reasonable request.

Author Contributions

XX and WY designed the research study. JP performed the research. SZ and YD collected the data. ZL analyzed the data. JP and ZL wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflicts of Interest

Dali Lesson Dairy Industry Co., Ltd. and Yunnan Lesson Dairy Industry Co., Ltd. did not affect the execution of this study, the interpretation of results, or the preparation and submission of the manuscript. These companies had no restrictions on the publication of the study. All authors had full access to all data in the study and assume full responsibility for the integrity of the data and the accuracy of the data analysis.

Declaration of AI and AI-Assisted Technologies in the Writing Process

During the preparation of this work, the authors used Deepl in order to check spelling and grammar. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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