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Physiological Adaptability of Yak in Extreme High-Altitude Habitat Using Serum Metabolite Analysis

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ABSTRACT

Comparative analysis of divergence in serum chemical derivatives among yaks living in ecological habitats at different altitudes was performed to determine the physiological adaptation basis in blood metabolites of yaks at extreme high altitudes due to long-term natural selection of plateau ecological environment. Twenty serum samples from yaks were collected, including 10 from those living on the Qinghai–Tibet Plateau at altitudes above 5000 m for a long time and 10 from those living in ecological regions at altitudes of ~4000m. Results revealed 592 metabolites in all samples, of which the highest proportion was attributed to amino acids and their metabolites (18.58%), followed by organic acids and their derivatives (16.20%). Meanwhile, 89 significantly different metabolites (SDMs) were identified between groups, of which 55 were significantly upregulated (e.g., 3-methylcrotonyl glycine, 2-hydroxy-3, and 5-dinitrobenzoic acid, etc.) in the yaks living at extremely high altitude. In addition, 28 metabolites (fumaric acid, serotonin, glycochenodeoxycholic acid, and all trans-retinal) were enriched in 55 KEGG signaling pathways and mainly attributed to organismal systems and metabolism. This work further clarified that long-term natural selection at extremely high altitude prompts animals to have visible physiological adaptations in serum. In addition, the serum of yak living at high altitudes is rich in a series of metabolites (e.g., lysophosphatidylethanolamine, γ-linolenic acid, and 12-hydrox) that are helpful for the treatment of cerebrovascular diseases and respiratory diseases and could contribute to the drug development of anti-plateau in the future.

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INTRODUCTION

Altitude adaptation refers to the ability of animals or plants developed by natural selection that allows them to habituate in a plateau environment (thin air, low oxygen content, strong ultraviolet rays, and low pressure) for long term. The key point of altitude adaptation is that the body can maximize the uptake and use of limited oxygen to satisfy daily physiological processes in a low-oxygen environment (Yi *et al*., 2010; Li and Zhang, 2021).

As one of the main livestock species in the Qinghai–Tibet Plateau, domestic yaks (*Bos grunniens*) were domesticated from wild yaks about 7,000 years ago (Qiu *et al*., 2015) and have become the main members of livestock development in this region with an important role in the local economy (Zeng *et al*., 2019). They have excellent plateau adaptability due to their long-term habitation in highland ecoregions by long-term natural selection. To date, the genetic and phenotypic adaptation basis of yak plateau adaptation has not been explored using genetics (Ji *et al*., 2021), cytogenetics (Wishart *et al*., 2022), and physiology (Ding *et al*., 2018).

As a new favorite tool of frontier omics research, metabolome has been widely applied for screening various disease markers and analyzing biological bases (Johnson *et al*., 2016; Wishart *et al*., 2022), especially blood metabolism analysis (Trifonova *et al*., 2020). In the livestock and agri-food section, metabolomics is widely used to analyze animal meat quality and nutritional content (Carraturo *et al*., 2020). Recent studies have

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attempted to explain the plateau adaptability of yaks using metabolomic analysis (Huang *et al*., 2022); however, they ignored evolutionary differences between species. The current work identified serum metabolite differences among yaks living at different altitudes using metabolomic analysis. The findings will help us deeply understand the physiological metabolic basis of the intraspecific altitude adaptability of yaks.

MATERIALS AND METHODS

Animal and sample collection

The study protocol was approved by the Institutional Animal Care and Use Committee of the Tibet Academy of Agricultural and Animal Husbandry Science (No. 20220301). Twenty male yaks (body weight = 200 ± 8.34 kg, 4–5 years old) were obtained from 4 local dominant populations living at differential altitudes (5 yaks per population), namely, Riwoqe (LWQ, altitude 4,000 m), Maizhokunggar (SB, altitude 3,900 m), Ngari (CWL, altitude 5,000 m), and Gerze (AL altitude 5,300 m). The yaks grazed on natural grassland all year round and did not receive any supplements. In July (warm season), 5 mL of jugular blood samples were collected using evacuated tubes without anticoagulant (Bokan Bio-engineering Co., Ltd., Shenyang, China) between 6:00 and 6:30 before grazing in the morning. The blood samples were centrifuged at 2,500 × g for 15 min at 4°C after being maintained in a slanted position for 15 min at room temperature. The obtained serum samples were stored in 1.5 mL plastic tubes (Eppendorf, Axygen, California, USA), snap-frozen in liquid N_2, and stored at −80°C for metabolomic analyses.

Metabolomic analyses

After the serum samples were thawed on ice, a mixture of 300 µL of methanol and 50 µL of thawed serum was prepared, vortexed for 3 min, and centrifuged at a rotation seeped of 12000 rpm for 10 min at 4°C. Afterward, 200 µL of supernatant was collected, maintained at −20°C for 30 min, and centrifuged at 4°C for 3 min at 12000 rpm. Finally, 150 µL of supernatant was taken for subsequent analysis. Liquid Chromatograph Mass Spectrometer (LC-MS) was carried out using an LC-ESI-MS/MS system consisting of an ultra-performance liquid chromatography (ExionLC AD, https://sciex.com.cn, AB SCIEX, US) with a tandem mass spectrometry (QTRAP® System, https://sciex.com, AB SCIEX, US). In brief, 2 µL aliquot of each sample was injected onto a 2.1 × 100 mm C18 1.8 µm column (Waters ACQUITY UPLC HSS T3), heated to 40°C, and gradient eluted using mobile phase A (0.1% formic acid in the water) and mobile phase B (0.1% formic acid in acetonitrile) at 0.4 mL/min. The gradient program was as follows: 95:5 V/V at 0 min, 10:90 V/V at 10.0 min, 10:90 V/V at 11.0 min, 95:5 V/V at 11.1 min, and 95:5 V/V at 14.0 min. Positive and negative modes were used in electrospray ionization with a triple quadrupole-linear ion trap mass spectrometer. The following parameters were used: source temperature 500 °C; capillary voltage, 5.5 kV for positive mode and 4.5 kV for negative mode; ion source gas I and II at 55 and 60 psi, respectively; and curtain gas at 25 psi with high-level collision-activated dissociation. For repeatability and accuracy, instrument tuning and mass calibration were performed with 10 and 100 µmol/L polypropylene glycol solutions in triple quadrupole and linear ion trap modes, respectively (He *et al*., 2017).

Data statistics

MS data were processed with Analyst 1.6.3 (AB SCIEX, US) and Multi Quant (3.0.3, AB SCIEX, US) software and further subjected to principal components analysis (PCA) and partial least squares-discriminant analysis (OPLS-DA) by statistics function prcomp 3.5.0 and Metabo Analyst R package 1.0.1 within R (1.0.1), respectively. Metabolites with variable importance (VIP) value ≥ 1 in the projection of OPLS-DA and absolute Log2FC (fold change) ≥ 1 or ≤ 0.5 were considered as differential metabolic regulators between groups. The identified metabolites were clustered using hierarchical cluster analysis (HCA) by Complex Heatmap package 2.2.0 with R, annotated using the KEGG compound database (http://www.kegg.jp/kegg/compound/), and mapped to KEGG Pathway database (http://www.kegg.jp/kegg/pathway.html) (Saleem *et al*., 2012; Wang *et al*., 2013; Artegoitia *et al*., 2017).

RESULTS AND DISCUSSION

A total of 592 metabolic-related chemical derivatives were identified from 20 samples (Supplementary Table I), and the highest proportion was attributed to amino acids and their metabolites (110/592), including L-citrulline, 4-hydroxy-L-glutamic acid, and N-acetyl-beta-alanine. The second highest proportion was ascribed to organic acids and their derivatives (93/574), such as 4-guanidinobutyric acid, quinoline-4-carboxylic acid, and 2-hydroxy-3,5-dinitrobenzoic. On the contrary, the lowest proportions were attributed to sphingolipids (1/592, sphingosine 1-phosphate) and glycerides (2/592).

Correlation analysis of metabolite expression among all individuals (Supplementary Table II and Fig. 1A) showed that the highest inter-individual correlation was recorded within population, indicating that the significant serum physiological difference between populations was due to their independent ecological habitat. PCA

results (Fig. 1B) also revealed that all CWL and AL yaks constructed a single cluster based on metabolite doses. Meanwhile, SB and LWQ yaks formed another cluster. This finding showed that habitat altitude difference led to visible divergence of metabolism types and doses in animals blood.

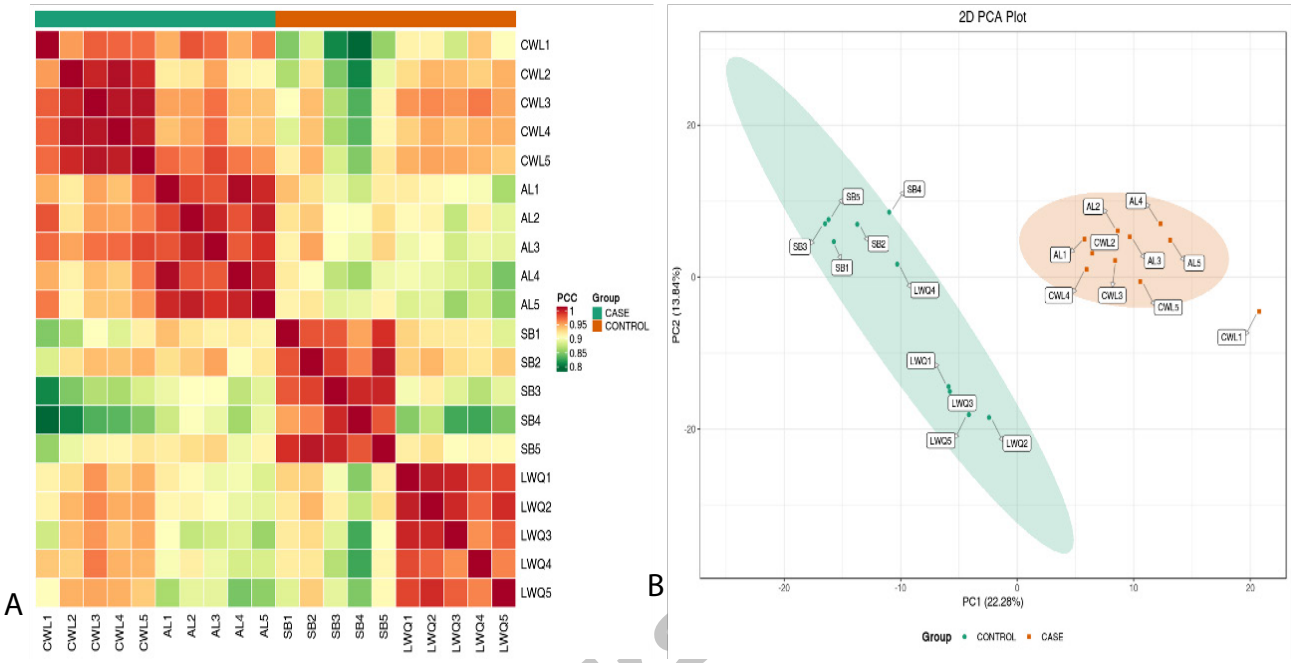

Fig. 1. Correlation among all individual samples and principal component analysis based on blood metabolite profiles. A is correlation analysis of all animals, PCC is the value of correlation coefficient r2, and B is the principal component analysis of all animals.

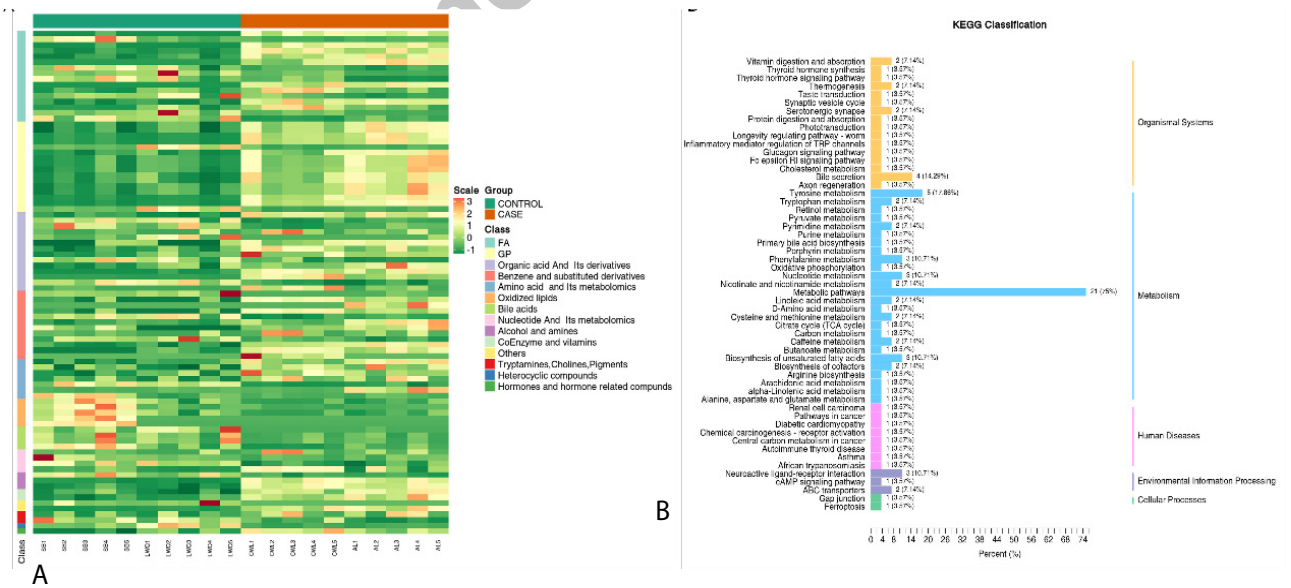

Fig. 2. KEGG functional enrichment of significantly differentially expressed chemical derivatives in the serum of wild blood yak vs. domestic yak. A is the heatmap of significantly different metabolites between groups and B is the KEGG enrichment of significantly different metabolites and their classification of KEGG pathways.

In this work, 89 significantly different metabolites (SDMs) were found between yaks living in an extreme high-altitude habitat and those living in a low-altitude habitat, including 55 upregulated (e.g., 3-methylcrotonyl glycine, 2-hydroxy-3, 5-dinitrobenzoic acid, and γ-linolenic acid (C18:3N6) and 34 downregulated (e.g., 8-epidermal glycoside, DL-carnitine, and 20-carboxyarachidonic acid) (Supplementary Table III and Fig. 2A). In particular, glycerophospholipids (14/89) accounted for the highest proportion, mainly including a series of lysophosphatidylcholine and lysophosphatidylethanolamine and their isomers.

Functional enrichment results of SDMs showed that 28 SDMs (e.g., fumaric acid, serotonin, glycochenodeoxycholic acid, and all trans-retinal) were enriched in 55 KEGG signaling pathways (Supplementary Table IV and Fig. 2B), mainly organismal systems (16/55), metabolism (26/55), human diseases 8/55), environmental information processing (3/55), and cellular processes (2/55). Only three pathways were significantly enriched (P<0.05), namely, tyrosine metabolism, biosynthesis of unsaturated fatty acids, and thermogenesis. A large number of pathways related to the digestion and metabolism of energy substances were identified, such as cholesterol metabolism, linoleic acid metabolism, pyruvate metabolism, glucagon signaling pathway, carbon metabolism, protein digestion and absorption, and arachidonic acid metabolism. Several immune and disease signaling pathways were also included, such as diabetic cardiomyopathy and autoimmune thyroid disease.

Identifying SDMs in the serum of yaks living in different altitude habitats will help us clearly understand the physiological evolutionary basis of plateau adaptation, especially the physiological role of some widely recognized metabolites, such as 3-methylindole, prostaglandin E2, and 12-hydroxydodecanoic acid.

3-Methylindole (3-methylindole, skatole), commonly known as stinky feces, is the tryptophan produced by anaerobic bacteria in the intestines of animals. It causes pulmonary edema in many animals, including goats and cattle (Carlson *et al*., 1975; Popp *et al*., 1998). In particular, 3-methylindole can selectively attack Clara cells and cause lung damage (Carlson and Bray, 1983). This compound is involved in tryptophan metabolism (TMB), and increased levels of TMB metabolites were found to be positively correlated with heart failure (Liu *et al*., 2017; Razquin *et al*., 2021). As the active ingredient in animal serum, 3,4,5-trimethoxycinnamic acid (TMAC) has been applied to treat insomnia, headache, and epilepsy (Duarte *et al*., 2008; Lee *et al*., 2013). In theory, TMCA has a sedative effect by inhibiting norepinephrine (NE) in murine blue spot (LC) (Kawashima *et al*., 2004). Rapid heart rate and arrhythmia are the most important clinical manifestations of altitude sickness in animals (Djarova *et al*., 2013; Ha *et al*., 2021). In this study, the yaks living at high altitudes had lower levels of 3-methylindole and higher 3, 4, 5-trimethoxycinnamic acid compared with those living at low altitudes.

Prostaglandin E2 (PGE2) is the main metabolize product of arachidonic acid (AA) through catalytic synthesis via epoxidation metabolic pathway (Lannoy *et al*., 2020). It regulates water reabsorption of the header with autocrine or paracrine system (Li *et al*., 2017; Li and Zhang, 2021). PGE2 also promotes water reabsorption in the collecting duct by inhibiting arginine vasopressin, which is consistent with the diuretic effect of PGE2 *in vivo* (Hassouneh *et al*., 2016; Li and Zhang, 2021). Clinical analysis showed that PGE2 has the effect of relieving asthma symptoms. When released from asthma airways, it induces bronchiectasis and inhibits allergen-induced bronchoconstriction and inflammatory mediator release (Towers *et al*., 2004; Chung, 2005). Endogenous pyrogen PGE2 promotes the firing rate of most temperature-insensitive neurons in the ventromedial preoptic area of the hypothalamus and suppresses that of temperature-sensitive neurons (Xu *et al*., 2000; Ranels and Griffin, 2005).

Lysophosphatidylethanolamine is produced by phosphatidylethanolamine (PE) through phospholipase A reaction and is one of the components of cell membrane (Makide *et al*., 2009). An increase in the activity of phosphatidylethanolamine acyltransferase is accompanied by the quality of phosphatidylethanolamine in heart cells, which is beneficial for the functional development of heart cells (Fotheringham *et al*., 2000). In addition, the high variability of lysophosphatidylethanolamine has been associated with the plaque volume of lipids and fibrosis (non-calcified), which may lead to coronary atherosclerosis (Tan *et al*., 2022). Recent studies have confirmed that lysophospholipids protect some cells from inflammation and oxidative stress by mediating the expression of related cytokines (Tsukahara *et al*., 2021). The corresponding chelate of lysophosphatidylethanol regulates mitochondrial membrane phospholipid metabolism to influence the level of oxidative stress during hepatic cell-associated metabolic processes (Dalton *et al*., 1984; Nagaraj *et al*., 2022). Therefore, lysophosphatidylethanolamine plays a crucial role in the development and related functions of cardiac cells.

γ-Linolenic acid γ (GLA) belongs to polyunsaturated fatty acids (PUFAs), which are components of the body's tissue biofilm and participate in various metabolisms and physiological and biochemical activities of cells (Ide *et al*., 2017). The balance of GLA and arachidonic acid derivatives can effectively affect the inflammatory

response (Sergeant *et al*., 2016). In addition, GLA can kills cancer cells by cancerous mitochondrial dysfunction and oxidative stress (Chen *et al*., 2021). The intake of GLA in erythrocyte plasm strongly increases the concentrations of GLA and dihigh GLA in plasma lipids, cholesterol esters, and erythrocyte membranes, greatly improving the oxygen transport capacity of red blood cells (Dawczynski *et al*., 2011). Supplementation of GLA changes the composition of fatty acids in plasma and erythrocyte membrane to improve the deformability of erythrocytes and thus enhance their ability to transport oxygen (Iijima *et al*., 2000).

As a saturated fatty acid, 12-hydroxydodecanoic acid is one of the metabolites of lauric acid by omega hydroxylation (Kandel *et al*., 2007; Zhang *et al*., 2022). Cytochrome P450 can inhibit omega hydroxylation to transport oxygen to the thermodynamically touch-difficult terminal carbon, suggesting that inhibiting omega hydroxylation could enhance the ability of red blood cells to transport oxygen to perform their normal cellular functions under a hypoxic condition (CaJacob *et al*., 1988). These findings remind us that the concentration of 12-hydroxydodecanoic acid in the blood of high-altitude yak populations is related to their ability to survive under a hypoxic environment. In addition, the oxidative decomposition of 12-hydroxydodecanoic acid in myocardial cells regulates the oxidation of mitochondrial fatty acids, generating rich energy to satisfy the ATP demand of maintaining the continuous contractive activity of the heart and preventing heart damage and heart failure caused by cardiac function changes under hypoxic conditions (Zhang *et al*., 2022).

Carnitine, abbreviated as carnitine, is a quaternary ammonium cationic complex (Steiber *et al*., 2004), which includes the isomeric form L-carnitine with biological activity and D-carnitine with abiotic activity (Liedtke *et al*., 1982). In general, carnitine refers to L-carnitine. Carnitine supplementation can improve the activity of mitochondrial respiratory chain enzymes, especially the activities of the starting and end enzymes and the terminal oxidase, thereby indirectly improving myocardial oxidative phosphorylation (Ferrari *et al*., 2004; Zhang *et al*., 2019). Clinically, carnitine supplementation can remarkably improve the work ability and oxygen consumption per stroke under the anaerobic threshold and significantly reduce respiratory entropy, blood lactic acid value after exercise, and carbon dioxide production, thus delaying exercise fatigue and improving the aerobic exercise ability of the body (Huang and Owen, 2012). This finding suggests that the relatively high carnitine content in the blood of yaks living at high altitudes is their adaptive evolution that ensures their long-term survival in a hypoxic environment.

Fumaric acid (FA) is a naturally occurring organic acid that has clinical effects on a variety of skin diseases and is an important intermediate metabolite of the TCA cycle (Song *et al*., 2013; Kuhn *et al*., 2017). FA is metabolized to succinic acid through oxidation and reduction pathways under hypoxia conditions to enhance myocardial contraction and reduce the release of lactate dehydrogenase (cell necrosis index) to protect the heart (Laplante *et al*., 1997; Ragavan *et al*., 2017). As an intermediate product of glycolysis, fructose-1,6-diphosphate (FDP) can effectively protect and promote the recovery of tissue function from hypoxia or ischemia. FA and FDP enter the cell through the same transporter. Under hypoxic conditions, increasing the FA level can significantly inhibit the transport and metabolism of FDP but does not protect hypoxic or ischemic tissues, thereby weakening the contractility of myocardium and leading to heart failure (Hardin *et al*., 2001). The current study found that the serum FA concentration in yaks living at high altitudes was significantly lower compared with that in yaks living at low altitudes, suggesting that the low blood FA levels may be an adaptive evolution of yaks living at an extremely high altitude under hypoxic conditions.

As the amide form of vitamin B3, niacinamide (NAM) is rich in many foods, including meat, fish, eggs, and legumes. Supplementation with NAM helps maintain the energy balance of cows and improve their immune function under heat stress by improving their antioxidant levels (Sun *et al*., 2014; Zeng *et al*., 2022). In addition, NAM is a precursor to nicotinamide adenine dinucleotide (NAD+), which is an important coenzyme for redox reactions in adenosine triphosphate (ATP) production and other metabolic processes (Rigoulet *et al*., 2020). Vitamin B3 deficiency mainly leads to pellagra and skin sun sensitivity. In animal models, NAD+ deficiency leads to ultraviolet sensitivity in the skin, impairs DNA damage response, and increases genomic instability and cancer incidence (Touat *et al*., 2018; Abdellatif *et al*., 2021). Hence, NAM prevents UV-induced ATP depletion and enhances cellular energy and DNA repair activity *in vitro* and *in vivo*. NAM has been clinically found to reduce UV-mediated immunosuppression levels and high skin cancer risk of non-melanoma incidence (Fania *et al*., 2019; Zhang *et al*., 2021). NAM may also reduce the release of pro-inflammatory mediators by inhibiting the MAPK and AKT/NF-κB signaling pathways to reduce lung damage (Zhang *et al*., 2021).

Triiodothyronine (T3) is normally found in animal serum; two-thirds of its secretion comes from the thyroid gland, and the remaining one-third comes from the conversion of thyroxine to T3. Hence, it can be used to

diagnose various thyroid diseases (Gharib, 1974). T3 can cause smooth muscle relaxation and affect the regulation of the cardiovascular system, indicating its possible use as a novel vasodilator (Danzi and Klein, 2014; Samuel *et al*., 2017). A series of studies suggested that T3 can enhance myelination in the brain by inducing oligodendrocyte maturation (Zendedel *et al*., 2016). Cattles exposed to cold stress have shown changes in the concentration of CORT, T3, and T4 in blood circulation to maintain temperature stability and then adapt to changes in the external environment through the regulation of hypothalamus pituitary adrenal and hypothalamus pituitary thyroid axes (Hu *et al*., 2019). The concentrations of T3 and T4 significantly increased in fat tailed sheep and rats exposed to low temperature (Nazifi *et al*., 2003; Park *et al*., 2017), suggesting that high T3 in the serum of yaks living at high altitudes can help them resist cold environments.

As an active metabolite produced in the intestinal microbiota of the host, trimethylamine oxide (TMAO) affects the health of the host (Salzano *et al*., 2020). An elevated TMAO level in the blood can be used to directly assess the prognosis of patients with heart failure, and the gut–TMAO–heart failure axis can be used as a new target for heart failure treatment (Bennett *et al*., 2013). At present, a large number of studies are attempting to reduce the level of TMAO by improving the intestinal microbiota to treat cardiovascular diseases (Coutinho-Wolino *et al*., 2021; He *et al*., 2021; Lv *et al*., 2022).

CONCLUSION

In this study, 89 SDMs were identified among yak populations distributed at different altitudes by serum metabolite analysis. Several chemical derivatives known to be related to cerebrovascular diseases and cardiopulmonary function were found to be enriched in the serum of yaks living at extremely high altitudes. Some examples include lysophosphatidylethanolamine, GLA, and 12-hydroxydodecanoic acid. This finding indicated that the physiologically adaptation of animals was developed due to natural selection by living in high-altitude environments for long term. This work helps us understand the natural selection and evolution of plateau adaptability in yaks and provides a theoretical basis for the future development of drug precursors to resist plateau environmental discomfort.

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Institutional review board statement

This study was approved by Institutional Animal Care and use Committee of Institute of Animal Husbandry and Veterinary Medicine, approval code: 20220301, approval date: March 6, 2022.

Data availability statement

The metabolome data of all samples are provided in supplementary documents.

Ethical statement

The study protocol was ap-proved by the Institutional Animal Care and Use Committee of the Tibet Academy of Agricultural and Animal Husbandry Science (No. 20220301).

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20230119050142

Statement of conflicts of interest

The authors have declared no conflict of interest.

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Supplementary Material

Physiological Adaptability of Yak in Extreme High-Altitude Habitat Using Serum Metabolite Analysis

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Table I. Detection of serum metabolites of 20 yak with different altitudes Habitat.

Table II. Correlation analysis of all yak individuals based on serum metabolites.

Table III. Significant difference expressed serum metabolites between different altitude inhabited yaks.

Table IV. KEGG enrichment of significant difference expressed Metabolites in serum of different altitude yaks.

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