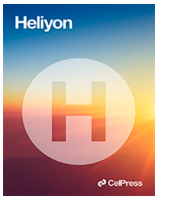
Heliyon 9 (2023) e18522

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Analysis of metabolites of bactrain camel milk in Alxa of China before and after fermentation with fermenting agent TR1 based on untargeted LC-MS/MS based metabolomics

Weisheng Xu [1], Qigeqi Dong [*,1], Guofen Zhao [**], Bing Han [***]

Key Lab of Germplasm Innovation and Utilization of Triticeae Crops at Universities of Inner Mongolia Autonomous Region, College of Life Sciences, Inner Mongolia Agricultural University, Hohhot 010011, People's Republic of China

ARTICLE INFO

Keywords:
Bactrain camel milk
Fermentation
LC-MS/MS
Untargeted metabolomics
KEGG

ABSTRACT

Camel milk produces many beneficial functional compounds and affects body health through metabolism. The differential metabolites of bactrain camel milk in Alxa before and after fermentation were identified by liquid chromatography-tandem mass spectrometry based metabolomics (LC-MS/MS). The differential metabolite pathway types were also identified in this paper. We obtained the following results that 148 and 82 differential metabolites were detected in positive and negative ion mode respectively, 85 differential metabolites were shown a significant upward trend and 63 with downward trend after fermentation in positive ion mode. Meanwhile, 32 differential metabolites characterized upward trend and 50 characterized downward trend in negative ion mode. The differential metabolites were mainly organic acids, amino acids, esters, vitamins and other substances contained in camel milk. Among them, most up-regulated substances had the functions of lowering blood pressure, lowering blood sugar, treatment of inflammation, antibiosis and other effects. Many harmful substances were significantly down-regulated after camel milk fermentation. However, there were also some metabolites whose prebiotic functions have been weakened by camel milk fermentation, which may provide reference values for healthcare function, exploitation and application of camel milk.

1. Introduction

Camels mainly exist in Asian and African countries such as China, Sudan, Somalia, Ethiopia, Mongolia, etc. Recognized as the "Hometown of Camels in China", Alxa in China has the largest population of bactrain camels with tall size and strong resistance to stress. Certain recent, the researches on camel industry and its products have gain great attention, with camel milk emerging as the most absorbing products. Being enrich in many essential nutrition such as peptides, lactoferrin, and fatty acids [1], etc., camel milk shows high medical value and antibacterial function. To date, the camel milk have been extensively studied in terms of medical value and microecology. Camel milk was used as a useful medicine for the treatment of acute food allergies in children long before. To date, it

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: dongqigeqi@imau.edu.cn (Q. Dong), guofenzhao@126.com (G. Zhao), hb_nmg@163.com (B. Han).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.heliyon.2023.e18522
Received 29 May 2023; Received in revised form 18 July 2023; Accepted 19 July 2023
Available online 21 July 2023

has been evidenced that camel milk can treat asthma, diabetes, tuberculosis, etc. A report by Muleta et al. demonstrated that camel milk intervention can reduce the incidence of anaemia in children in Somalia and eastern Ethiopia [2]. Additionally, camel milk is rich in phytochemicals with anti-cancer, anti-platelet and anti-thrombotic properties, and is beneficial in the treatment of chronic hepatitis and the improvement of liver function [3,4]. For instance, Korish and coworkers have demonstrated that full fat cow milk and camel milk have hypoglycemic and anticoagulant effects on diabetic rats induced by streptozotocin [5].

In addition to fresh camel milk, the fermented camel milk is more favorable in some countries for its contribution on beauty, whitening and health care. Typically, the camel milk fermentation was processed with the assistance of lactic acid bacteria (LAB) [6,7].

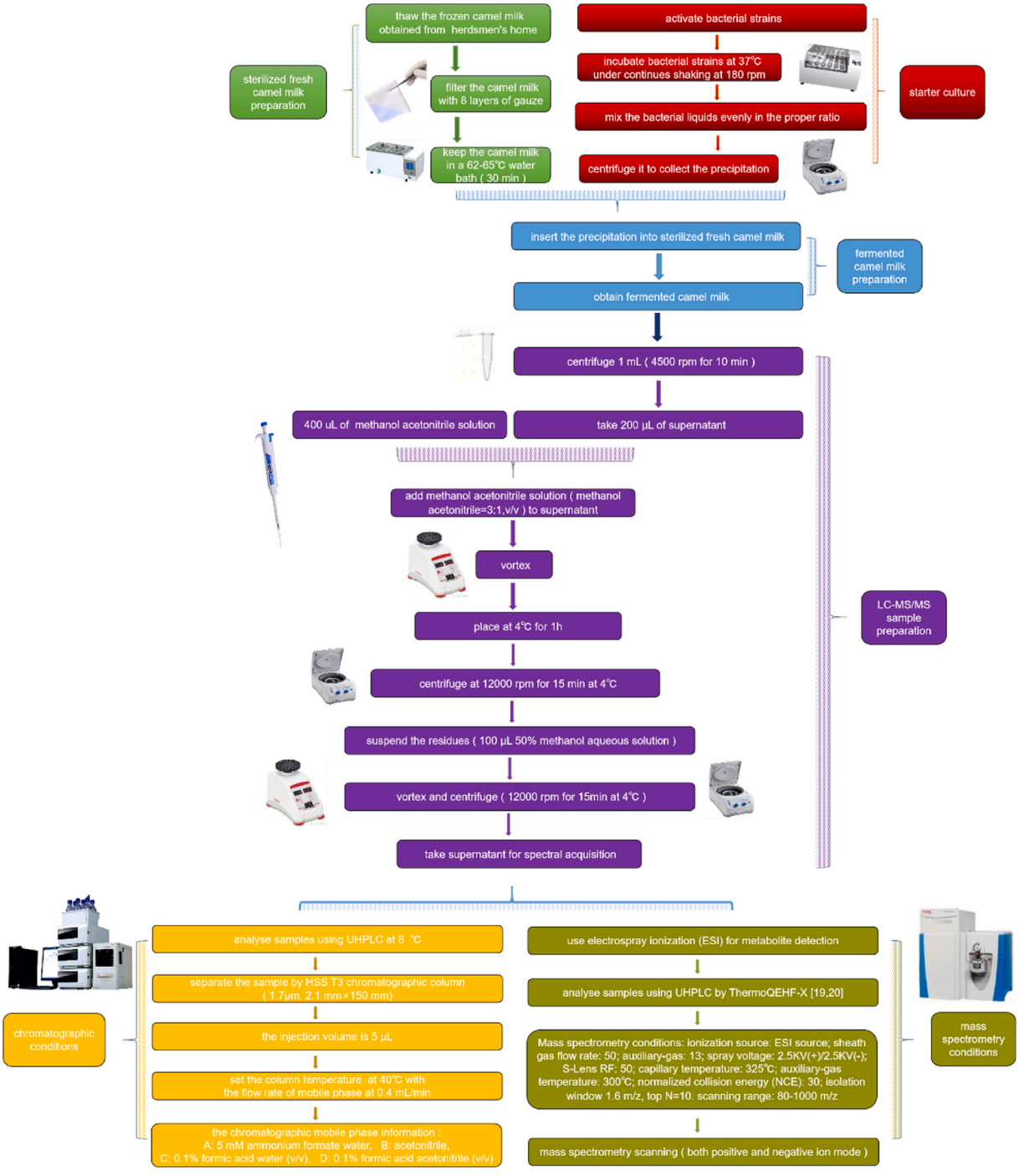


Fig. 1. Flow path of sterilized fresh camel milk preparation, starter culture, fermented camel milk preparation, LC-MS/MS sample preparation and spectral acquisition.

According to Ayyash's report [8], the medical and prebiotic effect of fermented camel milk were attributed to its LAB. LAB have been widely used as probiotics in food, which could improve the balance of human intestinal flora and promote digestion [9]. LAB is affiliated with the Gram-positive bacteria with no spore formation, can not only promote human intestinal peristalsis but also enhance intestinal absorption of nutrients and digestion. Most of the rest LAB play a very important role in the regulating intestinal micro-environment balance.

It is thus clear that there is an absolute relationship between so many biomedical functions of camel milk and its nutritional components. As a major branch with an omics technology, metabolomics [10] can solve the problem of food safety, quality and traceability [11]. Metabolomics is able to accurately evaluate the "phenotype" of disease, involving genes, transcripts and proteins [12], also has been used to study root exudates of plant seedlings [13] and animal intestinal microorganisms [14]. Untargeted metabolomics comprehensively examines the entire metabolome of an organism to detect as many molecules as possible in a sample [15]. The metabolic alterations genetic transformation of herbicide resistant buffalo grasses were characterized and evaluated after the comprehensive GC–MS based untargeted metabolomics by Boonchaisri et al., and found that herbicide resistant buffalo grasses were similar to their wild type, which guaranteed the safety of these herbicide resistant buffalo grasses [16]. Roberts and coworkers used metabolomics of ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) analysis to improve treatment efficiency of COVID-19 patients [17]. These findings fully confirmed the success of untargeted metabolomics in detecting metabolome of organisms, however metabolic analysis of beneficial components in camel milk especially bactrain camel milk in Alxa has been rare reported.

We had previously isolated and identified LAB from fermented camel milk in Alxa and found dominant bacteria, they were *Lactobacillus plantarum strain* KC28, *Lactobacillus plantarum strain* K25, *Lactobacillus sp.* D1501, *Leuconostoc mesenteroides strain* SHU1396. Among them, the former three LAB possessed the characteristics of acid and bile salt resistance [18]. The isolated strains and *Lactobacillus acidophilus strain* 2888 obtained from Beijing Chuanxiu Technology Co., Ltd. Were properly combined and developed a compound starter, namely, fermenting agent TR1, which have fermented the fresh camel milk.

In this contribution, the differences in the composition of camel milk in Alxa before and after fermentation was analyzed by LC-MS/MS based metabolomics. Multivariate statistical analysis combined with univariate statistical analysis was served to clarify the metabolic difference of camel milk before and after fermentation. Metabolites with significant differences were screened. The efficacy of camel milk will be explained from a deeper level to provide more favorable evidence and guarantee for the efficacy of camel milk.

2. Materials and methods

2.1. Samples

Fresh camel milk: fresh milk were acquired from bactrian camel aged seven years from herdsmen's home in Alxa area in June 2021 and was frozen and stored for experimental use. Frozen camel milk was thawed when needed at room temperature and filtered with 8 layers of gauze. After that, it was kept in a 62–65 °C water bath for 30 min to achieve sterilization. Then it was divided into sterilized blue cap bottles in a super-clean bench and finally stored at −20 °C in the laboratory, the main process was shown in Fig. 1 with green rounded rectangle.

Bacterial strains: Five LAB strains were frozen at 50% glycerol with MRS broth (1%, v/v) in −80 °C ultra-low temperature freezer in the Laboratory of Biochemistry and Molecular Biology of Inner Mongolia Agricultural University. They were used in the camel milk fermentation as starter culture. Of which four samples, *Lactobacillus plantarum strain* KC28, *Lactobacillus plantarum strain* K25, *Lactobacillus sp.* D1501, *Leuconostoc mesenteroides strain* SHU1396 were obtained from traditionally fermented camel milk through the isolation and purification, and another one was obtained from Beijing Chuanxiu Technology Co., Ltd, *Lactobacillus acidophilus strain* 2888.

Starter culture and fermentation: Bacterial strains above-mentioned were activated at MRS broth (1%, v/v) individually. They were incubated at 37°Cunder continues shaking 180 revolutions per minute (rpm) for 12–16 h. It was followed the same steps above to activate again. After that, *Lactobacillus plantarum strain* KC28, *Lactobacillus plantarum strain* K25, *Lactobacillus sp.* D1501, *Leuconostoc mesenteroides strain* SHU1396 were inoculated into UMRS broth, *Lactobacillus acidophilus strain* 2888 were inoculated into MRS broth to incubate for 24 h. Buffer solution was added to keep the medium pH stable. The bacterial liquids were mixed evenly in the ratio of 2:1:3:5:3 (v/v) in the above-mentioned order. It was centrifuged (6000 rpm, 5min), and washed by saline. A more one centrifugation (6000 rpm, 5min) was required to collect the precipitation, which was combined starter, namely, fermenting agent TR1. The precipitation was inserted into sterilized fresh camel milk to obtain fermented camel milk by orthogonal experiments, the main process was shown in Fig. 1 with red and sky blue rounded rectangle. After fermentation, the bacterial survival and pH were determined.

2.2. LC-MS/MS sample preparation

The camel milk was fermented according to the selected optimal fermenting conditions, from which 1 mL of sample was taken for the next analysis. The main experimental procedure was shown in Fig. 1 with purple rounded rectangle. Before use, organic solvent was pre-cooled in 40 °C. 400 μL of organic solvents was used for 200 μL of supernatant.

Quality Control (QC) samples were used to balance the next steps of the Chromatography-Mass Spectrometry System and determine the instrument status. Therefore, both experimental samples and QC samples were detected throughout the entire experimental procedure. There were two groups of experimental samples (fresh camel milk group and fermented camel milk group) with 6 samples in each group. Simultaneously, QC samples were prepared, which were a mixture of equal amounts of all experimental samples. For QC

samples, 3 technical replicates were performed.

2.3. LC-MS/MS spectral acquisition

Chromatographic conditions: we performed sample analyzing with UHPLC. The main experimental procedure and related information were shown in Fig. 1 with yellow rounded rectangle. The chromatographic gradient elution was as follows: negative ion mode: 0–1 min, linear change from 10% to 30% D; 1–19 min, linear change from 30% to 95% D; 19–20 min, 95% D; 20–21min, linear change from 95% to 10% D; 21–25min, 10% D. Positive ion mode: 0–1min, linear change from 10% to 30% D; 1–19 min, linear change from 30% to 95% D; 19–20 min, 95% D; 20–21 min, linear change from 95% to 10% D; 21–25min, 10% D. Mass spectrometry conditions were also shown in Fig. 1 with dark green rounded rectangle [19,20].

2.4. LC-MS/MS data processing and statistical analyses

The raw data were analyzed by compound discovery software. Metabolite structure identification was matched by exact molecular mass data (<10 ppm) using compound discovery software and the secondary mass spectrum for retrieving database.

The metabolomic mass spectrometry data of 12 experimental samples detected (2 groups, 6 samples in each group, "Tre" represents fermented camel milk sample, "Con" represents fresh camel milk sample) were analyzed by relevant software. The total ion chromatograms (TIC) were employed for QC, TIC in two ion detection modes were superimposed and compared. Data preprocessing [21] was completed, which were embodied data filtering, missing value processing, and normalization. Specifically, removing the data with no definite substance name and no spectral ratio for similarity, missing value recoding of less than 50% of the missing substances in the comparison group samples by k-nearest neighbor (KNN) algorithm and more than 50% of the missing substances were filtered directly, the internal standards (IS) or total ion current (TIC) of each samples were used for normalization.

In order to accurately excavate the potential information in the date, univariate analysis and multivariate statistical analysis were combined when analyzing metabolomics date and performed dimensionality reduction and classification on the collected multidimensional date. Through statistical analysis, differential metabolites between different kinds of samples were preliminarily screened and identified. The differential metabolites were screened by combining p-value and VIP value of OPLS-DA [22,23], and performed hierarchical cluster analysis of differential metabolites, kyoto encyclopedia of genes and genomes (KEGG) annotation of differential metabolites, metabolic pathway analysis [24], enrichment analysis of differential metabolite pathway. The processed data was obtained through the comparison with HMDB database (V4.0), KEGG compound database [25,26], and annotated metabolites. Software SIMCA (V14.1) was used to screen out the differential metabolites. The metabolomic analysis of this experiment was conducted by Genepioneer Biotechnologies, NANJING.

3. Results

3.1. Optimization of fermentation conditions

On the basis of previous study, the fermenting conditions of camel milk were optimized by orthogonal experiments of bacterial survival and pH individually by selecting three levels of three factors including bacterial inoculation amount, temperature, fermenting time. According to the experimental results, the optimized fermenting condition was fermenting time of 24 h with bacterial inoculation amount of 24 mL bacterial liquid precipitation into 20 mL fresh milk at 38 °C by both bacterial survival and pH orthogonal experiments. Bacterial survival during fermentation was up to 2.25×10^{10} CFU/mL (Table S1) and with a pH of 4.21 at the optimized fermenting condition (Table S2).

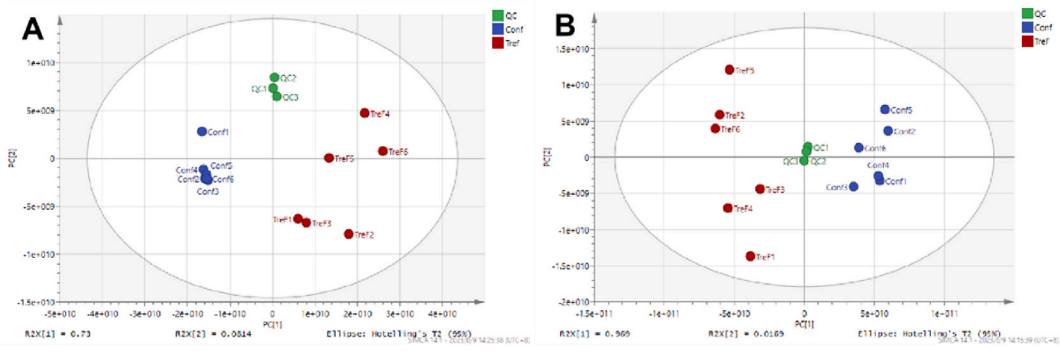


Fig. 2. Analysis of metabolites detected by LC-MS/MS. A: PCA score scatter plot in positive ion mode; B: PCA score scatter plot in negative ion mode.

3.2. Quality control analysis and raw data preprocessing

QC sample mass spectrometry TIC indicated that the response intensity and retention time of each color spectrum peak basically overlapped, the results were shown in Fig. S 1A and S 1B, taking the retention time as the abscissa and the ion intensity as the ordinate, indicating that the analysis method was stable and reliable, errors caused during operation was negligible in the whole experimental process, and the data can be used for further analysis.

After raw data preprocessing, 1813 and 1316 peaks were retained in positive and negative ion mode respectively after preprocessing the raw data. 259 and 147 components in positive and negative ion mode were identified respectively.

3.3. Multivariate statistical analysis

PCA and OPLS-DA were performed after obtaining the sorted data. In the PCA diagram, each sample in each group was represented by dots, the relationship between samples was expressed based on the aggregation or separation trend of samples in Fig. 2A–B, the more the dots gathered, the higher the similarity of the observed variables, the higher dispersion of dots, the more significant difference of observation variables. The PCA loading plot was shown in Fig. S 2 A and S 2 B. In both positive and negative ion modes, different samples can be well distinguished, and the biological information analysis of metabolomics can be carried out. In the PCA diagram (Fig. 2A–B), X-axis and Y-axis revealed the scores of the first and second principal components respectively. The parallel samples were in a state of aggregation on the graph, indicating clear separation between the two groups. The metabolites of the 6 samples in each group were highly similar. On the other hand, the samples in the control group and the experimental group were in a discrete state, and there was a large difference between the two groups, indicating that there was large difference in metabolites between the two groups, which provided a prerequisite for subsequent screening of differential metabolites. The PCA score chart indicated that the samples were all within the 95% confidence interval, therefore the data deserved further study.

In this experiment, the components of the two groups were predicted, as shown in Fig. 3A–B, which were the OPLS-DA score graphs in positive and negative ion modes. The Y-axis represented the orthogonal principal component score, and the X-axis represented the predicted principal component score of the first principal component, scatter colors represented different experimental groups. OPLS-DA showed that the samples of the same type were relatively concentrated, and different types of samples showed a dispersed state. That was, the six samples of fresh camel milk gathered together, the other six samples of fermented camel milk gathered together. The samples of fresh camel milk and fermented camel milk showed a dispersed state. The experimental data were true and reliable, and the metabolic differences can be screened later. The result of OPLS-DA permutation test were shown in Fig. 3C–D. It could be seen that the original model R2 was close to 1, indicating that the established model was in line with the real situation of the sample data; Q2 was close to 1, indicating that if a new sample was added to the model, an approximate distribution will be obtained. In general, the original model can also well explain the difference between the two groups of samples.

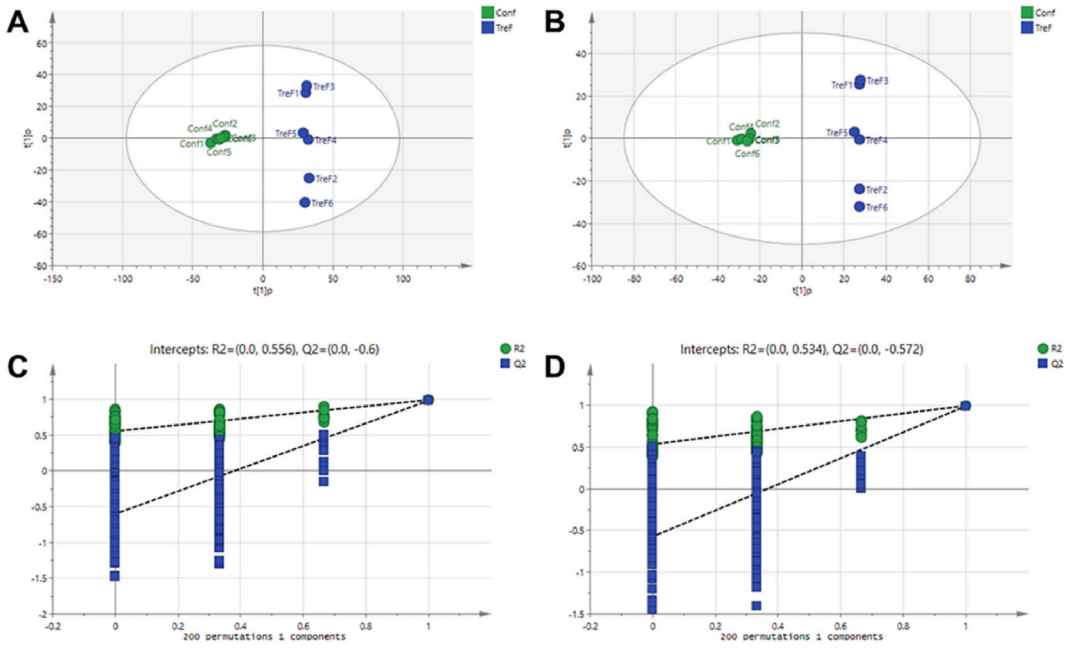


Fig. 3. Analysis of metabolites detected by LC-MS/MS. A: OPLS-DA in positive ion mode; B: OPLS-DA in negative ion mode; C: Permutation test of the OPLS-DA model in positive ion mode; D: Permutation test of the OPLS-DA model in negative ion mode.

3.4. Differential metabolites screening

We used OPLS-DA variable importance in project (VIP) to screen the differential metabolites preliminarily, then further screen was accomplished through combining with the p-value or fold change of univariate analysis. The screened metabolites were compared with the KEGG compound database to obtain metabolites that could be named, and the screened differential metabolite results were visualized in the form of volcano plots, as shown in Fig. 4A–B and Table S3, Table S4. The metabolites colored red were up-regulated significantly, metabolites colored blue were down-regulated, and ones colored gray had no significant difference. Among 148 differential metabolites detected, 85 presented upward trend and 63 presented downward trend in the positive ion mode. In the negative ion mode, among all 82 differential metabolites, 32 presented upward trend and 50 presented downward trend in the fermented camel milk group relative to the fresh camel milk group.

The differential components mainly included organic acids, amino acids, esters, vitamins, ketones, nucleosides and alkaloids. There were the most types of organic acids with a total of 47 kinds of components both in positive and negative ion mode, among them, 3-phenyllactic acid, creatine, cinnamic acid, 2-aminoadipic acid, linolenic acid, linoleic acid, palmitoleic acid were detected as main up-regulated differential metabolites, and ricinoleic acid, deoxycholic acid, lauric acid, phosphocreatine, pentadecanoic acid, citric acid were detected as main down-regulated differential metabolites. Amino acids such as *N*-acetyl-L-histidine, D-phenylalanine, phenylacetylglycine, 1-methylhistidine, atalanine were more important substance among down-regulated differential metabolites decreased significantly after fermentation. The up-regulated amino acids mainly included *N*-acetyl-L-leucine, L-phenylalanine and derivatives of lysine, methionine, leucine, histidine. A total of 16 esters have been found, including 5-guanidino-2-oxopentanoate, (R) - 10-hydroxystearate, 4-acetamidobutanoate, (1-ribosylimidazole)-4-acetate, phenol sulfate, 1-(5′-phosphoribosyl)-5-formamido-4-imidazolecarboxamide, which were significantly decreased after camel milk fermentation, and thiram, 8-amino-7-oxononanoate, chloramphenicol palmitate, 4-imidazolone-5-propanoate, (2 S)-2-{[1-(R)-Carboxyethyl]amino}pentanoate, 6-acetamido-3-aminohexanoate, (R)-3-amino-2-methylpropanoate, 7-dehydrologanin tetraacetate, D-4′-phosphopantothenate, and 3-(2-hydroxyphenyl) propanoate of up-regulated metabolites. Additionally vitamin B including B2, B3, B4, B5, B13 and H were also have been detected in camel milk. Vitamin B4 and B3 showed an upward trend, while the rest showed a downward trend.

3.5. Hierarchical cluster analysis

The differential metabolites were displayed by heatmap as shown in Fig. 5A–B. The X-axis represented the name of the samples and the clustering results of the samples, and the Y-axis represented the differential metabolites and clustering results of differential metabolites. Different columns in Fig. 5A–B represened different samples and different rows represented different metabolites. The color represened the relative content levels of metabolites in the sample. Up-regulated metabolites were represented by red and down-regulated once were blue in both the positive and negative ion mode. Cluster analysis showed that the experimental group and the control group were divided into two major categories, fermented camel milk samples had different metabolites compared with the control once.

3.6. KEGG annotation

All pathways mapped to partial differential metabolites of corresponding species were given in Tables S5 and S6 with a positive and negative ion mode through comparison with KEGG pathway database. The column with “KEGG pathway” in the Table includes the KEGG pathway database ID of metabolites enriched into the metabolic pathway, the name of the metabolic pathway and the number of differential metabolites in the pathway; the column with “Compounds” in the Table includes the KEGG Compound ID and the name of

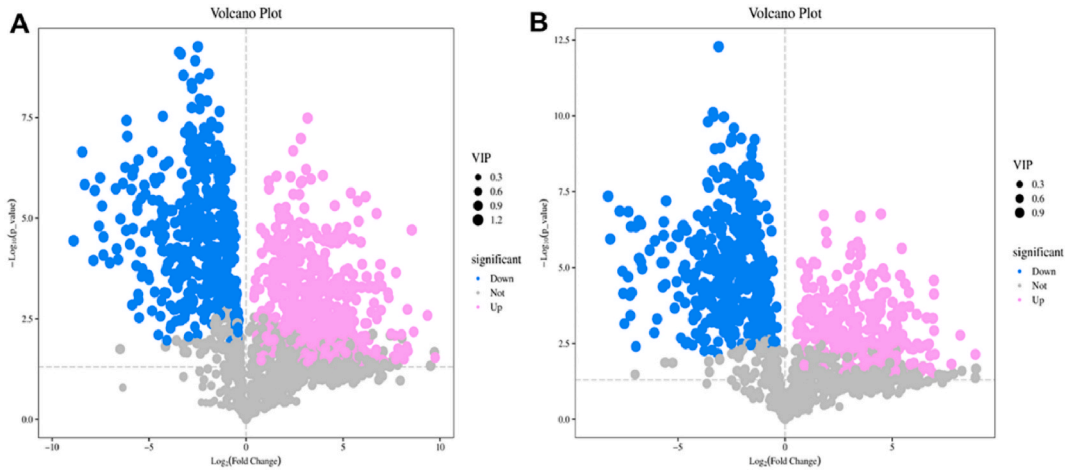


Fig. 4. Volcano plot of differential metabolites for fermented camel milk versus fresh camel milk in (A) positive ion mode, (B) negative ion mode.

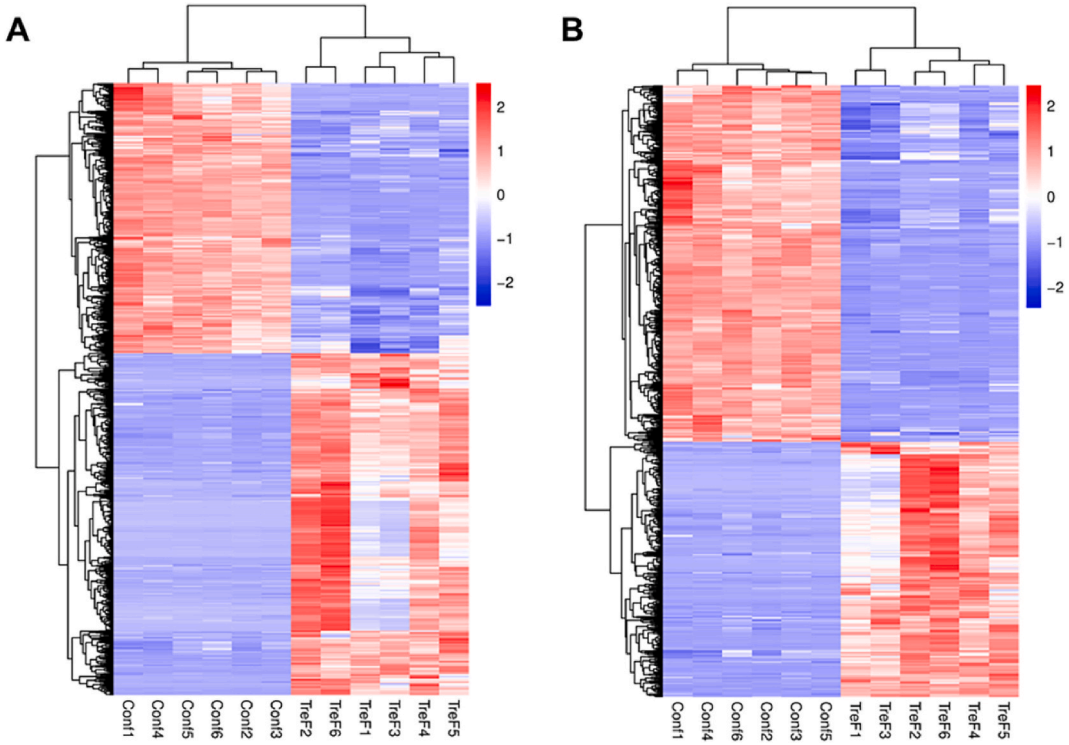


Fig. 5. Heatmap of the differential metabolites for fermented camel milk versus fresh camel milk in (A) positive ion mode, (B) negative ion mode.

the metabolites. After matching with KEGG pathway database, there were the most differential metabolites in metabolic pathways which includes 68 metabolites hit this pathway and 20 differential metabolites in biosynthesis of secondary metabolites pathway in a positive ion mode. However there were 40 differential metabolites in metabolic pathways and 27 differential metabolites in biosynthesis of secondary metabolites pathway in a negative ion mode.

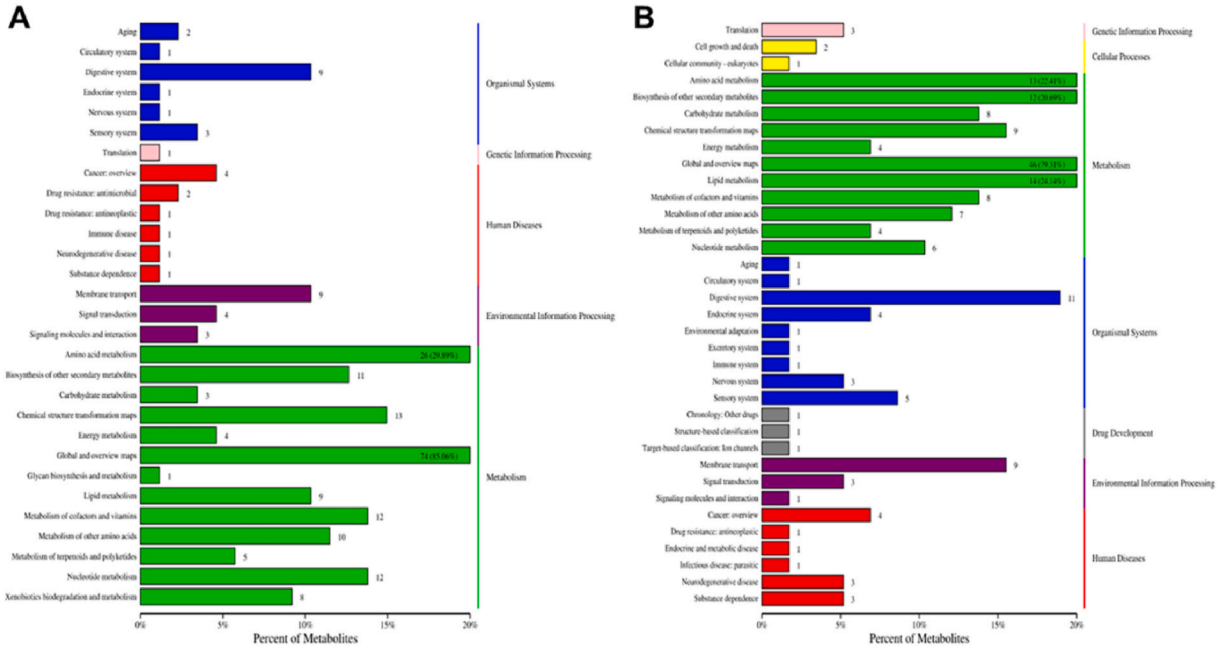


Fig. 6. KEGG pathway types analysis in (A) positive ion mode, (B) negative ion mode.

3.7. Differential metabolite pathway types analysis

The results of KEGG annotation of differential metabolites were classified according to the KEGG pathway, and Fig. 6A–B were obtained. The right side was the primary classification of KEGG pathway, and the left side was the secondary classification of KEGG pathway. Different colors represent different primary classifications. The X-axis represented the proportion of metabolites annotated to the secondary pathway in the total annotated metabolites, and those exceeding 20% were marked as a percentage. Under the primary classification, there were five types of metabolic pathways, of which about 45% were metabolism, while there was only 1 pathway of genetic information processing, 6 of organismal systems; 3 of environmental information processing, and 6 of human disease in the positive ion mode. In the negative ion mode, metabolism were also the most in the total metabolic pathways, there were 11 pathways accounting for 31% of the total, the other six metabolic types were 9 of organism systems, 3 of drug development, 3 of environmental information processing, 6 of human disease, only 1 of genetic information processing, and 2 of cellular processes respectively.

3.8. Enrichment analysis of differential metabolite pathway

By enriching and analyzing the pathways of different metabolites, we can further screen the pathways and find the key pathway with the highest correlation with differential metabolites. Firstly, mapping differential metabolites to authoritative metabolite databases such as KEGG and PubChem was done, the result was shown in Tables S7 and S8, after obtaining the matching information of differential metabolites, the corresponding pathway database was searched and the enrichment of metabolic pathways were analyzed, ensuring whether differential metabolites have over-presented on a certain pathway, which was differential metabolites enrichment analysis (Tables S9 and S10). Differential metabolites enrichment was shown by differential metabolite KEGG enrichment bubble map (Fig. 7A–B). KEGG enrichment was measured by enrichment factor, p value and the number of metabolites enriched in this pathway. The greater the enrichment factor, the greater the degree of enrichment, the p value range was [0, 1]. The smaller the p value, the greater the enrichment factor and the greater the reference value; vice versa. The first 20 pathways were selected to show the least significant enrichment of p value. Metabolic pathways were the most relevant pathway, followed by nicotinate and nicotinamide metabolism, lysine degradation, biosynthesis of unsaturated fatty acids, arginine and proline metabolism, vitamin digestion and absorption, phenylalanine metabolism, vancomycin resistance, longevity regulating pathway–worm, caffeine metabolism, D-arginine and D-ornithine metabolism, fatty acid biosynthesis, purine metabolism, tropane, piperidine and pyridine alkaloid biosynthesis, pyrimidine metabolism, vascular smooth muscle contraction, polycyclic aromatic hydrocarbon degradation, atrazine degradation, linoleic acid metabolism, autoimmune thyroid disease in positive ion mode; However, in the negative ion mode, biosynthesis of secondary metabolites was the most relevant pathway with the least p value, followed by taste transduction, secondary bile acid biosynthesis, phenylpropanoid biosynthesis, aminoacyl-tRNA biosynthesis, protein digestion and absorption, isoquinoline alkaloid biosynthesis, linoleic acid metabolism, porphyrin and chlorophyll metabolism, biosynthesis of alkaloids derived from histidine and purine, biosynthesis of unsaturated fatty acids, phenylalanine metabolism, 2-oxocarboxylic acid metabolism, biosynthesis of alkaloids derived from shikimate pathway, biosynthesis of alkaloids derived from ornithine, lysine and nicotinic acid, vitamin digestion and absorption, central carbon metabolism in cancer, biosynthesis of siderophore group nonribosomal peptides, glutathione metabolism,

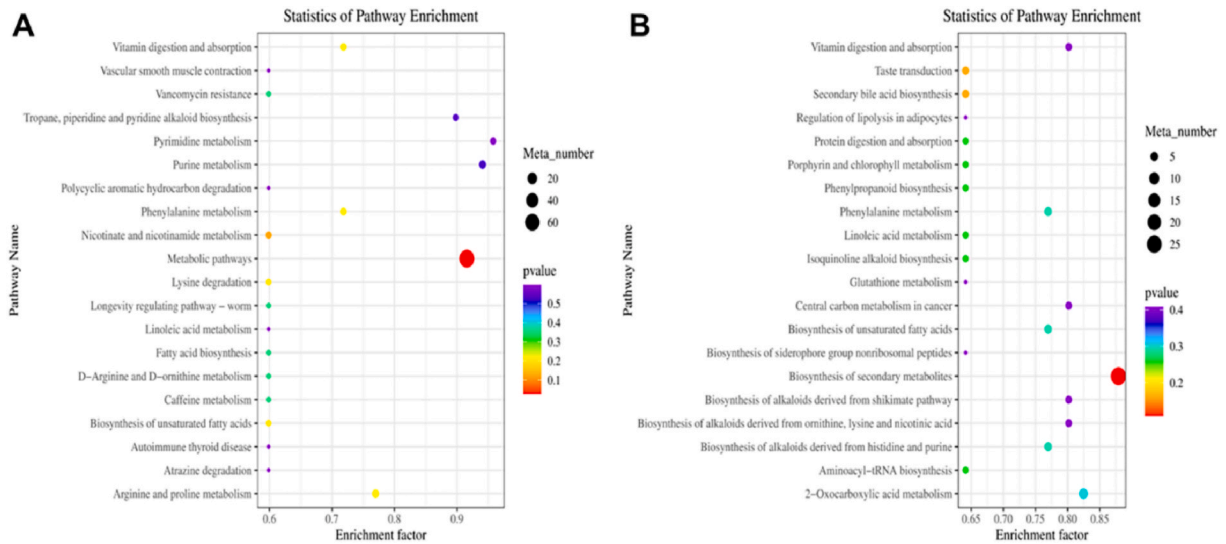


Fig. 7. Differential metabolite KEGG enrichment bubble map. (A) positive ion mode, (B) negative ion mode. Each row in the map represented a KEGG pathway. The X-axis was the enrichment factor. The larger the enrichment factor, the more significant the enrichment level of differential metabolites in this pathway. The color of the dot represented p value, and the bubble size represented the number of differential metabolites in the pathway.

regulation of lipolysis in adipocytes.

4. Discussion

Previous studies showed that LAB isolated from fermented bactrain camel milk in Alxa had excellent characteristics such as acid and bile salt resistance [18]. These LAB mixed with *Lactobacillus acidophilus strain* 2888 isolated from fermenting agent obtained from Beijing Chuanxiu Technology Co., Ltd. made the optimal fermenting agent. Therefore we optimized the mixing proportion and made the compound starter, TR1. After that, the camel milk before and after fermentation was detected by untargeted LC-MS/MS based metabolomics and univariate and multidimensional statistical analysis models of different groups such as TIC, PCA, volcano diagram and KEGG enrichment analysis were established. This paper aimed to determine metabolites and investigate various metabolic pathways in the process of camel milk fermentation. It has special significance and broad prospects for the medical and health care of dairy products, and provide an in-depth understanding.

Through raw data preprocessing, 259 components in 1813 peaks were identified in positive ion mode, 147 components in 1316 peaks were identified in all in negative ion mode. According to differential metabolite analysis of camel milk before and after fermentation, a total of 148 differential metabolites were screened out in the positive ion mode, and of which 85 were up-regulated and 63 were down-regulated after camel milk fermentation. In the negative ion mode, a total of 82 differential metabolites were screened out, of which 32 presented a rising tendency and 50 were shown a downward trend after camel milk fermentation. Metabolites mainly contained organic acids, amino acids, esters, vitamins and other substances in positive ion mode, which were similar to those detected in camel milk by UHPLC combined with quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF/MS) expressed by Li et al. [27]. In the negative ion mode, mainly detected metabolites were also organic acids, amino acids, esters, vitamins and other substances.

There were 47 types of organic acids before and after fermentation both in positive and negative ion mode, organic acids had the most differential metabolites compared with other substances. Among them, 20 organic acids were up-regulated and 27 organic acids were down-regulated. The up-regulation of organic acids constituted the main reason for the decline in pH of camel milk after fermentation. Up-regulated organic acids mainly included 3-phenyllactic acid, creatine, cinnamic acid, 2-aminoadipic acid, linolenic acid, linoleic acid, palmitoleic acid. 3-phenyllactic acid was the organic acid with the highest content, and the antibacterial activity of 3-phenyllactic acid could be enhanced when combined with nisin, presented high composited antibacterial properties against pathogens including *Staphylococcus xylosu (S.xylosus)* and *Micrococcus luteus (M.luteus)* and affording a bright prospect for the 3-phenyllactic acid-nisin co-expressing *Lactococcus lactis (L. lactis)* in food preservation owing to its significant antibacterial function and cost-efficient use [28]. Creatine also had the higher concentration in the differential metabolites, which was an indispensable amino acid derivative in body [29]. Studies have shown that when creatine was used as a dietary supplement, the amount of lean meat and exercise ability increased during short-term with high-intensity exercise. In addition to increased strength, postexercise injury and neurological recovery related to exercise can be achieved by supplementing creatine [30]. Studies have shown that cinnamic acid and its derivatives extracted from plants have antibacterial effects on many microorganisms [31], but there was no research on extracting this substance from animals. There was an upward trend of both cinnamic acid and its derivative 2-hydroxycinnamic acid in camel milk after fermentation, and new probiotic functions can be developed in the future. The content of 2-aminoadipic acid was significantly increased by more than 100 times after fermentation of camel milk. 2-aminoadipic acid increased the decomposition of fat through up-regulating PGC1α and UCP1 mediated by β3AR, and stimulate fat decomposition through enhancing hormone sensitive (HSL) expression by activating β3AR signal, so as to alleviate diabetes symptoms in diabetic mice [32], it was also found that 2-aminoadipic acid can regulate insulin secretion by them [32]. Therefore it was an important substance for treating obesity and diabetes and fermented camel milk may be better than fresh camel milk in treating obesity and diabetes.

In addition, linolenic acid and linoleic acid in differential metabolites were both detected as up-regulated substances. α-linolenic acid as one of the differential metabolites was considered to be essential fatty acid in the daily diet [33]. It was pointed out that the function of α-linolenic acid in reducing cardiovascular disease, inflammation, cancer, diabetes and hypertension was obvious [34]. This was also in line with previous studies that camel milk and fermented camel milk had the effect of reducing blood pressure [1]. Linoleic acid is a kind of polyunsaturated fatty acid (PUFA), which is consumed most in human diet [35], and was also recognized as an essential fatty acid. Studies have found that linoleic acid-rich fats can reduce the development of atherosclerosis in apolipoprotein E deficient mice [36], providing new evidence for the probiotic function of camel milk. Palmitoleic acid also has been identified as one of the more important differential metabolites, and was also detected as an up-regulated metabolite. In previous studies, it was pointed out that when palmitoleic acid was supplemented in mice fed high-fat diet, palmitoleic acid played an important role in improving metabolic function in liver [37]. Hussain et al. showed that camel milk significantly reduced glycosylated hemoglobin (HbA1c), some lipids and transaminase in diabetic mice, which can be used as appropriate substitute for the treatment of diabetes, which was consistent with our results [38].

In addition to up-regulation of many beneficial substances, many harmful substances also showed a downward trend after fermentation of camel milk. Down-regulated organic acids mainly included ricinoleic acid, deoxycholic acid, lauric acid, phosphocreatine, pentadecanoic acid, citric acid. Ricinoleic acid had a certain cytotoxicity on cells, and may lead to cell apoptosis [39]. The reduction of this substance after fermentation may significantly enhance the biological activity of LAB and thereby enhancing the probiotic function in fermented camel milk. It has been proved that, deoxycholic acid can lead to gastric cancer [40]. After fermentation, the reduction of deoxycholic acid content can effectively prevent gastrointestinal dysfunction, indicating that camel milk will have this effect after fermentation. Lauric acid that cause allergic reactions was down-regulated after fermentation. Therefore fermented camel milk has stronger probiotic functions than fresh camel milk in some aspects.

However, in other aspects, the efficacy of fresh camel milk is better than that of fermented camel milk. Phosphocreatine was the

most obvious down-regulated component. However, phosphocreatine has been used as a cardiac protective drug and was used to treat myocardial infarction [41]. Therefore fresh camel milk was better than fermented camel milk in recovering myocardial infarction. Pentadecanoic acid is an odd-carbon-saturated-fatty-acid, and can up-regulate the expression of pro-apoptotic protein Bax and inhibit the expression of anti-apoptotic protein Bcl-2, thus triggering the apoptosis of human breast cancer cell MCF-7 [42]. However, pentadecanoic acid was down-regulated in camel milk after fermentation, this study provides a theoretical basis for the use of odd-carbon-saturated-fatty-acids in the nutritional treatment of breast cancer patients, also provides evidence for the development of new probiotic functions related to anticancer effects in camel milk. Citric acid was down-regulated after fermentation of camel milk. Mustafa et al. found that the blood sugar levels in the group given citric acid presented a downward trend compared with those in the control group, achieving a prebiotic function by reducing triglycerides and blood sugar levels [43], indicating that hypoglycemic and lipid-lowering effects of citric acid in fresh camel milk was better than that in fermented camel milk.

The number of amino acids differential metabolites detected by LC-MS/MS based metabolomics ranked second, there was 28 amino acids screened out in all. Among them, 9 amino acids were down-regulated and 19 amino acids were up-regulated. The content of *N*-acetyl-L-histidine, D-phenylalanine, phenylacetylglycine, 1-methylhistidine, atalanine, which were more important substance among down-regulated differential metabolites decreased significantly after fermentation. *N*-acetyl-L-histidine significantly decreased after camel milk fermentation in all amino acids, with a content of only 5% before fermentation. *N*-acetyl-L-histidine had the lowest content among all down-regulated amino acids. Studies have shown that this substance can catalyze the synthesis of gangliosides [44], and high levels of certain specific gangliosides can lead to many genetic diseases. The significant decrease of *N*-acetyl-L-histidine in fermented camel milk indicated that fermented camel milk can effectively reduce the occurrence of many genetic diseases. D-phenylalanine was also a relatively important down-regulated substance, which is an isomer of L-phenylalanine. Both phenylalanines can reduce postprandial blood sugar by stimulating the release of insulin. However, the former was down-regulated many times, while the latter was up-regulated by 74 times. Amin et al. have shown that D-phenylalanine can control blood sugar more effectively then L-phenylalanine for a longer time [45], consistent with the hypoglycemic effect of camel milk. However, from this perspective alone, fresh camel milk was superior to fermented camel milk in reducing sugar. To sum up, camel milk and fermented camel milk have their respective advantages in terms of probiotic function, which can also lay the foundation for better guiding people to scientifically use camel milk or fermented camel milk. Phenylacetylglycine, 1-methylhistidine, and atalanine have not been found in the study of camel milk at present, and the mechanism and function of these substances are not very clear. In the future, it is possible to explore the role and function of these substances in fermentation metabolism.

The up-regulated amino acids mainly included *N*-acetyl-L-leucine, L-phenylalanine and derivatives of lysine, methionine, leucine, histidine. Hegdekar et al. have found that oral administration of *N*-acetyl-L-leucine can significantly improve exercise and cognition in mice with traumatic brain injury, and reduce the expression of neuroinflammatory markers [46]. *N*-acetyl-Leucine can effectively protect nerves, and it was expected that fermented camel milk will have a beneficial prebiotic function of protecting nerves in the future. The essential amino acids in the human body cannot be synthesized by itself and reach the requirement of normal metabolism of human body, and can only be obtained from external protein. In this study, a large number of essential amino acids in the human body also have been found, such as L-phenylalanine with the highest content. In addition, lysine, methionine, leucine, histidine, and other essential amino acids in the human body appear in the form of derivatives in the differential components. Moreover, most essential amino acid derivatives were significantly up-regulated in fermented camel milk, so fermented camel milk can provide essential amino acids to the human body more effectively than fresh camel milk.

A total of 16 differential esters components were screened. Among them, 5-guanidino-2-oxopentanoate, (R) - 10-hydroxystearate, 4-acetamidobutanoate, (1-ribosylimidazole)-4-acetate, phenol sulfate, and 1-(5′-phosphoribosyl)-5-formamido-4-imidazolecarboxamide significantly decreased after camel milk fermentation. Mindikoglu et al. found that the content of 4-acetamidobutanoate was higher in patients with severe liver and kidney diseases [47], with a significant decrease in content, which can reduce the adverse effects of fermented camel milk on renal function. 10 esters below were significantly up-regulated, namely, thiram, 8-amino-7-oxononanoate, chloramphenicol palmitate, 4-imidazolone-5-propanoate, (2 S)-2-{[1-(R)-Carboxyethyl]amino}pentanoate, 6-acetamido-3-aminohexanoate, (R)-3-amino-2-methylpropanoate, 7-dehydrologanin tetraacetate, D-4′-phosphopantothenate, and 3-(2-hydroxyphenyl) propanoate. Moreover, the content of 8-amino-7-oxononanoate and chloramphenicol palmitate increased 123 and 53 times. The significantly increased chloramphenicol palmitate demonstrated better probiotic function, which has been the same effect as chloramphenicol and can treat inflammation, which was consistent with previous studies that camel milk has antibacterial and anti-inflammatory effects [3]. There was no research on the use of thiram and many other esters in camel milk, and there is currently a lack of research on the toxicological or pharmacological effects of these substances. In the future, an in-depth study on these substances can be conducted.

In addition to the organic acids, amino acids, esters pointed out above, vitamins were also found in camel milk. As we all know, vitamins are indispensable trace elements for the body and have an important protective effect on the body. A total of 6 vitamins were obtained through differential metabolite analysis, they were all vitamins of B group, containing B2, B3, B4, B5, B13, and H. Vitamin B4 and B3 showed an upward trend, while the rest showed a downward trend. Vitamin B4 was the most significantly up-regulated, 16 times higher with a content after fermentation than before. Research has shown that vitamin B4 was beneficial for the development of fetuses and children, and preventing cognitive problems [48]. The up-regulation of this substance effectively enhances the prebiotic function after fermentation, providing new evidence for its beneficial function of improving memory. Studies have shown that supplementing with vitamin B3 can attenuate skin aging and hyperpigmentation as a cosmeceutical ingredient [49].

Among all down-regulated vitamins, vitamin B5 has the highest content, and although there was a downward trend, its content was more than 10 times that of other vitamins. Xu et al. found that lack of vitamin B5 was likely to cause neurodegeneration in Alzheimer's disease and dementia, leading to Alzheimer's disease [50]. Taking an appropriate amount of B5 by oral administration can prevent

leading to disease and even reversible in the early stages of Alzheimer's disease. Although the content of B5 in fermented camel milk decreased relative to camel milk, it still maintained at a high level, providing new evidence for the prebiotic function of camel milk. Other vitamins also have their own functions. To sum up, only considering the beneficial effects of vitamins on the human body, camel milk and fermented camel milk had different functions, each has its own merits.

In addition to the above mentioned organic acids, amino acids, esters and vitamins, there were also many other substances like fructose, nucleosides, alkaloids, ketones with prebiotic functions in camel milk. Fructose is a commonly known substance required by the human body. D-(−)-fructose was highly up-re gulated after camel milk fermentation, which can directly supply heat energy, supplement body fluid and nourish the whole body especially in fermented camel milk, and is easier to absorb and use than glucose. In addition, there are some substances whose functions need to be developed.

5. Conclusion

In summary, this study analyzed the metabolites of bactrain camel milk in Alxa before and after fermentation with fermenting agent TR1 based on untargeted LC-MS/MS based metabolomics. After camel milk fermentation, 148 differential metabolites were detected. Among them, 85 presented upward trend and 63 presented downward trend in the positive ion mode. In the negative ion mode, among all 82 differential metabolites detected, 32 presented upward trend and 50 presented downward trend in the experimental group relative to the control group. Differential metabolites mainly included organic acids, amino acids, esters, vitamins and other substances contained in camel milk. It was explained that camel milk especially after fermentation have prebiotic functions such as lowering blood pressure and sugar, antibacterial activity, and anti-inflammatory because of 3-phenyllactic acid, creatine, cinnamic acid, 2-aminoadipic acid, linolenic acid, linoleic acid, palmitoleic acid, *N*-acetyl-L-leucine, chloramphenicol palmitate etc. The significant decrease in harmful substances such as ricinoleic acid, deoxycholic acid, lauric acid, *N*-acetyl-L-histidine and 4-acetylaminobutyrate indicated that fermented camel milk was more beneficial to human health than fresh camel milk. There were also substances whose content in fresh camel milk was higher than fermented camel milk had their unique probiotic functions. Vitamins beneficial to body health were found both in fresh and fermented camel milk. However, there were also many substances with unknown functions that have been detected and have not been explained in this study, and providing a support in exploring new prebiotic functions in the future.

Ethical statement

Ethical approval was obtained from the Inner Mongolia Agricultural University Animal Ethics Committee ([2020]104).

Author contribution statement

Weisheng Xu: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Qigeqi Dong: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Guofen Zhao: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Bing Han: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This report was supported by "Inner Mongolia Autonomous Region Natural Science Foundation (2023MS03047 and 2023LHMS03029), Program for Improving the Scientific Reasearch Ability of Youth Teachers of Inner Mongolia Agricultural University (BR230131), Inner Mongolia Autonomous Region Scientific and Technological Achievements Transformation Project for R&D and Industrialization Demonstration of Compound Probiotics Suitable for Fermented Camel Milk (CGZ2018122), Key Lab of Germplasm Innovation and Utilization of Triticeae Crops at Universities of Inner Mongolia Autonomous Region, Innovation Team of Germplasm and Molecular Breeding of Forage Crops and Useful Microbiology (TD202103), and Inner Mongolia Agricultural University College of Life Science Foundation of Youth Fund Project (QN202103)".

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18522.

References

[1] A. Buchilina, K. Aryana, Physicochemical and microbiological characteristics of camel milk yogurt as influenced by monk fruit sweetener, J. Dairy Sci. 104 (2) (2021) 1484–1493, https://doi.org/10.3168/jds.2020-18842.

[2] A. Muleta, D. Hailu, T. Belachew, Camel milk consumption was associated with lower prevalence of anemia among preschool children in rural pastoral districts of Somali, eastern Ethiopia, Nutrition 86 (2021), 111170, https://doi.org/10.1016/j.nut.2021.111170.

[3] A.I. Hamed, et al., Electrospray ionization mass spectrometry characterization of ubiquitous minor lipids and oligosaccharides in milk of the camel (Camelus dromedarius) and their inhibition of oxidative stress in human plasma, J. Dairy Sci. 103 (1) (2020) 72–86, https://doi.org/10.3168/jds.2019-16710.

[4] R. Krishnankutty, et al., Anticancer activity of camel milk via induction of autophagic death in human colorectal and breast cancer cells, Asian Pac. J. Cancer Prev. APJCP 19 (12) (2018) 3501–3509, https://doi.org/10.31557/APJCP.2018.19.12.3501.

[5] A.A. Korish, A. Galil, A. Abdulqader, Comparison of the hypoglycemic and antithrombotic (anticoagulant) actions of whole bovine and camel milk in streptozotocin-induced diabetes mellitus in rats, J. Dairy Sci. 103 (1) (2020) 30–41, https://doi.org/10.3168/jds.2019-16606.

[6] A. Fugl, et al., Characterisaton of latic acid bacteria in spontaneously fermented camel milk and selection of strains for fermentation of camel milk, Int. Dairy J. 73 (2017) 19–24, https://doi.org/10.1016/j.idairyj.2017.04.007.

[7] C. Jans, J. Bugnard, P.M.K. Njage, C. Lacroix, L. Meile, Lactic acid bacteria diversity of African raw and fermented camel milk products reveals a highly competitive, potentially health-threatening predominant microflora, LWT–Food Sci. Technol. 47 (2) (2012) 371–379, https://doi.org/10.1016/j.lwt.2012.01.034.

[8] M. Ayyash, A.K. Ai-Nuaimi, S. Ai-Mahadin, S.Q. Liu, In vitro investigation of anticancer and ACE-inhibiting activity, α-amylase and α-glucosidase inhibition, and antioxidant activity of camel milk fermented with camel milk probiotic: a comparative study with fermented bovine milk, Food Chem. 239 (15) (2018) 588–597, https://doi.org/10.1016/j.foodchem.2017.06.149.

[9] J.N. Zhao, et al., Analyses of physicochemical properties, bacterial microbiota, and lactic acid bacteria of fresh camel milk collected in Inner Mongolia, J. Dairy Sci. 103 (1) (2020) 106–116, https://doi.org/10.3168/jds.2019-17023.

[10] M. Castro-Puyana, R. Pérez-Míguez, L. Montero, M. Herrero, Reprint of: application of mass spectrometry-based metabolomics approaches for food safety, quality and traceability, Trends Anal. Chem. 96 (2017) 62–78, https://doi.org/10.1016/j.trac.2017.08.007.

[11] L. Xu, Z.Z. Xu, I. Strashnov, X.J. Liao, Use of information dependent acquisition mass spectra and sequential window acquisition of all theoretical fragment-ion mass spectra for fruit juices metabolomics and authentication, Metabolomics 16 (8) (2020) 81, https://doi.org/10.1007/s11306-020-01701-2.

[12] M. Masarone, et al., Untargeted metabolomics as a diagnostic tool in NAFLD: discrimination of steatosis, steatohepatitis and cirrhosis, Metabolomics 17 (12) (2021) 1–13, https://doi.org/10.1007/s11306-020-01756-1.

[13] Y.J. Peng, N. Cheng, J.J. Li, Y.L. Kong, C.X. Sun, Effects of nitrogen fertilizer reduction on root exudates of maize seedling analyzed by rhizosphere metabonomics, Chin. J. Eco-Agric. 26 (6) (2018) 807–814, https://doi.org/10.13930/j.cnki.cjea.170858 (In Chinese).

[14] S.M. Qin, et al., Microbiome-metabolomics analysis insight into the effects of dietary resistant starch on intestinal integrity, Food Chem. 401 (1) (2023), 134148, https://doi.org/10.1016/j.foodchem.2022.134148.

[15] A.M. Evans, et al., Dissemination and analysis of the quality assurance (QA) and quality control (QC) practices of LC-MS based untargeted metabolomics practitioners, Metabolomics 16 (10) (2020) 113, https://doi.org/10.1007/s11306-020-01728-5.

[16] S. Boonchaisri, T. Stevenson, D.A. Dias, Utilization of GC-MS untargeted metabolomics to assess the delayed response of glufosinate treatment of transgenic herbicide resistant (HR) buffalo grasses (*Stenotaphrum secundatum* L.), Metabolomics 16 (2) (2020) 22, https://doi.org/10.1007/s11306-020-1644-9.

[17] I. Roberts, et al., Untargeted metabolomics of COVID-19 patient serum reveals potential prognostic markers of both severity and outcome, Metabolomics 18 (1) (2022) 6, https://doi.org/10.1101/2020.12.09.20246389.

[18] S. Fahad, H.Y. Xu, C. Zhi, H.M. Gong, H.P. Zhang, G.F. Zhao, Separation, identification, acid-resistent and halotolerant characterization of lactic acid bacteria from Shubat of Alashan bactrian camel, Food Res. Dev. 41 (16) (2020) 189–194, https://doi.org/10.12161/j.issn.1005-6521.2020.18.031 (In Chinese).

[19] G. Theodoridis, H.G. Gika, I.D. Wilson, LC-MS-based methodology for global metabolite profiling in metabonomics/metabolomics, Trends Anal. Chem. 27 (3) (2008) 251–260, https://doi.org/10.1016/j.trac.2008.01.008.

[20] B. Zhou, J.F. Xiao, L. Tuli, H.W. Ressom, LC-MS-based metabolomics, Mol. Biosyst. 8 (2) (2012) 470–481, https://doi.org/10.1039/C1MB05350G.

[21] W.B. Duan, et al., Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry, Nat. Protoc. 6 (7) (2011) 1060–1083, https://doi.org/10.1038/nprot.2011.335.

[22] J. Trygg, S. Wold, Orthogonal projections to latent structures (O-PLS), J. Chemometr. 16 (3) (2002) 119–128, https://doi.org/10.1002/cem.695.

[23] E. Saccenti, H.C.J. Hoefsloot, A.K. Smilde, J.A. Westerhuis, M.M.W.B. Hendriks, Reflections on univariate and multivariate analysis of metabolomics data, Metabolomics 10 (3) (2014) 361–374, https://doi.org/10.1007/s11306-013-0598-6.

[24] J.G. Xia, I.V. Sinelnikov, B. Han, D.S. Wishart, MetaboAnalyst 3.0-making metabolomics more meaningful, Nucleic Acids Res. 43 (2015) W251–W257, https://doi.org/10.1093/nar/gkv380.

[25] H. Ogata, S. Goto, K. Sato, W. Fujibuchi, H. Bono, M. Kanehisa, KEGG: kyoto encyclopedia of genes and genomes, Nucleic Acids Res. 27 (1) (2000) 29–34, https://doi.org/10.1093/nar/27.1.29.

[26] M. Kanehisa, Y. Sato, M. Kawashima, M. Furumichi, M. Tanabe, KEGG as a reference resource for gene and protein annotation, Nucleic Acids Res. 44 (D1) (2016) D457–D462, https://doi.org/10.1093/nar/gkv1070.

[27] R.R. Li, et al., Untargeted metabolomics allows to discriminate raw camel milk, heated camel milk, and camel milk powder, Int. Dairy J. 124 (2022), 105140, https://doi.org/10.1016/j.idairyj.2021.105140.

[28] J.C. Liu, et al., Combinational antibacterial activity of nisin and 3-phenyllactic acid and their co-production by engineered *Lactococcus lactis*, Front. Bioeng. Biotechnol. 9 (2021), 612105, https://doi.org/10.3389/fbioe.2021.612105.

[29] S.M. Ostojic, Creatine as a food supplement for the general population, J. Funct.Foods 83 (1) (2021) 104568, https://doi.org/10.1016/j.jff.2021.104568.

[30] M. Hall, E. Manetta, K. Tupper, Creatine supplementation: an update, Curr. Sports Med. Rep. 20 (7) (2021) 338–344, https://doi.org/10.1249/JSR.0000000000000863.

[31] S. Yilmaz, M. Sova, S. Ergun, Antimicrobial activity of trans-cinnamic acid and commonly used antibiotics against important fish pathogens and nonpathogenic isolates, J. Appl. Microbiol. 125 (6) (2018) 1714–1727, https://doi.org/10.1111/jam.14097.

[32] W.Y. Xu, Y. Shen, H. Zhu, J. Gao, Z.G. Wang, 2-Aminoadipic acid protects against obesity and diabetes, J. Endocrinol. 243 (2) (2019) 111–123, https://doi.org/10.1530/JOE-19-0157.

[33] G.O. Burr, M.M. Burr, A new deficiency disease produced by the rigid exclusion of fat from the diet, Nutr. Rev. 31 (8) (1973) 148–149, https://doi.org/10.1111/j.1753-4887.1973.tb06008.x.

[34] F.L. Chen, et al., Synthesis and application of novel silver magnetic amino silicone adhesive particles for preparation of high purity α-linolenic acid from tree peony seed oil under applied magnetic field, J. Chromatogr. A 1610 (11) (2020), 460450, https://doi.org/10.1016/j.chroma.2019.460540.

[35] J. Whelan, K. Fritsche, Linoleic acid, Adv. Nutr. 4 (3) (2013) 311–312, https://doi.org/10.3945/an.113.003772.

[36] M. Sato, K. Shibata, R. Nomura, D. Kawamoto, R. Nagamine, K. Imaizumi, Linoleic acid-rich fats reduce atherosclerosis development beyond its oxidative and inflammatory stress-increasing effect in apolipoprotein E-deficient mice in comparison with saturated fatty acid-rich fats, Br. J. Nutr. 94 (6) (2005) 896–901, https://doi.org/10.1079/BJN20051409.

[37] C.O. Souza, A.A.S. Teixeira, L.A. Biondo, E.A.L. Junior, H.A.P. Batatinha, J.C.R. Neto, Palmitoleic acid improves metabolic functions in fatty liver by PPARα-dependent AMPK activation, J. Cell. Physiol. 232 (8) (2016) 2168, https://doi.org/10.1002/jcp.25715.

[38] H. Hussain, et al., Camel milk as an alternative treatment regimen for diabetes therapy, Food Sci. Nutr. 9 (3) (2021) 1347–1356, https://doi.org/10.1002/fsn3.2078.

[39] A. Blaszczyk, S. Matysiak, J. Kula, K. Szostakiewicz, Z. Karkusiewicz, Cytotoxic and genotoxic effects of (R)- and (S)-ricinoleic acid derivatives, Chirality 32 (7) (2020) 998–1007, https://doi.org/10.1002/chir.23226.

[40] X. Xu, J. Cheng, S. Luo, X. Gong, H. Zhou, Deoxycholic acid-stimulated macrophage-derived exosomes promote spasmolytic polypeptide-expressing metaplasia in the stomach, Biochem. Biophys. Res. Commun. 524 (3) (2020) 649–655, https://doi.org/10.1016/j.bbrc.2020.01.159.
[41] C.X. Wang, et al., The protective effect of Cx43 protein-mediated phosphocreatine on myocardial ischemia/reperfusion injury, Cardiol. Res. Pract. 22 (2021), 8838151, https://doi.org/10.1155/2021/8838151.
[42] N.B. To, Y.T.K. Nguyen, J.Y. Moon, M.K. Ediriweera, S.K. Cho, Pentadecanoic acid, an odd-chain fatty acid, suppresses the stemness of MCF-7/SC human breast cancer stem-like cells through JAK2/STAT3 signaling, Nutrients 12 (6) (2020) 1663, https://doi.org/10.3390/nu12061663.
[43] A.M. Mustafa, M.N. Ashikeen, A.N. Hafidz, O. Baib, Effect of acid load (Citric acid) on diabetes-induced rats, Int. J. Med. Toxicol. Legal Med. 21 (3 and 4) (2018) 265, https://doi.org/10.5958/0974-4614.2018.00070.0.
[44] M. Pitto, P. Palestini, M. Masserini, Dependence of rat liver CMP-N-acetylneuraminate :GM1 sialyltransferase (SAT IV) activity on the ceramide composition of GM1 ganglioside, FEBS Lett. 383 (3) (1996) 223–226, https://doi.org/10.1016/0014-5793(96)00262-1.
[45] A. Amin, et al., Differential effects of L- and D-phenylalanine on pancreatic and gastrointestinal hormone release in humans: a randomized crossover study, Diabetes Obes. Metabol. 23 (1) (2021) 147–157, https://doi.org/10.1111/dom.14204.
[46] N. Hegdekar, M.M. Lipinski, C. Sarkar, N-Acetyl-L-leucine improves functional recovery and attenuates cortical cell death and neuroinflammation after traumatic brain injury in mice, Sci. Rep. 11 (1) (2021) 9249, https://doi.org/10.1038/s41598-021-88693-8.
[47] A.L. Mindikoglu, et al., Unique metabolomic signature associated with hepatorenal dysfunction and mortality in cirrhosis, Transl. Res. 195 (2018) 25–47, https://doi.org/10.1016/j.trsl.2017.12.002.
[48] D. Crucean, G. Debucquet, C. Rannou, A. Le-Bail, P. Le-Bail, Vitamin B4 as a salt substitute in bread: a challenging and successful new strategy. Sensory perception and acceptability by French consumers, Appetite 134 (2019) 17–25, https://doi.org/10.1016/j.appet.2018.12.020.
[49] Y.C. Boo, Mechanistic basis and c1inica1 evidence for the app1ications of nicotinamide (niacinamide) to contro1 skin aging and pigmentation, Antioxidants 10 (8) (2021) 1315, https://doi.org/10.3390/antiox10081315.
[50] J. Xu, et al., Cerebral deficiency of vitamin B5 (d-pantothenic acid; pantothenate) as a potentially-reversible cause of neurodegeneration and dementia in sporadic Alzheimer's disease, Biochem. Biophys. Res. Commun. 527 (3) (2020) 676–681, https://doi.org/10.1016/j.bbrc.2020.05.015.