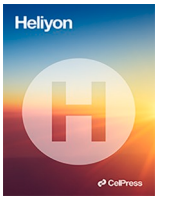
Heliyon 10 (2024) e36429

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Daily koumiss has positive regulatory effects on blood lipids and immune system: A metabolomics study

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ARTICLE INFO

Keywords:
Koumiss
Metabolomics
Lipid metabolism
Cardio-cerebrovascular

ABSTRACT

Koumiss, a traditional Mongolian beverage, is believed to possess high nutritional value and potential medical benefits. However, there is a lack of comprehensive research on its potential impact on the human body. Metabolomics, as a sensitive approach in systems biology, offers a new avenue for studying the overall effects of koumiss. In this work, metabolomics was utilized to identify potential biomarkers and pathways associated with koumiss using UPLC-MS detection, pattern recognition analysis, pathway enrichment, network pharmacology. The findings indicated that koumiss exerts a beneficial regulatory influence on lipid metabolism, neurotransmitters, hormones, phospholipids and arachidonic acid metabolism, besides up regulating the content of nutrients. It could reduce the risks of dyslipidemia and inflammatory responses. This study confirmed the benign regulatory effect of koumiss on normal organism from the perspective of endogenous metabolites, and provided objective support for the promotion and application of this ethnic food.

1. Introduction

Koumiss is a traditional fermented dairy product originating from central Asia. It is made from fresh horse milk fermented by lactic acid bacteria and yeasts spontaneously. Described in the Principles of Correct Diet (Yin Shan Zheng Yao) of the Yuan Dynasty (1271 CE–1368), koumiss is characterized by its light and warm nature, with a taste profile encompassing sweet, sour and some astringent notes. It has the functions of invigorating spleen, nourishing stomach, moistening lung, promoting blood circulation and improving sleep. As a traditional fermented dairy product, koumiss is rich in amino acids, unsaturated fatty acids, lactose, vitamins, minerals and other essential components of the human body, which are beneficial to health [1,2]. Additionally, research suggests that koumiss may have therapeutic properties, including enhancing immune function [3] and serving preventive or adjuvant effect on cardiovascular [4], digestive and kidney diseases [5].

Currently, research on koumiss primarily centers on its probiotic bacterium, including the isolation, identification and activity of microbiota in koumiss [6–10]. However, the effect of koumiss as a daily diet for the normal body remains unclear. Metabolomics is a

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https://doi.org/10.1016/j.heliyon.2024.e36429
Received 4 November 2023; Received in revised form 5 August 2024; Accepted 15 August 2024
Available online 17 August 2024

correlation between data-intensive chemical analysis and chemometrics, used for metabolic profiling in intricate systems [11–13]. It could reflect changes in endogenous metabolites that are the most sensitive to disturbance of external impact on the organism, providing a new perspective on assessing the holistic influence of koumiss. In this study, metabolomic strategy was approached to explore the disturbance of this traditional fermented food on normal body metabolism, accordingly to provide basic data for the daily use of koumiss, and to provide a potential mechanism for its further application in dairy therapy.

2. Materials and methods

2.1. Instruments and reagents

Waters UPLC (Waters, USA); AB SCIEX Triple TOF 5600 (AB Sciex, USA); Milli-Q ultrapure water instrument (Millipore, USA); Velocity 14R high-speed refrigerated centrifuge (Dynamic, Australia); Chromatographic-grade acetonitrile and formic acid (Merck, Germany); AB Sciex APCI calibration solution for Triple TOF (AB Sciex, USA). Koumiss, bought from the Ili basin market in Xinjiang, is kept in a refrigerator at temperatures below −20 °C.

2.2. Animal experiments

Twenty male specific pathogen-free Sprague-Dawley (SD) rats (weight, 180 ± 20 g) were provided by the Experimental Animal Centre of Southern Medical University (certificate number, 44002100013523). All tests were conducted following the globally recognized standard protocols for animal usage. The research was carried out in compliance with the national laws of China and local regulations, and the Ethical Committee of Guangdong Provincial Hospital of Chinese Medicine also approved the treatment and management of the rats. The rats were housed under standard environmental conditions (23 ± 2 °C, 55 % ± 5 % humidity and 12 h/12 h light/dark cycle) and were allowed to eat freely.

After 3 days of adaptive feeding, the rats were randomly divided in two groups: the control group (feeding saline) and the SMN group (feeding koumiss). Koumiss or saline were given at the dose of 22.5 mL/kg/d (calculated as 250 mL of Koumiss per person everyday) by gastric administration for 8 weeks. Rats were sacrificed after being anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg of body weight) (GBCBIO Technologies, Guangzhou, China) and about 3 mL blood was obtained from the abdominal aorta in each rat. Blood was centrifuged at 1500 rpm for 10 min at normal temperature. Serum was collected and immediately stored at −80 °C.

2.3. Sample collection and preparation

Acetonitrile (1200 μL) was combined with thawed serum (300 μL) and stirred for a duration of 2 min. After centrifugation at 15,000 rpm for 15 min at 4 °C, the supernatant was filtered by a microporous filter (0.22 μm) and transferred to HPLC vial. Prior to the sample detection, the precision of the instrument, the stability of the sample and the repeatability of the method were investigated firstly. The RSD values of retention time and peak area were calculated to assess the methodology of metabolomics analysis. During sample sequence detection, QC samples and calibration solution were detected at every 5 injection intervals.

2.4. Instrumentation and conditions

The separation condition for chromatography in UPLC-Q/TOF MS was carried out using an Acquity UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm), with the temperature set at 35 °C. The mobile phase was water (0.1 % formic acid) as phase A and acetonitrile as phase B. Gradient elution was used and set as follows: 0–2 min, 2 % B; 4 min, 25 % B; 10 min, 50 % B; 12 min, 65 % B; 22 min, 85 % B; 28–30 min, 98 % B. The volume of the injection was 5 μL, while the velocity of the flow was 0.4 mL per minute. Each wash cycle consisted of 200 μL of strong wash solvent (80 % MEOH-H2O, 8:2, v/v) and 600 μL of weak wash solvent (10 % MEOH-H2O, 1:9, v/v).

The mass spectrum condition in AB SCIEX Triple TOF 5600 was performed on the Duo Spray source (AB Sciex, UK), as ESI probe for sample analysis and APCI probe for calibration solution. MS spectra were acquired both in positive and negative ion mode with high resolution, and information dependent acquisition (IDA) was used to acquire fragment information of the top 6 compounds automatically. The desolvation and auxiliary gas flow were both 50 mL/min, and curtain gas was 35 mL/min. The ion spray voltage was 5500 V in positive ion mode and 4500 V in negative ion mode respectively at temperature 500 °C. For MS scan, the declustering potential voltage was 80 V in positive mode and 100 V in negative mode with accumulation time of 0.1 s. For product ion scan, the collision energy was 35 V with a spread of 15 V. Dynamic background subtractions were used throughout the acquisition process.

2.5. Instrumentation and conditions

The high stability of the tool and technique offered significant confidence in the dependability and precision of the experimental analysis. Therefore, a range of method validation tests, including precision, repeatability, and stability, were conducted to evaluate the UPLC-Q/TOF MS method. The procedures for preparing and determining the samples were carried out as per the previously mentioned section. The accuracy was evaluated by performing six successive injections of the sample solutions for analysis. In terms of repeatability, six example solutions were concurrently extracted. The stability was examined by studying a single sample over a 24-h duration (at intervals of 0, 4, 8, 12, 16, 20, and 24 h) [13].

2.6. Data processing

The original mass spectrum data were conducted by MarkerView software (version 1.2.1, AB Sciex) to align peaks, reduce noise, normalize and correct missing values. The data acquisition range was 1–30 min, and the minimum peak width was 50 ppm. The deviation of retention time and *m/z* are within 0.05 min and 25 ppm respectively.

The processed data was then exported into SIMCA-P 13.0 and processed by the principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). PCA was used first to determine the general interrelation between the groups as unsupervised analysis, and PLS-DA was subsequently performed to maximize the difference in metabolic profiling as supervised analysis. The fitness qualities of the PLS-DA model were evaluated with value of R2 (the percentage of variation being explained by the model) and Q2 (denoting the predictive ability of the model). A typical 7 round cross validation was used to validate the model against over fitting. The variables with VIP value (variable importance in projection) of PLS-DA over 1.5 and p value of t-test between groups less than 0.05 were deemed to be potential metabolite biomarkers.

Qualitative analysis of compounds was performed using Peak View software 1.2 version (AB Sciex, USA), by searching the databases of HMDB (www.hmdb.ca/spectra/ms/search), Pubchem (pubchem.ncbi.nlm.nih.gov), NIST (www.nist.gov/srd/nist1a.html), MassBank (massbank.eu/MassBank/) and KEGG (www.genome.jp/kegg/) according to the information of MS information, isotope peak ratio and MS/MS fragments. Metabolic pathway analysis was performed based on identified metabolites in the system of MetaboAnalyst (www.metaboanalyst.ca/).

2.7. Statistics and data availability

SPSS software (Version 18.0, USA) was used for statistical analyses. Data were presented as the mean ± standard deviation for continuous variables with a normal distribution. Assumptions of normality and homogeneity of variance were first checked, and independent samples t-test was used to analyze the differences between groups. Statistical differences were acknowledged for p values below 0.05, while p values below 0.01 were deemed to have significant differences.

The entirety of the data produced throughout this research is incorporated in the published paper, and the unprocessed data can be obtained from the author responsible, given a reasonable request.

3. Results

3.1. General observations

In this study, the weight, organ index and some biochemical indicators were monitored to observe the effects of koumiss on the body by daily intervention. Compared to control group, the spleen of rats in SMN group was significantly enlarged ($p < 0.05$), and the blood triglyceride content was significantly reduced ($p < 0.05$). In weight, other organ indexes and blood indicators, no significant differences were observed. It is worth noting that the weight and blood glucose content of SMN rats showed a downward trend, no statistical difference though. The spleen index and triglyceride were shown in Fig. 1A and B, and the other indicators were listed in supplementary materials.

3.2. Metabolic profiling

In this study, metabolic profiling of serum samples was conducted in both positive and negative ionization modes due to the detection of numerous chromatographic peaks in both modes. Serum samples were conducted UPLC-MS under the optimized conditions and representative profile is shown in Fig. 2A and B.

The paired retention time_m/z of these peaks: 1.35_204.1228, 5.42_608.3840, 12.47_415.2123, 14.01_520.3390, 14.89_496.3390, 16.20_510.3544, 17.09_524.3702, 17.72_524.3705, 23.31_703.5737 were selected in positive ion mode, and 1.34_167.0253, 5.38_324.9539, 11.64_604.3388, 13.99_564.3423, 14.44_540.3422, 14.89_540.3416, 17.08_568.3737, 17.71_568.3726,

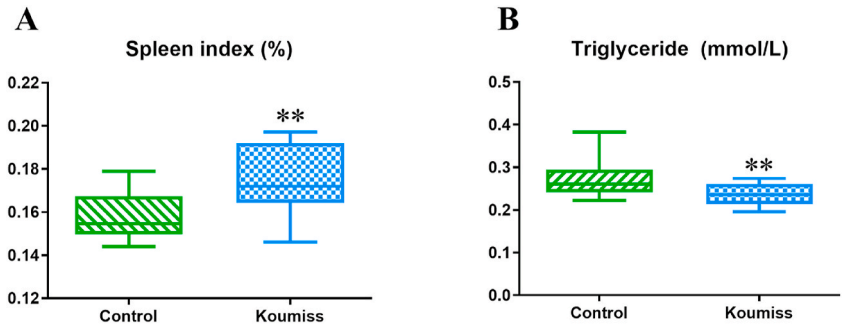

Fig. 1. The spleen index and blood triglyceride on rats. (A) Spleen index of rats; (B) Blood triglyceride (CON vs SMN: $^{**}p < 0.05$).

20.68_303.2384 were selected in negative ion mode to conduct methodological investigation. The variance of their retention time and peak intensity were calculated respectively. It was found RSD values of retention time and peak intensity were were less than 1 % and 10 %, indicating a reliable and reproducible metabolomics analysis method.

3.3. Pattern recognition

In this study, the significance of the intensity was reduced by Pareto scaling to improve equal importance of variables regardless of the magnitude. PCA was conducted first on the normalized UPLC-MS data to give the comprehensive view of the clustering trend (Fig. 3A and B). In PCA score plot, the control and SMN group were separated clearly both in positive and negative ion mode, indicating that koumiss drinking daily could alter endogenous metabolism of rats significantly.

Subsequently, PLS-DA was conducted to maximize the difference in metabolic profiling and to find the metabolites with a significant concentration change as shown in Fig. 3C and D. In the positive mode, the R^2Y and Q^2Y values calculated by SIMCA-P package were 0.999 and 0.949 respectively, meaning 99.9 % of data fit the model and 94.9 % of data could be predicted by this model. In the negative mode, the R^2Y and Q^2Y values were 0.994 and 0.929 respectively, meaning 99.4 % of data fit the model and 92.9 % of data could be predicted by this model. Both the Q^2Y and R^2Y close to 1 indicate an excellent model which is good to fitness and prediction.

3.4. Potential biomarkers and related pathway

Metabolites that significantly contributed to the clustering and discrimination were identified, with VIP >1.5 in PLS-DA analysis and $p < 0.05$ in independent samples *t*-test. Finally, 42 potential biomarkers were identified and listed in Table 1. As shown in Fig. 4A, the biomarkers mainly included nutrients (amino acids, vitamins, DHA, hormones and related productions), metabolites involved in arachidonic acid and cholesterol regulation (bile acids, carnitines and ubiquinol) and cardiovascular and cerebrovascular disease (ceramide, neuromedin, derivatives of linoleic acid, as well as some phospholipids). The relative levels of biomarkers were also analyzed, and the heat map was constructed as shown in Fig. 4C. Metabolic pathway analysis indicated that the influence of koumiss daily on normal rats is mainly related to pathways of glycerophospholipid metabolism, bile acids biosynthesis and metabolism, amino acids metabolism and degradation, as well as vitamins and purine metabolism (Fig. 4B). Besides, network pharmacology was constructed to build "marker-class-activity" topological map in this study. The results showed that the 42 metabolites are mainly involved in lipid metabolism, cardiovascular and nervous system, inflammation and oxidative stress, in addition to some nutrients (Fig. 4D).

4. Discussion

4.1. Koumiss contains a variety of nutrients that the body needs

Koumiss is a beverage that is abundant in essential nutrients required by the body, including protein, fat, lactose, vitamins and

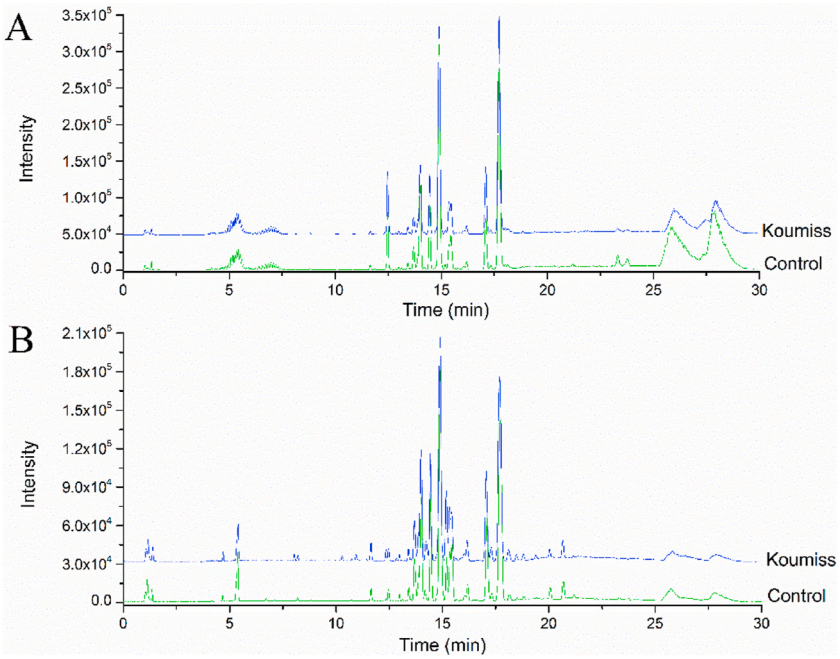

Fig. 2. Represented UPLC/MS BPC profiles on serum samples of rats. (A) Positive ionization mode; (B) Negative ionization mode.

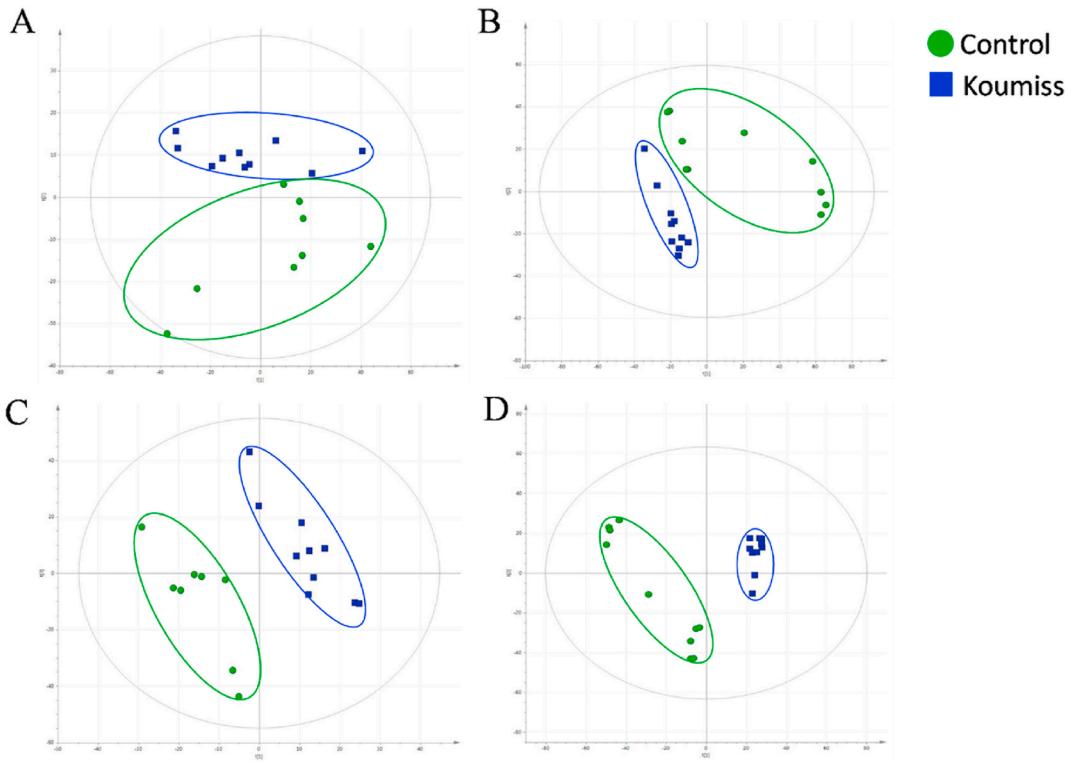

Fig. 3. The metabolic path of different rat clusters in the pattern recognition score plot. (A) PCA score plot in positive ionization mode; (B) PCA score plot in negative ionization mode; (C) PLS-DA score plot in positive ionization mode, Q^2Y (cum) = 0.949 , R^2X (cum) = 0.618 , R^2Y (cum) = 0.999; (D) PLS-DA score plot in negative ionization mode, Q^2Y(cum) = 0.929 , R^2X(cum) = 0.453 , R^2Y(cum) = 0.994.

minerals. Research indicates that koumiss contains higher levels of essential amino acids, unsaturated fatty acids, lactose and vitamins compared to other types of domestic animal milk [14,15]. In this study, metabolomics investigation screened thirteen nutrients or related metabolites among the 42 potential biomarkers: Valine, D-Erythro-imidazole-glycerol-phosphate, Arabinofuranobiose, DHA, 5-methyltetrahydrofolic acid, 2-Hydroxyestrone-1-S-glutathione, 23S,25-dihydroxyvitamin D3, 13′-Carboxy-alpha-tocopherol, Pipecolic acid, Indole-3-propionic acid, 11′-Carboxy-alpha-tocotrienol, L-Cystine and 5-Aminopentanamide.After administration of daily koumiss on normal rats, the contents of amino acids and vitamins, such as Valine, DHA, 5-methyltetrahydrofolicacidand 13′-Carboxy-alpha-tocopherol, increased significantly in serum of murine (Fig. 5). Koumiss plays a positive effect on cardio-cerebrovascular and nervous system. These findings provide straightforward evidence for the high nutritional value of koumiss based on endogenous terminal metabolism.

4.2. Koumiss influents inflammation and immune regulatory of rats

Consuming Koumiss on a daily basis has been shown to enhance the immune system, as approximately 80 % of the body's tissues are located in the intestines. The immune system experiences a notable decline following the elimination of intestinal bacteria. Research has demonstrated that animals lacking gut bacteria exhibit reduced levels of crucial white blood cells and protective chemicals in their bloodstream. Upon reintroduction of naturally occurring bacteria into the intestinal tract of these animals, white blood cells are activated, leading to a reinforcement of the immune system. Bacteria present in fermented foods have been found to produce chemicals that can traverse the intestinal barrier and induce the generation of immune cells within the immune system [16]. Research on the impact of Koumiss on the immune system and its support of antibacterial properties has demonstrated significant enhancements in the immune response of test subjects. Consumption of fresh mare milk has been shown to increase the thymus and spleen indices, enhance macrophage function, and elevate hemolysin levels in the blood serum. In addition, koumiss has been shown to enhance the weight of immune organs in BALB/c mice, improve normal immune functions, regulate cell immune capabilities, and modulate abnormal body fluid immune responses [17].

The results of this experiment also suggest that koumiss influents inflammation and immune regulatory of rats. There are five components belonging to arachidonic acids (AA) and linoleic acids (LA) screened as potential biomarkers in this experiment. Arachidonic acid and linoleic acid are implicated immune regulatory, pro-inflammatory, inflammation resolving, platelet aggregation and neuronal signaling [18,19], that are correspondingly related to the pathophysiology of metabolic and cardio-cerebrovascular [20,21]. Tetranor 12-HETE is the major β-oxidation product resulting from peroxisomal metabolism of 12(S)-HETE, which plays a role in inflammation, immune cell recruitment, vasoconstriction, and neurological function [18,22,23]. 20-Hydroxy-leukotriene E4 is a

Table 1
Identification of significantly differential expressed endogenous metabolites in the serum of rats[a].

No.	Identified potential biomarkers	VIP	Concentration (Intensity/1000) [b]		F [c]	Sig. [c]	*p* value [d]
			CON	SMN			
BM01	Valine	1.6218	57.49 ± 13.05	76.04 ± 8.33	3.166	0.092	1.34×10^{-3}
BM02	D-Erythro-imidazole-glycerol-phosphate	1.9156	7.48 ± 1.16	5.77 ± 0.79	1.148	0.298	1.16×10^{-3}
BM03	Arabinofuranobiose	1.7494	56.70 ± 4.83	46.81 ± 5.67	0.754	0.397	5.42×10^{-4}
BM04	9(S)-HODE	1.6464	20.81 ± 3.71	17.32 ± 2.56	1.272	0.274	2.51×10^{-2}
BM05	3-Hydroxyhexadecanoylcarnitine	1.6937	89.94 ± 21.23	117.63 ± 13.15	2.321	0.145	2.52×10^{-3}
BM06	DHA	1.6588	46.66 ± 11.01	61.69 ± 8.32	0.876	0.362	2.90×10^{-3}
BM07	5-methyltetrahydrofolic acid	1.7986	133.57 ± 34.02	183.43 ± 22.2	2.558	0.127	1.09×10^{-3}
BM08	2-Hydroxyestrone-1-S-glutathione	1.6781	252.63 ± 73.23	342.07 ± 46.89	4.166	0.056	4.42×10^{-3}
BM09	3-O-Sulfogalactosylceramide	1.6276	159.97 ± 44.24	209.14 ± 25.68	4.703	0.044	8.57×10^{-3}
BM10	23S,25-dihydroxyvitamin D3	1.8214	25.87 ± 6.97	36.67 ± 5.69	0.030	0.864	1.32×10^{-3}
BM11	13′-Carboxy-alpha-tocopherol	1.7835	38.37 ± 9.95	57.08 ± 12.38	0.468	0.502	1.55×10^{-3}
BM12	Neuromedin N (1–4)	1.6988	38.34 ± 11.20	56.77 ± 12.74	0.097	0.759	2.95×10^{-3}
BM13	Ubiquinol-6	1.7079	39.57 ± 11.34	57.04 ± 13.14	0.466	0.504	5.14×10^{-3}
BM14	Pipecolic acid	1.5759	35.87 ± 9.05	23.56 ± 9.04	0.226	0.640	7.03×10^{-3}
BM15	Indole-3-propionic acid	1.6222	52.74 ± 13.80	33.44 ± 12.72	0.585	0.454	4.43×10^{-3}
BM16	Creatinine	1.5306	41.21 ± 5.67	31.71 ± 2.50	3.632	0.072	1.30×10^{-4}
BM17	Taurocholic acid	1.8613	1679.74 ± 177.20	4656.77 ± 1853.36	10.473	0.005	6.47×10^{-4}
BM18	11′-Carboxy-alpha-tocotrienol	1.8793	93.90 ± 11.55	231.42 ± 75.54	10.068	0.005	2.51×10^{-4}
BM19	LysoPC(16:1)	1.7688	34.49 ± 2.10	29.46 ± 4.86	4.729	0.043	1.08×10^{-2}
BM20	Allantoin	1.8919	340.50 ± 82.05	498.20 ± 39.15	10.542	0.004	1.08×10^{-4}
BM21	TG(24:0)	1.5438	63.66 ± 15.06	82.77 ± 8.95	0.564	0.462	2.86×10^{-3}
BM22	LysoPC(P-16:0)	1.6293	81.97 ± 18.33	100.86 ± 8.58	1.81	0.195	8.54×10^{-3}
BM23	Dihydrothymine	1.7401	23.92 ± 8.33	14.60 ± 3.70	6.666	0.019	6.89×10^{-3}
BM24	LysoPC(18:0)	1.6055	94.30 ± 15.67	70.62 ± 11.95	0.576	0.458	1.31×10^{-3}
BM25	L-Cystine	1.7164	194.62 ± 33.42	144.52 ± 26.54	0.291	0.596	1.60×10^{-3}
BM26	Adrenoyl ethanolamide	1.7359	366.93 ± 48.07	279.79 ± 41.08	0.379	0.546	3.79×10^{-4}
BM27	Deoxycholic acid	1.8089	164.85 ± 22.73	209.20 ± 17.43	0.417	0.526	1.16×10^{-4}
BM28	Tetranor 12-HETE	1.7192	341.23 ± 39.23	277.04 ± 27.13	1.641	0.217	4.76×10^{-4}
BM29	8(R)-Hydroperoxylinoleic acid	1.5482	197.54 ± 47.24	263.72 ± 17.63	9.912	0.006	$1.48 \times 10\text{-}3^{-}$
BM30	LysoPE(22:6)	1.5006	324.37 ± 104.63	187.82 ± 53.81	5.212	0.035	2.68×10^{-3}
BM31	20-Hydroxy-leukotriene E	1.6787	198.64 ± 30.58	138.83 ± 15.69	11.621	0.003	9.03×10^{-5}
BM32	LysoPC(26:1)	1.6427	9.05 ± 2.15	12.63 ± 2.01	0.010	0.920	1.16×10^{-3}
BM33	LysoPE(18:2)	1.5617	20.69 ± 6.37	41.33 ± 14.71	6.108	0.024	1.48×10^{-3}
BM34	LysoPI(18:0)	1.5380	1.89 ± 0.63	2.81 ± 0.72	0.685	0.419	6.87×10^{-3}
BM35	Isoacitretin	1.6593	36.53 ± 4.79	30.14 ± 4.32	0.171	0.684	5.69×10^{-3}
BM36	9-OxoODE	2.0438	4.11 ± 0.98	5.24 ± 0.88	0.005	0.943	1.40×10^{-2}
BM37	5-HETE	1.6259	17.23 ± 2.02	13.10 ± 1.58	0.284	0.600	7.62×10^{-5}
BM38	5-Aminopentanamide	1.6725	37.80 ± 2.64	31.11 ± 1.46	2.574	0.126	1.50×10^{-6}
BM39	Homocysteine thiolactone	2.0244	6.59 ± 0.81	9.38 ± 1.59	15.229	0.001	2.43×10^{-4}
BM40	Leukotriene C5	1.7129	266.23 ± 39.35	316.19 ± 21.94	4.243	0.054	2.52×10^{-3}
BM41	Hypoxanthine	1.5229	101.61 ± 21.24	74.84 ± 15.99	0.861	0.366	5.13×10^{-3}
BM42	Cholesterol sulfate	1.9862	50.40 ± 10.60	16.04 ± 7.80	0.802	0.382	1.57×10^{-7}

[a] The presence of metabolites was verified using Rt and *m/z*, compared to genuine chemicals.
[b] The mean ± standard deviation is used to represent the concentration of potential biomarkers in rat serum.
[c] The F and Sig. values were derived from the *t*-test of independent samples. The F value represents the ratio of the variance among the groups to the variance within the group.
[d] *p* values were calculated from the *t*-test (CON: control group; SMN: koumiss group).

metabolite that can originate from the lipid oxidation of leukotriene E4 (LTE4), which is a kind of cysteinyl leukotriene as potent inflammatory mediators [18,24]. Leukotriene C5 is a slow reacting substance derived from eicosapentaenoic acid and has similar biological activity as leukotriene C4, which is also a kind of cysteinyl leukotriene [25]. 9-OxoODE belongs to the class of organic compounds known as lineolic acids and derivatives, and 5-HETEis an endogenous eicosanoid involved in the pathway of leukotriene synthesis [18]. After koumiss administration, the level of Tetranor 12-HETE, 5-hydroxyeicosatetraenoic acid (5-HETE) and Leukotriene C5 increased and 20-Hydroxy-leukotriene E, 9-OxoODE decreased significantly. The contents of above five metabolites showed different trends.

Research conducted on healthy human adults has shown that higher consumption of AA or LA does not lead to an increase in levels of inflammatory markers. Additionally, epidemiological studies have indicated a potential link between ARA and LA and reduced inflammation [18]. While inflammation is a normal process that is part of host defence and tissue healing [26], the effect of koumiss on inflammation called further targeted researches. Considering the reports on immune regulatory function of koumiss [3], enzyme linked immunosorbent assay was conducted to detect the level of immune cytokines in this work. The results showed that the content of IFN-γ, IgA and IgG in serum increased significantly in SMN group ($p < 0.05$), confirming the effect of koumiss on enhancing immunity.

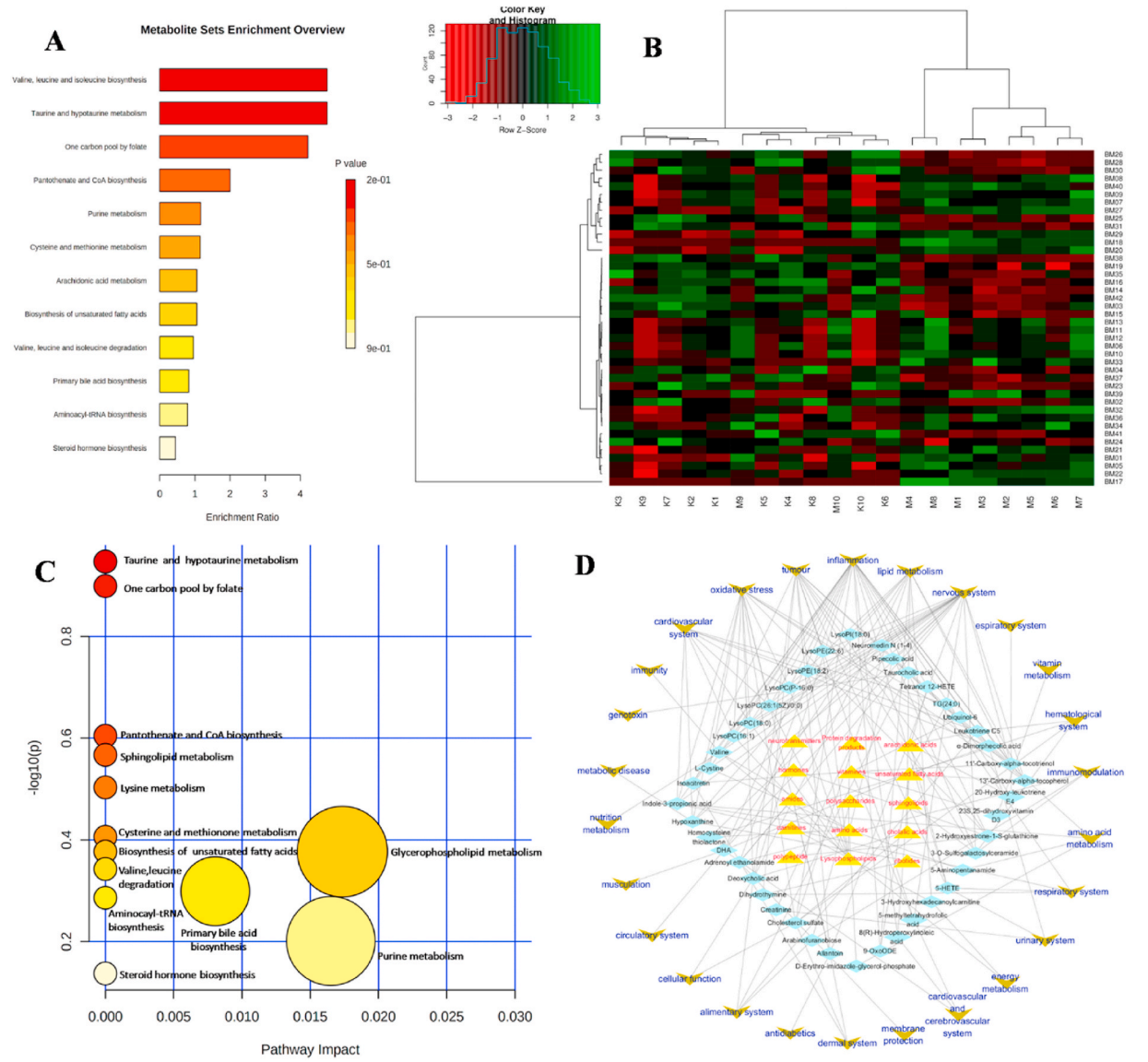

Fig. 4. Analysis of potential biomarkers and related pathway. (A) Overview of enriched metabolite sets (top 25); (B) Heat map of 42 potential biomarkers between groups (red, up regulated; green, down regulated). Rows: metabolites; Columns: samples (K: control group; M: koumiss group); (C) Pathway analysis; (D) Network topology on class and bioactivity of potential biomarkers (diamond: potential biomarkers; triangle: class; inverted triangle: bioactivity).

4.3. Koumiss regulates lipid metabolism of normal rats

Recent years, there has been a growing emphasis on scientific research regarding natural food products that have the potential to effectively reduce serum cholesterol levels with minimal or no adverse effects. Specifically, the probiotic properties of LAB cultures have been under evaluation. One notable benefit of probiotic-rich foods is their ability to lower serum cholesterol levels [27]. There is a continued interest in the development of bioactive compounds from food sources, such as traditional dairy products or dairy products fortified with Lactobacillus acidophilus like Koumiss, that have cholesterol-lowering properties [28]. Consistent intake of dairy products offers three essential antihypertensive nutrients (calcium, whey-derived peptides, and casein phosphopeptides) as well as additional active peptides that have been shown to reduce blood pressure [29].

After the intervention of koumiss, the contents of the above metabolites in rats were statistically differently, indicating that daily drinking of koumiss can regulate the lipid metabolism of normal healthy body. There are six potential biomarkers involved in lipid metabolism: 3-Hydroxy-hexadecanoylcarnitine, DHA, Ubiquinol-6, Taurocholic acid, Deoxycholic acid and 8(R)-hydroperoxylinoleic acid. Besides, seven lysophospholipids, as a class of functional lipids with high abundance in blood [30], were also screened as potential biomarkers in this work.

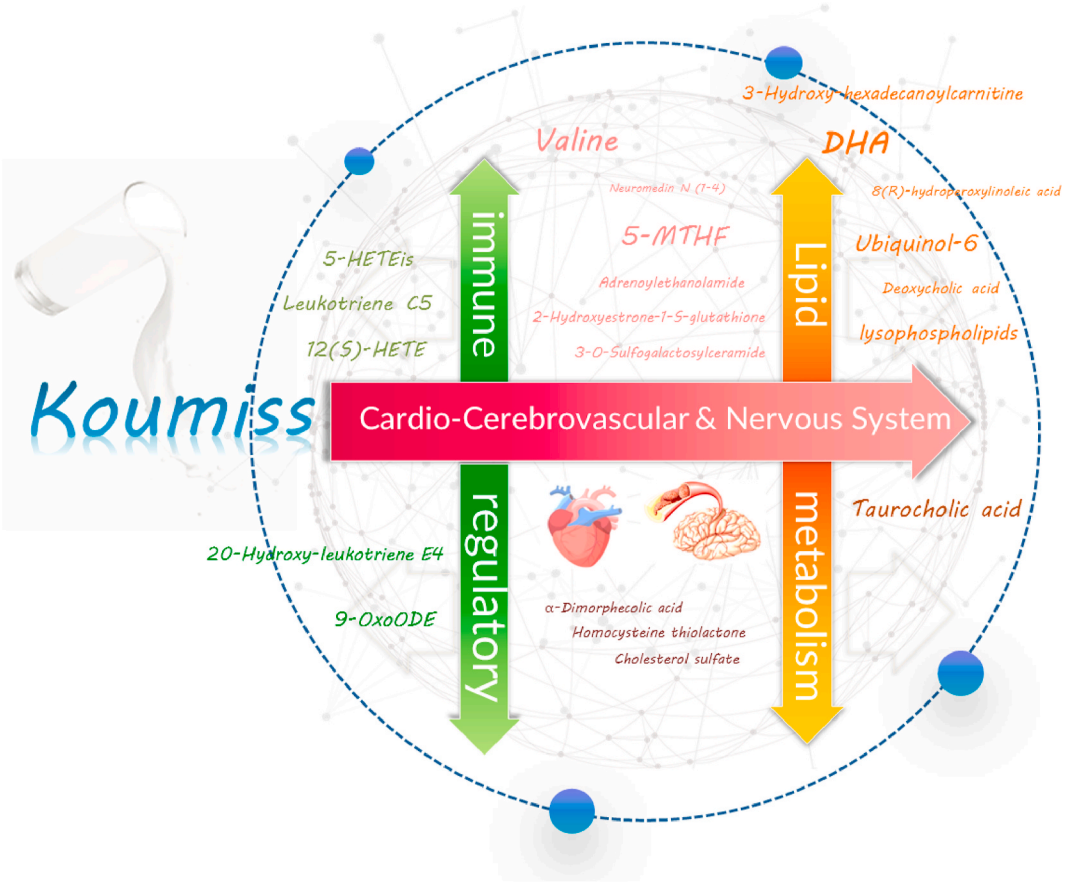

Fig. 5. The underlying mechanism of Koumiss ameliorating Cardio-Cerebrovascular Systems.

3-Hydroxy-hexadecanoylcarnitine is a long chain acylcarnitine that generated from long-chain fatty acids in the diet, and its function is to ensure long-chain fatty acids transport into the mitochondria [31]. It is thought as a feedback inhibition mechanism of insulin resistance [32,33]. The level of long chain acylcarnitine in blood increased when the oxidation of fatty acids is incompletely or the metabolism of carbohydrate and lipid altered [34]. In chronic heart failure patients, long chain acylcarnitine metabolite level was reported to increase in several studies [35–37]. DHA is an omega-3 essential fatty acid. It can reduce the levels of triglycerides in the blood and prevent them from lining the arterial walls, leading to decrease chances of contracting heart diseases considerably [38–40]. Ubiquinol-6 is an effective antioxidant in a number of membrane and biological systems by preventing peroxidative damage to lipids [41,42]. Taurocholic acid, Deoxycholic acid and 8(R)-hydroperoxylinoleic acid belongs to bile acids and oxidative product, playing an important role in lipid metabolism [43–45].

Lysophospholipids, including lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE) and lysophosphatidylinositol (LPI), have been reported in many metabolomics researches [46]. They are highly abundant in plasma and could bind to protein carriers in the extracellular milieu. The amphiphilic properties of some of them enable their extracellular secretion and significance as signaling molecules [47]. For example, has been recently recognized as a crucial contributor to the biosynthesis of neuronal membranes [48]. It has also been documented to have inflammatory, anti-coagulant, and cytotoxic functions [49]. Moreover, LPC has the ability to enhance oxidative stress and trigger abnormalities in cell structure and function, which eventually lead to hemodynamic disturbances [50,51]. Elevated amounts of LPC might intensify the production of nitric oxide, leading to a rise in the creation of reactive oxidative species (ROS). This could further cause damage to endothelial cells in atherosclerosis and cardiovascular diseases [52,53]. Numerous researches have shown that LPE participates in a variety of cell functions, especially in the differentiation and migration of PC-12 neuronal cells [54]. It was reported as a neurotrophic activator via activation of MAPK cascade, of which induced neuronal differentiation and suppressed serum deprivation-induced apoptosis. LPI is recognized for triggering signaling routes related to cell growth, movement, and tumor formation [55]. Of note, LPI has the ability to act as a lipid signaling molecule, participating in the control of fat accumulation and glucose balance [56], indicating a physiological role of LPI in regulation and metabolism.

4.4. Koumiss has therapeutic potential in cardio-cerebrovascular and nervous system

There are nine potential biomarkers involved in regulating of cardio-cerebrovascular and nervous system: Valine, α-Dimorphecolic

acid, 5-methyltetrahydrofolic acid (5-MTHF), 2-Hydroxyestrone-1-S-glutathione, 3-O-Sulfogalactosylceramide, Neuromedin N (1–4), DHA, Hypoxanthine and Cholesterol sulfate. The observed decrease in risk metabolites and increase in metabolites with beneficial regulatory functions suggest that koumiss may have a positive impact on the cardiovascular, cerebrovascular, and nervous systems in healthy individuals.

α-Dimorphecolic acid is synthesized from linoleic acid. It can increase plasminogen activator inhibitor type-1 (PAI-1) expression by activates peroxisomal proliferator-activated receptor-gamma (PPARγ) in human endothelial cells, leading to increases risk for myocardial infarction and venous thrombosis [57,58]. Elevated level of Hypoxanthine is a risk factor for cardiovascular diseases, neurodegenerative diseases, and neural tube defect [59,60]. Cholesterol sulfate is an endogenous steroid and the C3β sulfate ester of cholesterol. In terms of quantity, it is the most significant sterol sulfate identified in human plasma, serving as a part of cell membranes. Although Cholesterol sulfate has neuroprotective effects in brain [61]. It is a potent inhibitor of both plasma thrombin and plasmin in blood which is closely related to thrombosis [62,63]. The results showed that the serum contents of the above three risk metabolites decreased in SMN group.

Valine is one of the essential amino acids for body ，and its deficiency is marked by neurological defects in the brain [64,65]. It is one kind of branched chain amino acids (BCAAs), that play critical roles of nutrition physiological functions in glucose and lipid metabolism, protein synthesis, as well as intestinal health and immune [66]. 5-MTHF is a form of external supplementation for folate, whose deficiency has been linked with an increased risk of cardiovascular disease [67], neural tube defects [68], cancer and cognitive dysfunction [69,70]. Compared to folic acid, 5-MTHF can be absorbed well even when gastrointestinal pH is altered and its bioavailability won't be impacted by congenital deficiency of methyl tetrahydrofolate reductase. Besides, 5-MTHF can also prevent the potential negative effects of unconverted folic acid in the peripheral circulation [71,72]. 2-Hydroxyestrone-1-S-glutathioneis a glutathione conjugate derivative of estrogen that is a kind of steroid compound with extensive biological activity in cardiovascular system, endocrine system, body metabolism, bone growth and maturity and skin [73–75]. In addition, 3-O-Sulfogalactosylceramide, Neuromedin N (1–4) and DHA play an important regulatory role in nerve signal transduction, membrane protection, blood pressure and immune regulatory [76–79]. The results showed that the serum contents of the above six metabolites with positive regulatory function increased in SMN group.

5. Conclusion

Koumiss is a conventional fermented item with a rich nutritional value and beneficial features. It has therapeutic efficacy on regulating lipid metabolism and enhancing immunity. This study confirmed the benign regulatory effect of koumiss on normal organism from the perspective of endogenous metabolites, and provided objective support for the promotion and application of this ethnic food.

Funding

This research was funded by Major Science and Technology Projects in Xinjiang Uygur Autonomous Region, grant number 2017A01002.

Data availability statement

Data available on request from the corresponding author upon reasonable request.

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CRediT authorship contribution statement

Leqi Wang: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Formal analysis, Data curation. **Yuanfang Sun:** Software, Investigation, Data curation. **Lijing Du:** Methodology, Formal analysis, Data curation. **Qian Wang:** Investigation. **Min Zhan:** Validation. **Shasha Li:** Writing – review & editing, Supervision, Resources. **Xue Xiao:** Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Xue Xiao reports financial support was provided by Major Science and Technology Projects in Xinjiang Uygur Autonomous Region. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

All authors thank Jun Luo for the information of Koumiss, and the members of Xinjiang Xinziyuan Biopharmaceutical Co., Ltd for the help in supplying the Koumiss.

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