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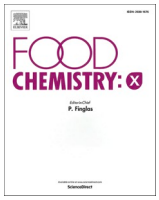

Effects of *Lacticaseibacillus casei* Zhang addition on physicochemical properties and metabolomics of fermented camel milk during storage

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ABSTRACT

Lacticaseibacillus casei Zhang (*L. casei* Zhang) was used as an auxiliary starter culture to explore its application in camel milk fermentation. This study evaluated the effects of *L. casei* Zhang supplementation on viable cell count, acidity, texture, insulin-like growth factor 1 (IGF-1) retention, and metabolite profiles over a 21-day storage period. *L. casei* Zhang enhanced the retention rate of active IGF-1 from 52.95% to 59.13% and mitigated the progression of acidity (from 125 °T to 97.5 °T) compared with the control group. Additionally, *L. casei* Zhang significantly improved viscosity and promoted the formation of gel structures. Furthermore, its addition significantly influenced the production of key metabolites, including adenosine diphosphate, oleuropein, and threonine–tryptophan ($P < 0.05$). These findings highlight the potential of *L. casei* Zhang as an effective auxiliary starter culture for camel milk fermentation, enhancing its physicochemical properties and modulating its metabolomic profile.

1. Introduction

The production and consumption of camel milk has a rich history, tracing back to ancient times. Due to its geographical constraints, camel milk is predominantly produced in the desert regions of the Middle East, Africa, and Central Asia, where it is referred to as 'desert gold' by indigenous communities. With the growing development of the camel milk industry, the camel milk market has gradually expanded to Europe and North America. Camel milk is rich in vitamins, minerals, unsaturated fatty acids, and lactoglobulin compared with bovine milk (Alhaj et al., 2022). Additionally, its high concentrations of lysozyme, lactoperoxidase, and N-acetyl-D-glucosaminidase provide protection against spoilage and pathogenic bacteria, contributing to an extended shelf life (Hamed et al., 2024). Recent advances in the production of camel dairy products have highlighted their health-promoting properties. Researchers have identified anti-diabetic, hypoallergenic, anti-cancer, and immune-protective effects that drive increased consumer interest (El-Kattawy et al., 2021). Fermented camel dairy products exhibit extended shelf life, improved digestibility, enhanced flavor, and elevated levels of vitamins and short-chain fatty acids compared with fresh camel milk (El Hatmi et al., 2018; Ho et al., 2022). However, camel milk presents challenges, such as weak and fragile curd formation during coagulation and prolonged fermentation time. Notably, Bulca et al. demonstrated that microbial transglutaminase can enhance the gel structure and sensory properties of camel milk yogurt (Bulca et al., 2022). Furthermore, studies have shown that *Lactobacillus helveticus* MB2–1 serves as an effective starter culture, and *in situ* exopolysaccharides may act as probiotic stabilizer substitutes in fermented dairy products (Ge et al., 2022). These findings suggest that incorporating probiotics and microbial enzymes is a promising strategy for improving gel stability and overall quality of camel milk-based products.

Lacticaseibacillus casei Zhang (*L. casei* Zhang) is a probiotic strain isolated from Inner Mongolian kumiss that exhibits exceptional gastrointestinal fluid tolerance, which is a prerequisite for the viability and effectiveness of probiotics. A second critical criterion for probiotics is achieving a 'sufficient quantity'. To exert beneficial effects, the

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concentration of viable cells in fermented dairy products must exceed 10^6 CFU/mL. The final essential attribute of probiotics is 'efficacy'. Previous research has demonstrated that *L. casei* Zhang harbors a rich repertoire of potential probiotic genes, including those involved in glutathione synthesis, riboflavin production, acid resistance, and extracellular polysaccharide secretion (Sun et al., 2023). These genetic characteristics provide robust theoretical support for its application in functional foods, fostering the development of diverse probiotic functionalities, including antioxidant activities. In addition to its genetic advantages, *L. casei* Zhang exhibits significant bioactivity in practical applications. Studies indicate that oral administration of *L. casei* Zhang may offer therapeutic potential by modulating short-chain fatty acid (SCFAs) and nicotinamide metabolism, thereby mitigating kidney injury and slowing the progression of renal decline (Zhu et al., 2021). Furthermore, *L. casei* Zhang possesses protein hydrolysis capabilities that can be applied in the preparation of fermented soymilk, where it contributes to the regulation of histidine and lysine biosynthesis (Wang et al., 2012). In conclusion, *L. casei* Zhang demonstrates outstanding biological properties and multiple health benefits, highlighting its potential for applications in functional food products.

The field of fermenter strains in China is experiencing rapid development, integrating both traditional and modern expertise to unlock promising opportunities. Among these strains, *L. casei Zhang*, which has been recognized for its probiotic properties in numerous studies, can be incorporated as a primary or secondary starter in various products to confer probiotic benefits. However, it is crucial to acknowledge that the beneficial effects of *L. casei* Zhang are not universally consistent across applications. For instance, the inclusion of *L. casei* Zhang in Minas Frescal cheese resulted in changes in multiple parameters and negatively impacted its sensory acceptance (Dantas et al., 2016). Consequently, it is necessary to investigate the specific effects of *L. casei* Zhang on different products. Bai et al. utilized *L. casei* Zhang as an auxiliary starter to examine its application in stirred yogurt and reported improvements in yogurt viscosity, gel strength, and extracellular polysaccharide synthesis (Bai et al., 2020). Similarly, researchers have observed that the addition of *L. casei* Zhang enhanced the production of SCFAs in yogurt during storage (Peng et al., 2022). These findings suggest that *L. casei* Zhang can significantly contribute to the production of extracellular polysaccharides and SCFAs in fermented dairy products, thereby improving yogurt texture and exerting prebiotic effects.

To further explore its potential, *L. casei Zhang* was incorporated as the primary starter alongside YoFlex® Mild 1.0, in the preparation of fermented camel milk. The resulting products were analyzed for pH, TA, IGF-1 levels, water-holding capacity, viable cell counts, viscosity, texture, and non-targeted metabolomic profiles to assess the impact of *L. casei* Zhang during storage.

2. Materials and methods

2.1. Materials and instruments

The freeze-dried *L. casei* Zhang bacterial powder used in this study was sourced from the Lactic Acid Bacteria Culture Collection at Inner Mongolia Agricultural University, China. The strain has also been deposited at the China General Microbiological Culture Collection Center (CGMCC) under the strain number CGMCC No. 1697. The commercial starter culture, YoFlex® Mild 1.0, was purchased from Kohansen (Beijing, China). Camel milk was collected from the Inner Mongolia Autonomous Region of the Alxa Left Banner Bayanhot City Pastoral Area. MRS and M17 media were obtained from Haibo Company in Qingdao, and vancomycin was purched from Luqiao (Beijing, China). Acetonitrile, methanol, and formic acid were purchased from Thermo Fisher Scientific (Shanghai, China).

2.2. Preparation of fermented camel milk

The fermented camel milk was prepared and divided into two groups. The samples in both groups were inoculated with YoFlex® Mild 1.0 at a rate of 0.03‰, and with *L. casei* Zhang at a concentration of 5.0×10^6 CFU/mL in the LcZ group. The production process of the fermented camel milk samples was as follows. Initially, camel milk was heated to 60 °C and homogenized at 20 MPa using an SRH 60–70 high-pressure homogenizer (China). Subsequently, the mixture was then pasteurized at 95 °C for 5 min and immediately cooled to 37 °C in a water bath. The samples were incubated at 37 °C for constant-temperature fermentation. Fermentation was terminated when the pH reached 4.50, and the samples were stored at 4 °C for 21 days. Three parallel samples were prepared for each group. Samples were collected on days 1, 7, 14, and 21 to measure various indicators.

2.3. Determination of viable cell counts

The method used was adapted from Sun et al. (Sun et al., 2023). The sample (1.0 g) was accurately weighed and placed in a sterilized glass bottle, to which 9 mL of 0.9 % sterile NaCl saline was added. The mixture was agitated for 15 min, after which 1 mL of the diluent was transferred into 9 mL of saline for gradient dilution. Subsequently, an appropriate dilution was then selected for pour-plating to determine the viable cell count. *Streptococcus salivarius* subsp. *thermophilus* (*S. thermophilus*) was cultured on M17 solid medium supplemented with 1.0% lactose and incubated at 42 °C for 48 h. *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. delbrueckii* subsp. *bulgaricus*) was cultured on MRS solid medium (pH = 5.2) at 37 °C for 48 h. *L. casei* Zhang was cultured in an MRS-V solid medium (pH = 6.2) supplemented with 0.01‰ vancomycin at 37 °C for 48 h. All the aforementioned solid plates were incubated under anaerobic conditions.

2.4. Determination of pH and acidity

2.4.1. pH

pH was measured using an SJ-3F pH meter (Shanghai Yi Electrical Scientific Instruments Co., Ltd., China).

2.4.2. Ta

A precisely measured 10.00 g sample of fermented camel milk was transferred into a 100 mL conical flask, followed by the addition of 20 mL of distilled water. The mixture was thoroughly agitated to ensure complete dissolution of the solid components. Subsequently, 2 drops of phenolphthalein indicator were added, and the solution was agitated until the phenolphthalein indicator transitioned from colorless to red, indicating a shift in pH from acidic to alkaline. Titration was performed using the standard sodium hydroxide titration method (0.1 mol/L) until the solution exhibited a slight red coloration that persisted for 30 s, signaling that the endpoint was reached. Finally, the volume of sodium hydroxide consumed was recorded and used to calculate TA using the following formula:

$$\mathrm{TA}\ (^{\circ}\mathrm{T}) = \mathrm{V}_{NaOH} \times C_{NaOH} \times 1000 / m_{sample}$$

2.5. Determination of insulin-like growth factor 1 (IGF-1) content

The IGF-1 content of fermented camel milk was determined according to the manufacturer's protocol provided with the kit (Shanghai Enzyme-Linked Biology Co., China). After storage, the fermented camel milk was centrifuged at 3000 × *g* for 10 min to remove suspended solids and large particles. Subsequently, 200 μL of whey from the fermented camel milk was combined with 800 μL of an acid/ethanol solution (comprising 12.5 mL of 2 mol/L HCl and 87.5 mL of anhydrous ethanol). The mixture was then incubated at 25 °C for 30 min. Following incubation, the mixture was centrifuged at 3500 *g* for 10 min. After

centrifugation, 500 μL of supernatant was collected. The solution was subsequently neutralized with 200 μL of 0.855 mol/L Tris solution. Following neutralization, the solution was diluted with IGF-1 buffer solution in accordance with the ratio specified in the manufacturer's instructions. Finally, the IGF-1 content was quantified by measuring the optical density (OD) using a spectrophotometer and comparing the results to a standard curve.

2.6. Viscosity analysis

The fermented camel milk sample was equilibrated at 25 °C to minimize temperature fluctuations that could influence viscosity measurements. Its viscosity was determined using a #2 rotor attached to a viscometer (Brookfield DV-1, Brookfield Company, USA), operated at a fixed speed of 100 rpm, with a torque range of 10–100%, over a measurement duration of 30 s. The rotor was meticulously cleaned before and after each test to ensure precision. Each sample was measured three times to ensure repeatability and reliability of the results.

2.7. Water-holding capacity analysis

The water holding capacity of fermented camel milk was determined according to a previously described method (Sahan et al., 2008). A 20 g sample of fermented camel milk was weighed and placed in a funnel lined with qualitative filter paper. The sample was maintained at 25 °C for 120 min, after which the filtrate was collected and weighed immediately.

$$\text{Water holding capacity } (\%) = 1 - \left(\frac{\text{Filtrate weight}}{\text{Sample weight}}\right) \times 100$$

2.8. Texture analysis

The hardness, viscosity index, consistency, and cohesion of the fermented camel milk samples were measured using a texture analyzer (TA. XT.plus, Stable MicroSystems Company, UK). The appropriate test probe was selected with the following parameters: pre-test speed of 1.5 mm/s, mid-test speed of 1.0 mm/s, post-test speed of 1.5 mm/s, initial force of 2.0 g, compression degree of 20%, compression time of 5 s, and test distance of 20 mm.

2.9. Untargeted metabolomics analysis

2.9.1. Sample preparation

Fermented camel milk samples were stored at −80 °C and thawed at 4 °C. Subsequently, 3 mL of the sample and 1 mL of acetonitrile (containing 400 ppm of 2-Chloro-L-phenylalanine) were added to a 5 mL centrifuge tube and thoroughly homogenized. The mixture was centrifuged at 10000 × *g* for 10 min, and the supernatant was transferred to a 2 mL centrifuge tube. The supernatant was concentrated to near dryness using a vacuum concentrator, and 500 μL of 40% (*v*/v) acetonitrile solution was added to resuspend the material. After filtration through a 0.22 μm organic microporous membrane, metabolites were determined by LC-MS (SCIEX, TripleTOF 6600, USA). Quality control (QC) samples were prepared by mixing equal volumes of each sample. QC samples were injected into the chromatographic system to ensure system stability and consistent conditions. After every 10 samples were analyzed, a QC sample was used to ensure accuracy. In addition, the effects of instrument drift and other potential issues have been corrected.

2.9.2. Experimental conditions

Pretreated fermented camel milk samples were analyzed using a UPLC system coupled with a SCIEX Q-TOF mass spectrometer (AB SCIEX 6600, USA), and each sample was analyzed in triplicate. A 1 μL aliquot of each sample was injected onto an ACQUITY HSS T3 column (Waters Corp, 2.1 mm × 100 mm × 1.8 μm; Waters, USA). The analysis was conducted at a flow rate of 0.4 mL/min and column temperature of 40 °C. *mobile* phases A (water: acetonitrile = 5:95 [v/v]) and B (acetonitrile) were prepared and stored at 4 °C prior to use. The gradient elution system employed was as follows: 1.0 min, 95% A; 6.0 min, 60% A; 18.0 min, 15% A; 18.5 min, 10% A; 22 min, 10% A; 22.5 min, 95% A; 25.0 min, 95% A, with a total run time of 25.0 min. The MS analysis was performed according to the method described by Shang et al. (Shang et al., 2022) with minor modifications. The declustering voltage was set at 40 V, gases 1 and 2 were maintained at 60 psi, and the curtain gas was set at 30 psi. The source temperature was maintained at 600 °C, and the ion spray voltage was set at 5000 V for the positive mode and −4500 V for the negative mode.

2.10. Data analysis

The raw metabolome data were imported into Progenesis QI software (Waters Corporation, USA) to generate a data matrix comprising retention time, mass-to-charge ratio, and peak intensity. To enhance the accuracy and reliability of the metabolomics data, the 'tidyverse' and 'dplyr' packages in R were used for data filtering. This process involved the following steps: (1) removing features with identical intensities across all samples, and (2) excluding features detected in more than 30 % of samples within the same experimental group. The final features were subsequently identified and classified by referencing HMDB (http://www.hmdb.ca/), Metlin (https://metlin.scripps.edu/), and other internal databases. The Human Metabolome Database (HMDB) was used for substance classification, while the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used for pathway annotation. All experiments were performed in triplicate, and one-way analysis of variance (ANOVA) was performed using R (v 4.3.0) to determine statistical significance.

3. Results

3.1. Determination of viable cell counts during storage time

The primary fermentation agent used in this experiment was YoFlex® Mild 1.0, which contained *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*. Consequently, changes in the viable cell counts of these strains were monitored throughout the storage period. As storage time increased, the viable cell counts of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* gradually decreased, with significant differences observed between the strains at different storage points ($P < 0.05$). On D21, the viable cell counts of *L. delbrueckii* subsp. *Bulgaricus* in the Control and LcZ groups were 6.39 ± 0.15 and $1.73 \pm 0.01 \times 10^6$ CFU/mL, respectively, whereas the corresponding counts for *S. thermophilus* were 1.14 ± 0.03 and $1.04 \pm 0.10 \times 10^8$ CFU/mL, respectively. Notably, on D1, the viable cell counts of *L. casei* Zhang in the LcZ group reached $6.05 \pm 0.10 \times 10^7$ CFU/mL, significantly exceeding the threshold required to confer expert-recognized probiotic benefits. Additionally, notable observations were made regarding viable cell counts of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, which were lower in the LcZ group than in the control group. This phenomenon may be attributed to the presence of *L. casei* Zhang and the competitive survival among these three strains, which utilized nutrients in the fermented camel milk, resulting in a reduced number of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* in the LcZ group compared with the control group. Interestingly, the number of *L. casei* Zhang exhibited a gradual upward trend as the storage time increased, with significant differences observed among the various time points ($P < 0.05$), warranting further investigation.

3.2. Changes of acidity and IGF-1 during storage time

Changes in acidity significantly influence the acceptability of fermented camel milk products (Ayyash et al., 2022). Throughout the 21-

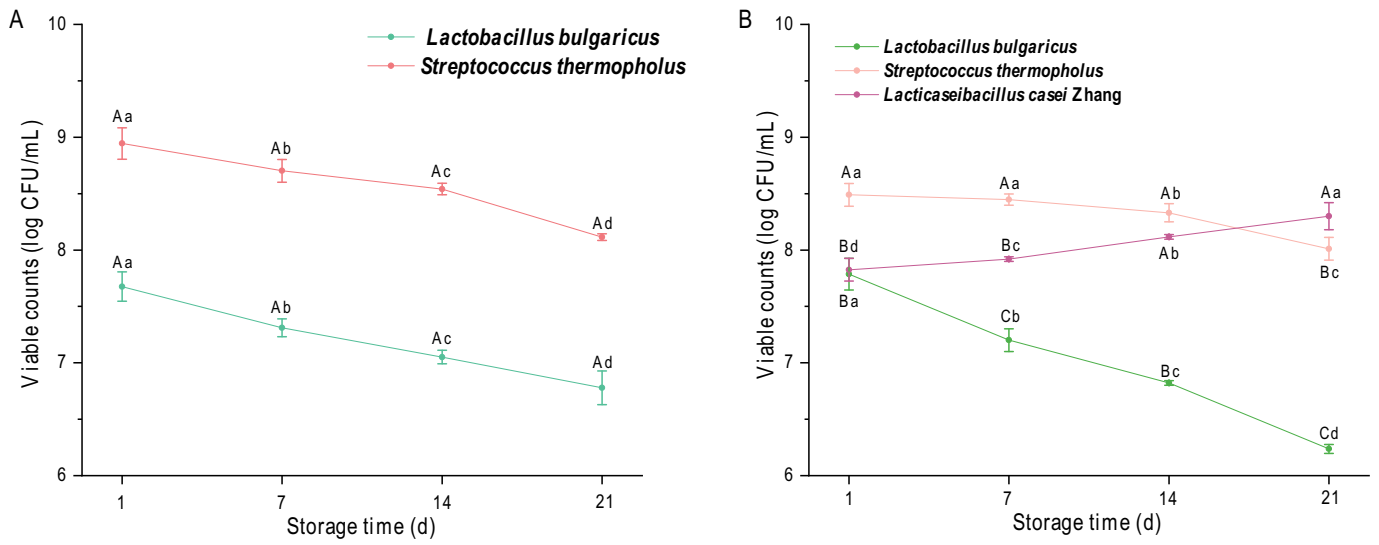

Fig. 1. Changes in viable cell counts during storage of fermented camel milk. Fig. 1A and B represent the Control and LcZ groups, respectively. Different capital letters represent differences between groups and different lowercase letters represent differences within groups. Statistical significance was set at $P < 0.05$.

day storage period, the pH of both groups of fermented camel milk exhibited a downward trend, with statistically significant differences ($P < 0.05$). The pH changed substantially in the initial storage phase (1–7 days), with the rate of decline decelerating after 14 days of storage, as illustrated in Fig. 2A. The pH of the LcZ group was higher than that of the control group, although the difference was not statistically significant ($P > 0.05$), indicating that the addition of *L. casei* Zhang mitigated the downward trend in pH. The TA of both groups of fermented camel milk exhibited an increasing trend throughout the storage period, reaching peak values at D21 of 125 ± 2.13 and 97.5 ± 1.39 °T for the Control and LcZ groups, respectively. Notably, significant differences in TA were observed between the two groups throughout the storage period ($P < 0.05$). Donkor posited that 70–110 °T was the optimal range for fermented milk (Donkor et al., 2006), and fermented milk exhibited superior organoleptic properties at this point. This suggests that the fermented camel milk in the control group may not meet consumer expectations. In contrast, the incorporation of *L. casei* Zhang appeared to mitigate the formation of acidic compounds, maintaining the acidity within an optimal range, thereby improving its stability and sensory acceptability throughout the storage period.

IGF-1 content in each sample of the Control and LcZ groups during storage was quantified. The IGF-1 content of camel milk was established as the baseline (100%) and the activity retention rate of IGF-1 after various storage periods was calculated. Changes in IGF-1 activity retention rate during storage were also investigated. This primary hypoglycemic effect of camel milk and fermented dairy products is attributed to their IGF-1 content. The results indicated that, as storage time increased, the IGF-1 activity retention rate in fermented camel milk gradually decreased. After 21 days of storage, the IGF-1 activity retention rate in the control group decreased to 54.95 ± 1.68%, whereas that in the LcZ group was 59.13 ± 2.58%. Throughout the storage period, IGF-1 activity retention rate in the LcZ group consistently exceeded that in the control group. Although this difference was not statistically significant ($P > 0.05$), the addition of *L. casei* Zhang enhanced the IGF-1 activity retention rate.

3.3. Changes in viscosity and water-holding capacity during storage time

Viscosity is a crucial parameter for assessing the quality of fermented camel milk, as high-quality fermented milk typically exhibits a semi-solid consistency with a certain degree of firmness. However, camel milk inherently resists coagulation, presenting a significant challenge for its utilization in the production of fermented dairy products. The changes in the viscosity of the two groups of fermented camel milk samples during storage are shown in Fig. 3A. As the storage duration increased, the viscosity of the fermented camel milk decreased to varying degrees, remaining below 80 mPa·s, whereas the viscosity of the control group remained below 20 mPa·s throughout the storage period. This study revealed that the addition of *L. casei* Zhang facilitated the formation of a gel structure in fermented camel milk, significantly enhancing its viscosity ($P < 0.05$). However, this improvement has limited practical significance, owing to the distinctive properties of camel milk. To address this limitation, future studies should explore the incorporation of additives, such as pectin to enhance the viscosity of fermented camel milk. Notably, the gel structure of fermented milk plays a crucial role in its water-holding capacity (Zhang et al., 2024). Insufficient water-holding capacity can lead to whey precipitation in fermented camel milk, consequently affecting the texture and organoleptic properties of the product. As depicted in Fig. 3B, the water-holding capacity of both groups of fermented camel milk samples exhibited a declining trend from days 1 to 7, with no significant changes observed thereafter ($P > 0.05$). Additionally, the incorporation of *L. casei* Zhang reduced the water-holding capacity of fermented camel milk; however, this alteration did not result in excessive syneresis of the whey.

3.4. Textural changes in fermented camel milk during storage time

This study assessed the texture indices of fermented camel milk samples in the Control and LcZ groups during storage and investigated the effect of adding *L. casei* Zhang on the quality of fermented camel milk. The measured parameters included the hardness, cohesion, consistency, and viscosity index. These indicators reflect the textural characteristics of fermented milk and influence its organoleptic properties. The fat and protein content of camel milk, type and concentration of fermentation agent, and fermentation time all significantly impact the quality of fermented camel milk. However, in this study, only the post-acidification of fermented camel milk and the effect of *L. casei* Zhang on its texture during storage were considered. The addition of probiotic *L. casei* Zhang enhances the hardness of fermented camel milk, potentially due to the production of extracellular polysaccharides, which improve texture (Bai et al., 2021). After 21 days of storage, the viscosity index of fermented camel milk samples in the Control and LcZ groups reached peak values of 1.59 ± 0.01 and 1.54 ± 0.05 g·s, respectively. A higher viscosity index indicates that liquid viscosity is less affected by fluctuations in temperature, whereas a lower viscosity index indicates that viscosity is more sensitive to temperature, resulting in significant

Table 1
Pathways enriched by differential metabolites at each storage time point.

Enrichment pathway	Class	Time
Amino sugar and nucleotide sugar metabolism	Carbohydrate Metabolism	D1
Fructose and mannose metabolism	Carbohydrate Metabolism	D1
Biosynthesis of unsaturated fatty acids	Lipid Metabolism	D7
Butanoate metabolism	Other	D7
Citrate cycle (TCA cycle)	Carbohydrate Metabolism	D7
Propanoate metabolism	Other	D7
Tryptophan metabolism	Amino Acid Metabolism	D7
Glycerophospholipid metabolism	Lipid Metabolism	D21
beta-Alanine metabolism	Amino Acid Metabolism	D1,D7
Cysteine and methionine metabolism	Amino Acid Metabolism	D1,D7
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	Other	D1,D7
Pantothenate and CoA biosynthesis	Metabolism of Cofactors and Vitamins	D1,D7
Retinol metabolism	Metabolism of Cofactors and Vitamins	D1,D21
Riboflavin metabolism	Metabolism of Cofactors and Vitamins	D1,D21
Caffeine metabolism	Other	D7,D14
Alanine, aspartate and glutamate metabolism	Amino Acid Metabolism	D1,D7, D14
Arginine and proline metabolism	Amino Acid Metabolism	D1,D7, D21
Galactose metabolism	Carbohydrate Metabolism	D1,D7, D21
Pentose phosphate pathway	Carbohydrate Metabolism	D1,D7, D21
Phenylalanine metabolism	Amino Acid Metabolism	D1,D7, D21
Phenylalanine, tyrosine and tryptophan biosynthesis	Amino Acid Metabolism	D1,D7, D21
Tyrosine metabolism	Amino Acid Metabolism	D1,D7, D21
Ubiquinone and other terpenoid-quinone biosynthesis	Lipid Metabolism	D1,D7, D21
Purine metabolism	Nucleotide Metabolism	D1,D7, D14,D21
Pyrimidine metabolism	Nucleotide Metabolism	D1,D7, D14,D21

Enrichment pathway, pathways enriched through the KEGG database; Class, classification of pathways; Time, D1, D7, D14, and D21 present the difference pathways between Control and LcZ groups at day 1, day 7, day 14, and day21.

changes. This suggests that the addition of *L. casei* Zhang increased the viscosity of fermented camel milk but reduced its viscosity index, rendering it more sensitive to temperature.

3.5. Metabolomics analysis of fermented camel milk during storage

Based on previous results, the addition of *L. casei* Zhang improved the IGF-1 retention rate, decelerated the decrease in pH during storage, and enhanced the water-holding capacity and viscosity of fermented camel milk. To elucidate the mechanisms underlying these changes, metabolomic alterations in fermented camel milk in the control and LcZ groups were investigated throughout the storage period. This study primarily examined the changes induced by the addition of *L. casei* Zhang to fermented camel milk, focusing on the differences between the control and LcZ groups at various storage time points, and identifying the metabolites responsible for these differences. As illustrated in Fig. 5E, 61 differential metabolites (VIP > 1.0, $P < 0.05$) appeared only once during storage, with the highest number of unique metabolites (45) observed on D7. As depicted in Fig. 5A-D, after 1, 7, 14, and 21 days of storage, the addition of *L. casei* Zhang resulted in 69, 102, 23, and 59 metabolites, respectively, which differed significantly from the control group ($P < 0.05$). According to the HMDB database classification, these compounds primarily belong to amino acids, peptides, and analogs, as well as carbohydrates and carbohydrate conjugates. Previous research has demonstrated that when *L. casei* Zhang reaches the late stage of growth in milk, the majority of the significantly altered genes are related to sugar metabolism, amino acid transport, and metabolism (Wang et al., 2012). Subsequently, KEGG pathway enrichment analysis was performed for the differential metabolites. As shown in Table 1, the incorporation of *L. casei* Zhang influenced 25 metabolic pathways, including carbohydrate, lipid, amino acid, cofactor, vitamin, and nucleotide metabolism. These findings align with those of previous studies, suggesting that the addition of *L. casei* Zhang enhances amino acid and carbohydrate metabolism. Furthermore, this study identified significant impacts on vitamin and nucleotide metabolism, a result that distinguishes this study from previous studies. Further analysis of these metabolic pathways revealed that purine and pyrimidine metabolism consistently appeared at each storage time point, suggesting that these two pathways may play a central role in the continuous changes induced by the addition of *L. casei* Zhang.

3.6. Core differential metabolites

The addition of *L. casei* Zhang induced alterations in the metabolome of fermented camel milk. To elucidate the relationship between the changes in numerous differential metabolites, this study focused on the metabolites that differed at each storage time point following the addition of *L. casei* Zhang, which were defined as core differential metabolites. In total, 6 core differential metabolites were identified: adenosine diphosphate (ADP), oleuropein, arginyl-arginine (Arg-Arg), isoleucyl-proline (Iso-Pro), lysyl-lysine (Lys-Lys) and threoninyl-tryptophan (Thr-Try). The latter four metabolites are dipeptides, which are typically derived from protein digestion, absorption, and degradation, as well as from a combination of free amino acid enzymatic reactions (Minen et al., 2023). As illustrated in Fig. 6A and F, the ADP and Thr-Try levels in the LcZ group were significantly higher than those in the control group at each storage time point ($P < 0.001$), indicating that the addition of *L. casei* Zhang significantly increased ADP and Thr-Try levels in fermented camel milk. The Thr-Try levels in the LcZ and control groups followed a similar trend, reaching their peak at D7 and subsequently decreasing to 118.52 ± 3.59 and 11.71 ± 2.10, respectively. Except for oleuropein, the other core differential metabolites exhibited an overall upward trend following the addition of *L. casei* Zhang to the fermented camel milk.

4. Discussion

During the fermentation and storage periods, the pH of the fermented milk decreased due to the continuous production of lactic acid by *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*. After one day of storage, the pH of the control and LcZ groups fell below 4.50, which is outside the optimal range for the growth of these strains. Consequently, the viable cell counts of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* gradually decreased throughout the storage period. The combined fermentation of the two strains led to an increased in the production of amino acids and oligopeptides, which are precursors of flavor compounds. This, in turn, promoted the synthesis of additional flavor compounds, enhancing the flavor and taste of yogurt (Kaneko et al., 2014). Previous research has demonstrated that the addition of *L. casei* Zhang, either alone or in combination with *Bifidobacterium lactis* V9, can effectively shorten the fermentation time, reduce the loss of IGF-1 (Wang et al., 2024), and contribute to functional products targeting hypertension and diabetes. Furthermore, these strains have been shown to significantly influence purine metabolism, offering valuable insights into the role of *L. casei* Zhang in driving metabolic changes in fermented camel milk during storage. For fermented camel milk to confer the health benefits of *L. casei* Zhang, it must contain a minimum of one million viable cells per gram of product. This level must be maintained throughout the shelf life, rather than at a specific point in time. Our

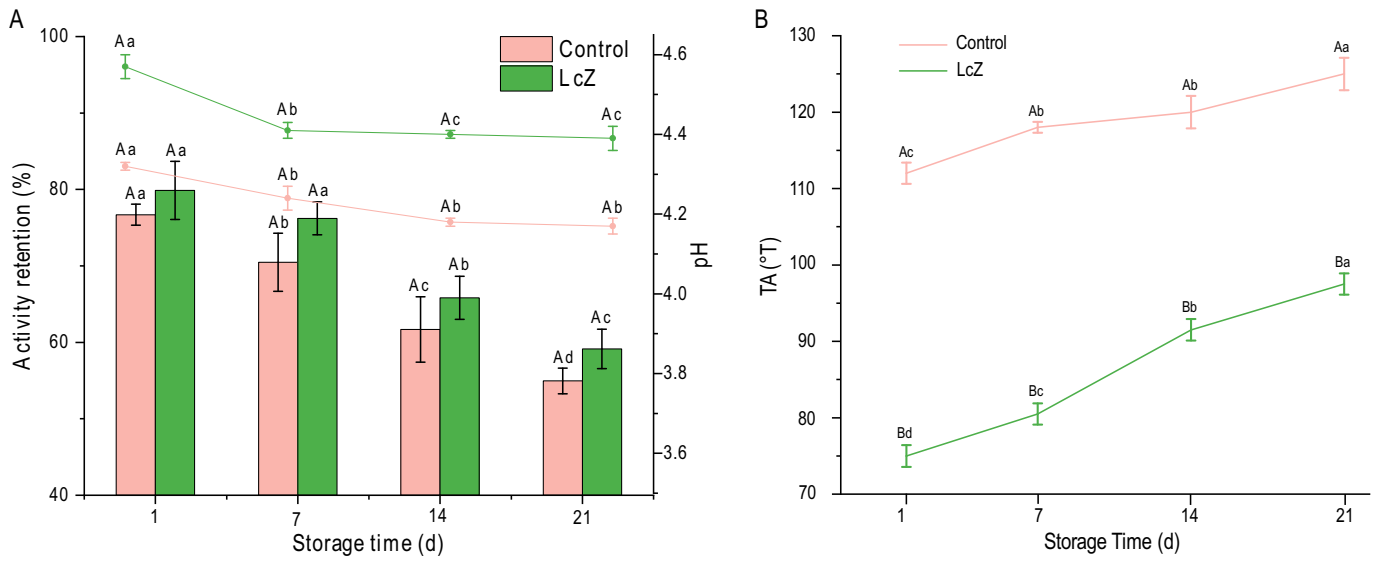

Fig. 2. Changes in pH, TA, and IGF-1 during storage of fermented camel milk. The broken line in Fig. 2A represents pH, the bar graph represents the IGF-1 activity retention rate (with camel milk set at 100 %), and Fig. 2B represents the trend of TA changes during storage. Different capital letters indicate differences between groups, whereas different lowercase letters indicate differences within groups. Statistical significance was defined as $P < 0.05$.

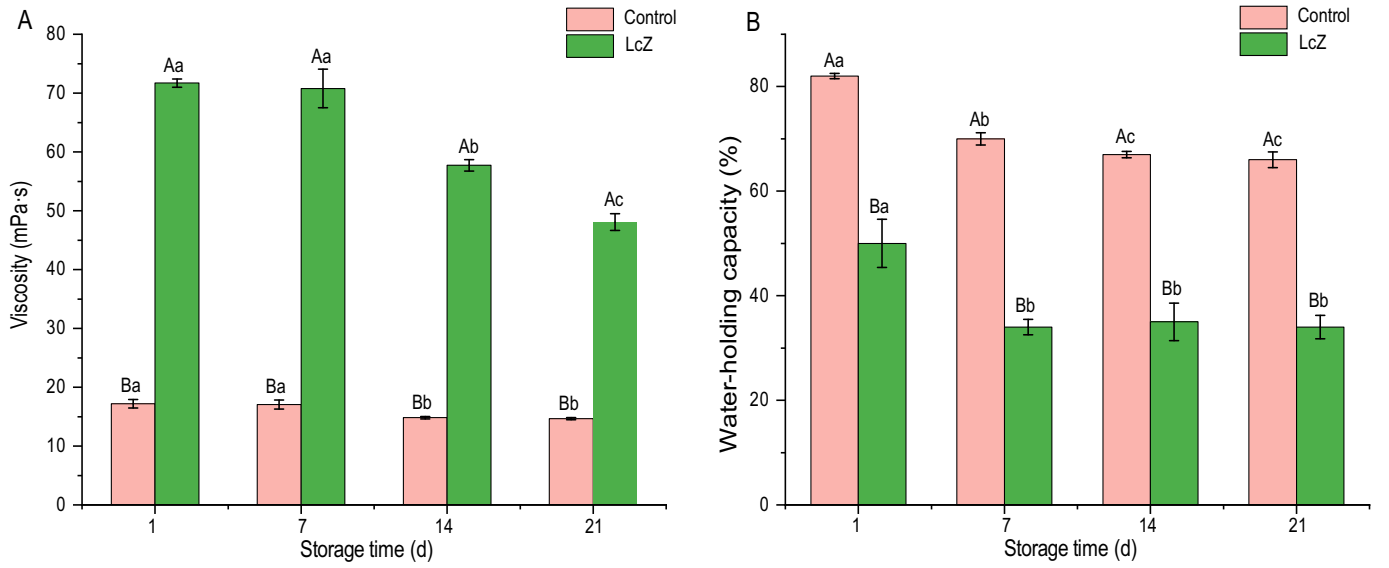

Fig. 3. Changes in viscosity and water-holding capacity of fermented camel milk during storage. Fig. 3A and Fig. 3B show the changes in the viscosity and water-holding capacity of fermented camel milk during storage. Different capital letters represent differences between groups, and different lowercase letters represent differences within groups. Statistical significance was defined as $P < 0.05$.

study demonstrated that the incorporation of *L. casei* Zhang decreased the number of viable *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*. This phenomenon occurs because *L. casei* Zhang competes these strains for nutrients in camel milk, such as lactose, amino acids, and vitamins, leading to reduced growth due to nutrient limitation during storage. Previous research has demonstrated that with increased storage time, regardless of the matrix being milk, goat milk, or a mixture thereof, the viable cell counts of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* exhibit a downward trend (Dimitrellou et al., 2019), which is consistent with our findings. In the later stages of storage, as the viable cell counts of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* and the pH decreased, the growth of *L. casei* Zhang was not significantly inhibited. As a result, the viable cell counts of *L. casei* Zhang exhibited a consistent upward trend throughout the storage period.

Our findings revealed that not all strains exhibited beneficial effects on fermented camel milk. For instance, when certain strains were used as starter cultures, the pH remained consistently low (around 3.3) throughout the storage period (Ayyash et al., 2018), rendering the product unsuitable for consumer consumption. These observations underscore the importance of thoroughly investigating the changes in fermented camel milk during storage to ensure its quality and acceptability. Throughout the storage period, the pH exhibited a decreasing trend (Fig. 2A). At the end of storage, the pH of the LcZ group was 4.39 $\pm$ 0.03, which was significantly higher than that of the control group ($P < 0.05$), indicating that *L. casei* Zhang can mitigate post-acidification in fermented camel milk when co-fermented with the commercial starter YoFlex® Mild 1.0. The researchers found that after adding *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus casei*, or *Lactiplantibacillus plantarum*, the pH of fermented camel milk dropped more rapidly, but its antioxidant capacity and flavor were better (Shori, 2024), indicating that the addition of *L. casei* Zhang may also help improve the probiotic properties of fermented camel milk. This may be due to the higher

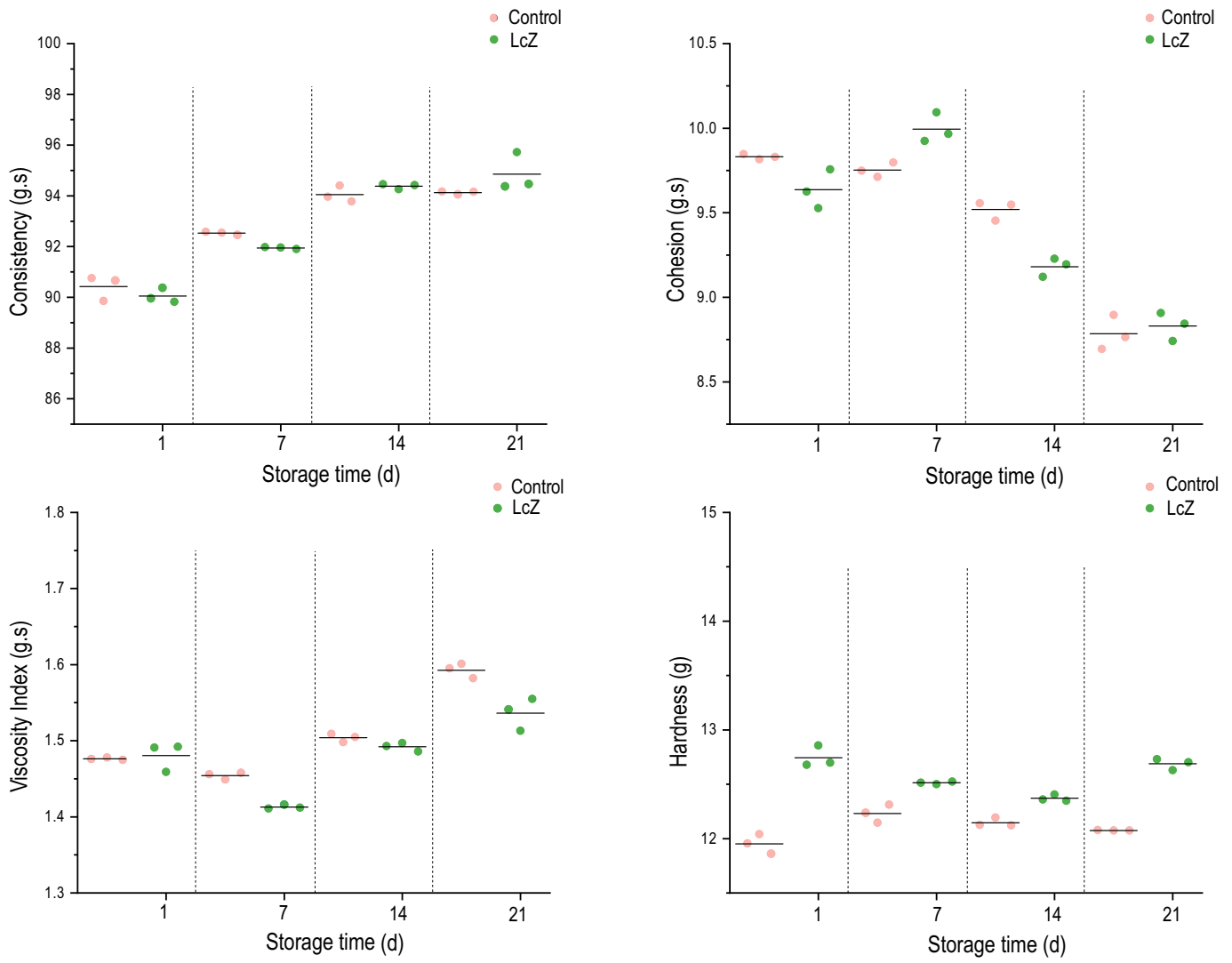

Fig. 4. Texture changes in fermented camel milk during storage. Fig. 4A-D display the consistency, cohesion, viscosity, and hardness. No significant differences were observed between the two groups at any time point ($P > 0.05$).

abundance of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* in the control group, which is capable of producing a larger number of acidic compounds, leading to a continuous decrease in pH, lower than that of the LcZ group. The pH of most fermented milk products ranges from 4.0 to 4.6. This pH range is ideal for fermenting camel milk, as it not only imparts a distinctive flavor to the milk, but also inhibits contamination by undesirable strains owing to the lower pH. Therefore, the pH of the both groups remained within the optimal range for shelf life throughout the storage period. As shown in Fig. 2B, titrated acidity (TA) in both groups increased over time, with statistically significant differences ($P < 0.05$), consistent with pH changes. Previous studies utilizing *L. casei* Zhang in yogurt observed that as storage time increased, pH decreased, whereas TA increased (Wang et al., 2021), which aligns with the findings of this study. The pH and TA results indicate that *L. casei* Zhang exhibits weak acid-producing ability, which may contribute to extending the shelf life of fermented camel milk and possesses potential application value. IGF-1 is structurally like insulin, promotes glucose utilization, and enhances insulin sensitivity, thereby aiding in blood sugar regulation. They also play indispensable roles in growth and development (Bailes & Soloviev, 2021). Under acidic conditions, IGF-1 undergoes protein denaturation, which results in decreased activity (Bhalla et al., 2022). Therefore, exploring the activity of IGF-1 during the storage of fermented camel milk is essential as it is a prerequisite for its efficacy. Throughout the storage period, the IGF-1 activity retention rate in the LcZ group was higher than that in the control group, although the difference was not statistically significant ($P > 0.05$). This observation indicated that when the pH was between 4 and 6, there was no significant effect on the activity of IGF-1. The activity of IGF-1 in both groups remained above 50%, demonstrating that fermented camel milk can serve as an effective carrier for IGF-1. The researchers found that *Lactococcus lactis* KX881782, when applied to fermented camel milk, can effectively inhibit α-glucosidase and help reduce blood sugar (Ayyash et al., 2018). This finding is consistent with the results of this study, indicating that the addition of *L. casei* Zhang can reduce the loss of IGF-1 and contribute to its hypoglycemic effects.

Camel milk exhibits difficulty in curd formation during fermentation because of its low casein and calcium ion content. This study incorporated *L. casei* Zhang into YoFlex® Mild 1.0 to investigate the effects of *L. casei* Zhang on the viscosity and water-holding capacity of fermented camel milk. The viscosity of both groups decreased during storage, with the viscosity of the LcZ group being significantly higher than that of the control group ($P < 0.05$), indicating that the addition of *L. casei* Zhang facilitated the formation of a stable gel structure in the fermented camel milk. The *Lactiplantibacillus plantarum* MWLp-12 and *Limosilactobacillus fermentum* MWLf-4 strains were introduced into milk, and it was observed that the addition of these two strains promoted protein

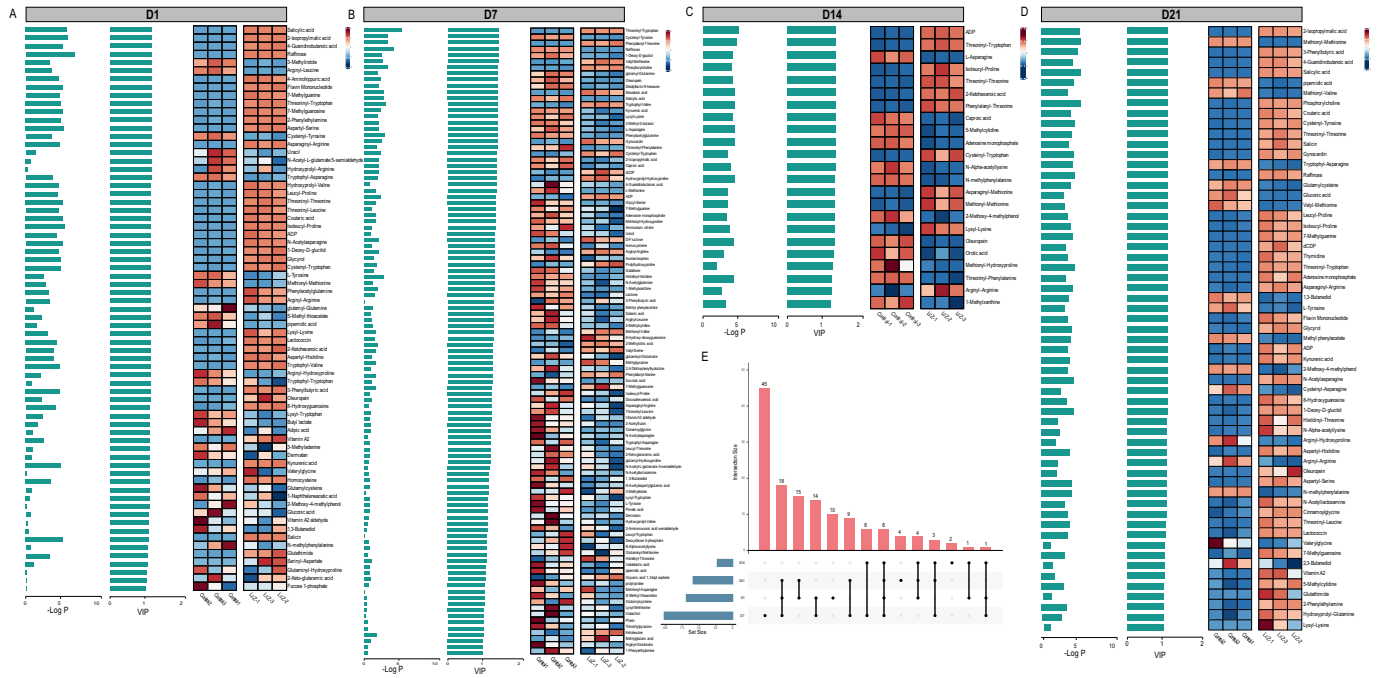

Fig. 5. Differential metabolites identified in fermented camel milk after 1, 7, 14, and 21 d of storage. Figs. 5A-D display the heat maps of differential metabolites between the Control and LcZ groups on days 1, 7, 14, and 21, whereas Fig. 5E presents the upset plot of differential metabolites between the Control and LcZ groups at each time point.

hydrolysis, influenced the water retention and network structure of the protein, and enhanced the textural characteristics of fermented milk (Wang et al., 2022). In this study, the gradual decrease in pH throughout storage suggests that *L. casei* Zhang possesses a limited capacity to produce acid, resulting in a gradual decrease in pH throughout the storage period. As the pH decreases, the colloidal calcium phosphate in fermented camel milk continues to dissolve, causing alterations in the cross-linked structure of the protein, thereby leading to whey syneresis and ultimately manifesting as a decrease in viscosity. However, the viscosity of the fermented camel milk prepared in this study was lower than 80 mPa·s, substantially below 900 mPa·s, which is considered to have the optimal viscosity according to Li et al. (Li et al., 2020). This discrepancy can be attributed to the inherent characteristics of camel milk. Future research should consider the addition of coagulants to increase the viscosity of fermented camel milk.

The three-dimensional network structure of casein undergoes continuous modifications in its gel structure during fermentation and storage of fermented camel milk, consequently altering its water-holding capacity. Whey precipitation occurs when the water holding capacity is insufficient, thereby affecting the texture and organoleptic properties of the product. Previous research has demonstrated that yogurt subjected to high-amplitude ultrasound treatment exhibits a significantly enhanced water-holding capacity and viscosity, as well as reduced syneresis compared with non-sonicated samples (Wu et al., 2000). Consequently, we incorporated high-pressure homogenization into the experimental protocol to ensure that the fermented camel milk maintained desirable organoleptic properties during the storage period. As shown in Fig. 3B, the water holding capacity of the control group ranges between 65 and 85 %, which was significantly different from that of the LcZ group ($P < 0.05$). The water-holding capacity of the LcZ group was lower than that of the control group, likely due to the stable network structure formed by the interaction of casein and microbial metabolites in the gel. As the storage duration increased, the bacterial content in fermented camel milk decreased. The bacterial content (including viable and non-viable bacteria) was higher in the LcZ group than in the control group. The accumulation of bacteria increases the viscosity of the sample, which can be readily disrupted in an acidic environment, thereby reducing the water-holding capacity of the fermented camel milk. Additionally, the degradation of hydrophobia subunits by lactic acid bacteria enhances the electrostatic repulsion within the gel, resulting in uneven distribution of gel particles and a coarse microstructure, which further diminishes the water-holding capacity of the gel.

These results demonstrated that the incorporation of *L. casei* Zhang enhanced the viscosity of fermented camel milk, increased the retention of IGF-1 activity, and reduced the rate of acidity change. To explore the mechanisms underlying these effects, we investigated the metabolites present in fermented camel milk. The study revealed that the addition of *L. casei* Zhang primarily altered the production of amino acids, peptides, and analogs, as well as carbohydrates and carbohydrate conjugates. During camel milk fermentation, *S. thermophilus* metabolizes lactose to produce lactic acid, thereby lowering the pH and facilitating the growth and protein utilization of *L. delbrueckii* subsp. *bulgaricus*. Concurrently, *L. delbrueckii* subsp. *bulgaricus* promotes the growth of *S. thermophilus* through the secretion of nutrients, such as amino acids. In this process, the protease produced by the strain hydrolyzes the proteins into amino acids and peptides. The proteases secreted by different strains exhibit varying specificities, resulting in the production of distinct final products. This phenomenon may account for the observed differences in amino acids, peptides, and analogs as differential metabolites in the present study. These strains exhibit varying utilization efficiencies and metabolic pathways for carbohydrates, leading to a diversity of metabolites that subsequently influence the pH and texture of fermented camel milk (Li et al., 2020). *L. casei* Zhang, an extensively studied probiotic strain, has been shown to produce various beneficial substances, including capric acid, caprylic acid, caproic acid, and valeric acid when incorporated into yogurt (Peng et al., 2022). The present study found that the addition of *L. casei* Zhang resulted in increased concentrations of Thr-Try, ADP, Thr-Thr, Iso-Pro, Cys-Try, and Arg-Arg in fermented camel milk.

KEGG enrichment analysis was performed on the differential metabolites at each time point (Table 1). The results indicated that two metabolic pathways, purine, and pyrimidine, were consistently present at each storage time point, suggesting that the addition of *L. casei* Zhang

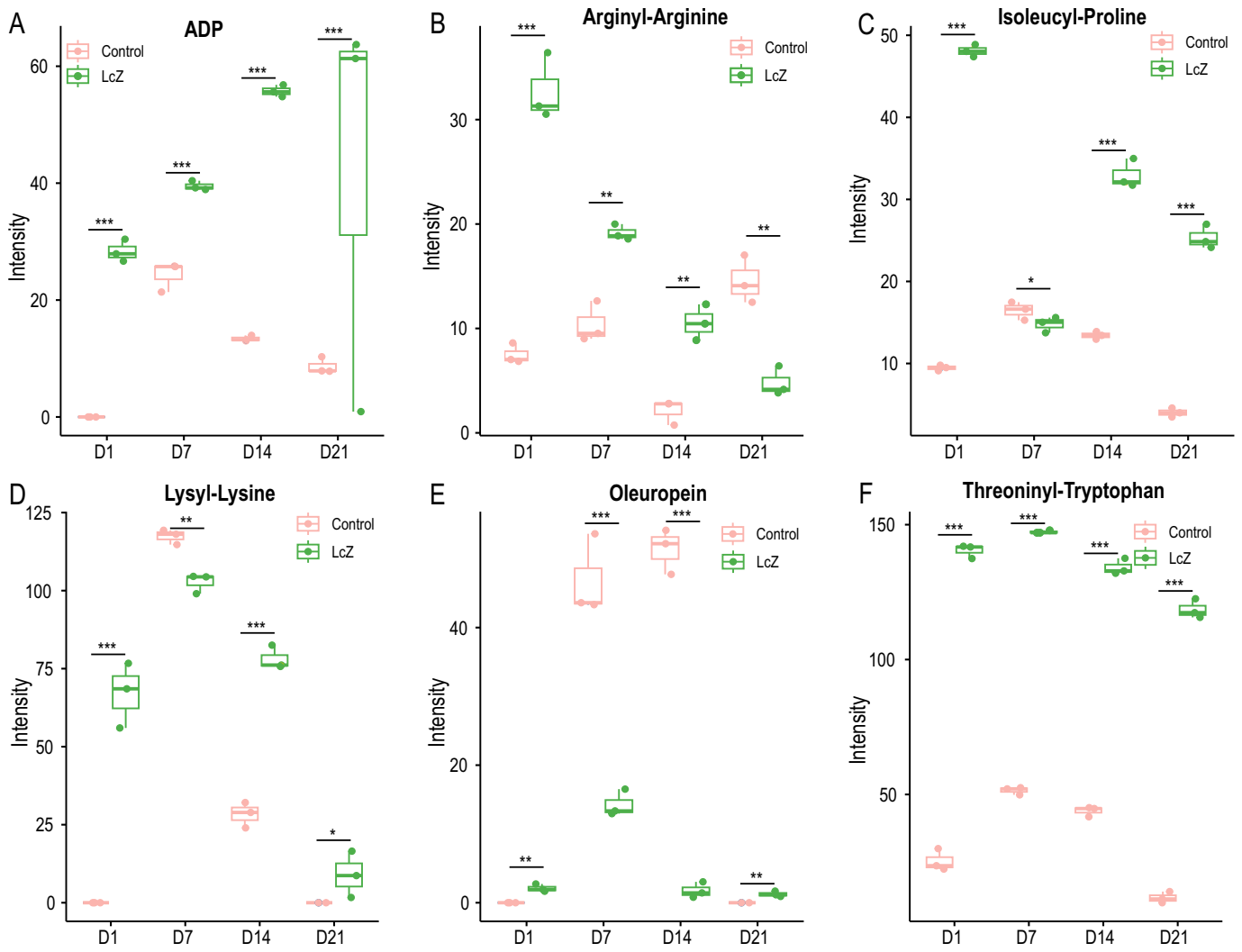

Fig. 6. Core differential metabolites identified after 1, 7, 14, and 21 days of storage. Fig. 6A-F show ADP, Arg-Arg, Iso-Pro, Lys-Lys, oleuropein, and Thr-Try, respective. Significant differences were evaluated using *t*-tests; *: $P < 0.05$, **: $P < 0.01$, and ***: $P < 0.001$.

significantly influenced these pathways in fermented camel milk. Purine metabolism is a critical biochemical metabolic pathway involved in the synthesis, degradation, and recycling of purine, which provides energy for cells and participates in intracellular signal transduction (Yin et al., 2018). A detailed analysis of all metabolites within this pathway, it was determined that the difference in purine metabolism was attributable to changes in ADP production (Fig. 6A), with the addition of *L. casei* Zhang significantly increased the ADP content of the fermented camel milk ($P < 0.05$). This phenomenon may be attributed to the larger total number of viable cells in the LcZ group, which consumed more nutrients, thereby promoting the conversion of ATP to ADP and leading to ADP accumulation. ADP is a central molecule in cellular energy metabolism, in which ATP is converted to ADP during the energy consumption processes, subsequently releasing energy. An increase in ADP content leads to a reduction in cellular ATP levels, consequently inhibiting purine synthesis, which may have beneficial implications for patients with uric acid-related disorders and other conditions (James et al., 2023). Pyrimidine metabolism plays a crucial role in nucleic acid synthesis and cellular metabolic regulation. Disruptions in this metabolic pathway can result in various pathological conditions including cancer and immunodeficiency. Hao et al. (Hao et al., 2023) observed that purine metabolism, pyrimidine metabolism, and ABC transporters were involved in the storage of fermented milk beverages. Purine metabolism is essential for LAB growth, as well as the transformation and synthesis of primary and secondary metabolites, which aligns with the core differential metabolic pathways identified in this study.

Subsequently, the differential metabolites of the Control and LcZ groups at various storage time points were analyzed (Fig. 5). These results indicated that the addition of *L. casei* Zhang altered the metabolic profile of fermented camel milk. Notably, several differential metabolites were observed at the four storage time points, specifically ADP, oleuropein, Arg-Arg, Ile-Pro, Lys-Lys, and Thr-Try (Fig. 6). Arg-Arg, Ile-Pro, Lys-Lys, and Thr-Try are dipeptides formed by the linkage of two amino acids through peptide bonds. These molecules serve as the basic building blocks of proteins and polypeptide chains and are prevalent, particularly in partially hydrolyzed or fermented foods, such as fermented dairy products and soy sauce. These dipeptides have potential applications as therapeutic agents for the prevention and mitigation of diabetes and its associated complications (Freund et al., 2018). Furthermore, they are widely utilized because of their distinctive flavor profiles and nutritional properties, as exemplified by aspartame. Cakmak et al. hypothesized that Ile-Pro is associated with the activity of proline enzymes involved in collagen degradation during airway remodeling (Cakmak et al., 2009). Concurrently, Ile-Pro possesses antioxidant properties and enhances bone mineral density. Notably, changes in Ile-Pro concentrations exhibited contrasting trends between the two groups. The control group initially increased to its highest value before subsequently declining, whereas the LcZ group showed an initial

decrease followed by an increase. The underlying mechanism of this phenomenon was not investigated in the present study and can be explored in subsequent experiments. The potential function of Thr-Try remains undetermined and merits investigation by additional researchers in future studies. Alanine, tyrosine, phenylalanine, and tryptophan have all been identified as amino acids that occupy terminal positions in antihypertensive peptide structures (Aguilar-Toalá et al., 2020). The addition of *L. casei* Zhang significantly increased Thr-Try content, which may confer health benefits.

The findings of this study demonstrated that the addition of *L. casei* enhanced IGF-1 activity and facilitated the production of beneficial metabolites. These results suggest that *L. casei* Zhang holds significant potential for application in fermented camel milk beverages and other dairy products, warranting further evaluation of its performance and adaptability. To achieve this, an in-depth investigation of the relationship between specific metabolites and their health effects is essential, necessitating comprehensive animal and human studies. Our ultimate goal is to develop *L. casei* Zhang as a commercially viable starter culture for fermented dairy products. However, challenges, such as cost management and broad consumer acceptance, must be addressed. Although this study represents a significant step forward, ongoing research will be crucial to overcoming these hurdles and further advance the field.

5. Conclusion

This study evaluated the effect of *L. casei* Zhang supplementation on the quality attributes and metabolomic profiles of fermented camel milk during storage. This study demonstrated that the concentration of *L. casei* Zhang in fermented camel milk consistently reached the recommended standard concentration (10^6 CFU/mL) of probiotics in functional dairy products. Additionally, supplementation with *L. casei* Zhang enhanced the IGF-1 activity retention rate from 54.95% to 59.13%, suggesting that fermented camel milk could effectively serve as a carrier for IGF-1. Supplementation with *L. casei* Zhang also had a minimal impact on post-acidification and did not negatively affect the viscosity, water-holding capacity, or texture of the camel milk. Subsequently, the metabolomics of fermented camel milk was explored. Metabolomic analysis further revealed significant alterations in the production of amino acids, peptides, analogs, and carbohydrate conjugates, indicating modulation of the metabolic profile of fermented camel milk. Moreover, the application of *L. casei* Zhang to commercial starter cultures further verified its potential as an auxiliary starter for augmenting ADP, Thr-Try, and Iso-Pro levels in fermented camel milk. In summary, *L. casei* Zhang has broad application prospects in the development of fermented camel milk products with enhanced nutritional and therapeutic properties.

CRediT authorship contribution statement

Dandan Wang: Writing – review & editing, Writing – original draft, Conceptualization. **Wusigale:** Visualization, Investigation. **Lu Li:** Software, Methodology. **Lu Bai:** Methodology, Data curation. **Yongfu Chen:** Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this study.

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Data availability

Data will be made available on request.

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