



Suckling calves (*Bos taurus*) with pica exhibit blood metabolome alterations

L CHEN, J ZHANG, N LI, L ZHANG and X F XU*

Ningxia University, Xixia District, Yinchuan Ningxia 750 021 PR China

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ABSTRACT

The aim of this study was to evaluate the changes of blood metabolomics in calves with pica, which causes serious harm to livestock. Two groups, each comprising 12 calves of approximately 3 weeks old and of similar weight were selected as subjects; Group A calves were control animals in good condition and Group B calves (pica animals) had rough hair, emaciation, flaccid forestomach, diarrhoea, and stunted development. Blood samples were collected from the tail root vein. Masslynx 4.1 software (Waters Company) was used to pre-process data, which were then analysed by principal component analysis, partial least squares discriminant analysis, and orthogonal partial least squares discriminant analysis. Twenty potential biomarkers were closely related to the occurrence of pica, viz. GDP-glucose, UDP-glucose, proline, creatine, arginine, glutamine, citrulline, urea, alanine, methionine, serine, glycerate, cysteine, spermine, spermidine, carnitine, xanthurenic acid, kynurenine, and thyroxine. Metabolic pathway analysis showed that, in calves, pica resulted in decreased antioxidant capacity; disruption of the mutual transformation between pentose and glucuronic acid; abnormal metabolism of cysteine, methionine, serine, arginine, and proline; impairment of lipid metabolism; reduced immunity; increased intestinal permeability; and elevated central nervous excitability. Calves with pica exhibit disruption of various metabolic pathways.

Keywords: LC-MS, Metabolomics, Pica, Suckling calves

Pica is an intractable eating disorder disease caused by abnormal metabolism and feeding, resulting in the phenomena of consuming non-food materials. The main feature of pica is the intake of foreign bodies. The symptoms of pica in cows are usually first exhibited as dyspepsia, followed by abnormal feeding. Diseased cows often exhibit biting, feeding, and swallowing of contaminated forage or litter, and consumption of foreign materials, such as sand and cow hair. Prolonged morbidity leads to continuous loss of appetite, weight loss, and eventually even death due to severe organ failure. Young animals can suffer from pica, which prevents their growth and development of young animals and causes symptoms of anaemia (Qi *et al.* 2016). The mechanisms underlying pica are not yet understood, although it has been associated with parasitism and deficiencies of phosphorus, salt, and protein. Serious complications of pica include cannibalism; foreign bodies penetrating the alimentary tract as traumatic reticuloperitonitis; poisonings, particularly by lead; botulism; or obstruction of the digestive tract by accumulations of wool, fibre, or sand (Constable *et al.* 2016). There are many reasons for pica. Living organisms need minerals, as well as essential nutritional elements, to sustain their normal development, and deficiencies of mineral elements are reported to cause consumption of soil, surface licking, a desire to eat non-food objects, weight

loss, and decreased resistance to infections, because effects on the immune system (Aytekin and Kalinbacak 2011). “Boredom”/under stimulation and closely confined housing can predispose animals to pica. Chronic abdominal pain, due to peritonitis or gastritis and central nervous system diseases, including rabies, ketosis/acetonemia, and lead poisoning, may also cause pica. Further, stimulation of an organ system by its specific external environment, can alter metabolite levels *in vivo*. Metabolomics studies can identify and monitor changes in metabolites, which may be useful as biomarkers, to determine disordered metabolic pathways and reveal the mechanisms underlying physiological and pathological changes.

MATERIALS AND METHODS

Blood collection: The experimental procedures used in this study were in accordance with the University’s Guidelines for Animal Research. The experiment was performed at dairy cattle breeding farm in Ningxia Province of China. Calves of approximately 3 weeks old and of similar weight were selected as subjects. A total of 12 calves in good condition, that did not feed on cattle hair and sand, were selected as the normal control group (Group A). Further 12 calves with rough coats, emaciation, flaccid forestomach, diarrhoea, and stunted development were selected as the pica Group (Group B). On day 21 after birth, 5 mL blood samples were obtained via the tail root vein by vacuum collection and centrifuged for 20 min at

*Corresponding author e-mail: xuxiaofengnd@126.com

13,000 × g. Serum samples were transferred into 1.5 mL centrifuge tubes and stored at −80°C until analysis.

Sample pre-treatment: Samples were thawed at room temperature and 100 µL aliquots transferred into centrifuge tubes (1.5 mL) by pipette. Next, they were extracted using 300 µL methanol and 10 µL internal standard (2.9 mg/mL, DL-2-chlorophenylalanine) added, followed by vortexing for 30 sec and centrifugation at 13,000 × g at 4°C for 15 min. Finally, 4.2 µL aliquots of the resulting supernatants were transferred to vials for LC-MS analysis.

Chromatography conditions: The analysis platform was the ACQUITY™ UPLC QTOF, with Waters ACQUITY UPLC HSS T3 columns (2.1 mm × 100 mm, 1.8 µm). For chromatographic separation, column temperature was 40°C, mobile phase A was water + 0.1% formic acid, mobile phase B was acetonitrile + 0.1% formic acid, the flow rate was 0.35 mL/min, and an injection volume of 6 µL was used.

MS parameters: Positive ion mode (ESI+): capillary voltage, 1.4 kV; sampling cone, 40 V; source temperature, 120°C; desolvation temperature, 350°C; cone gas flow, 50 L/h; desolvation gas flow, 600 L/h; collision energy, 10–40 V; ion energy, 1 V; scan time, 0.03 sec; inter scan time, 0.02 sec; scan range, 50–1500 m/z.

Negative ion mode (ESI−): capillary voltage, 1.3 kV; sampling cone, 23 V; source temperature, 120°C; desolvation temperature, 350°C; cone gas flow, 50 L/h; desolvation gas flow, 600 L/h; collision energy, 10–40 V; ion energy, 1 V; scan time, 0.03 sec; inter scan time, 0.02 sec; scan range, 50–1500 m/z.

Data analysis: Feature extraction and pre-processing of data were conducted using Masslynx 4.1 software (Waters). Data were then normalised and edited into a two-dimensional data matrix using Excel 2010 software, including retention time (RT), mass, observations (samples), and peak intensity. Subsequent data editing included multivariate analysis using SIMCA-P 13.0 software (Umetrics AB, Umea, Sweden).

Identification of differential metabolites: Various metabolites were identified according to their MS values, using the online databases HMDB (<http://www.hmdb.ca/>), KEGG (<http://www.genome.jp/kegg/>), and METLIN (<http://metlin.scripps.edu/>), and then compared and corrected

using the Bovine Metabolome Database, DMDb (<http://www.cowdb.ca/>).

RESULTS AND DISCUSSION

Metabolic spectrum analysis: Principal component analysis (PCA) demonstrated that the six quality control samples clustered together well, indicating that the LC-MS metabolite detection system exhibited good stability and generated reliable metabolite detection data. Although there was overlap among data from the samples in the different treatment groups, variants between the groups could be clearly separated. The influence of metabolite differences between the pica and normal control groups was subjected to further, in-depth analysis.

PCA analysis: Principal component analysis was performed on data from Groups A and B. In the positive mode, two principal components were obtained by this analysis, with cumulative $R^2X = 0.49$ and $Q^2 = 0.267$. Similarly, two principal components were obtained using negative mode data, with cumulative $R^2X = 0.388$ and $Q^2 = 0.0323$. Positive and negative mode data showed specific trends of separation.

Partial least squares discriminant analysis: The main parameters for discriminating the quality of the model are R^2Y (which represents the interpretation rate of the model) and Q^2 (which is the prediction rate of the model). The quality parameters for the model were as follows: in positive mode, there were two principal components, where $R^2X = 0.482$, $R^2Y = 0.991$, $Q^2 = 0.963$. In negative mode, there were also two principal components, $R^2X = 0.352$, $R^2Y = 0.999$, $Q^2 = 0.917$. Hence, for both modes, R^2Y and Q^2 were > 0.5. These data demonstrate that the model had a high explanatory rate and predictive ability, and was stable and reliable. There were some differences between the pica and normal control groups.

Orthogonal partial least squares discriminant analysis: Orthogonal partial least squares discriminant analysis (OPLS-DA) data was applied for modelling analysis. One principal component and one orthogonal component were obtained in the positive mode, with $R^2X = 0.482$, $R^2Y = 0.991$, and $Q^2 = 0.954$. Similarly, in negative mode, one principal component and one orthogonal component were

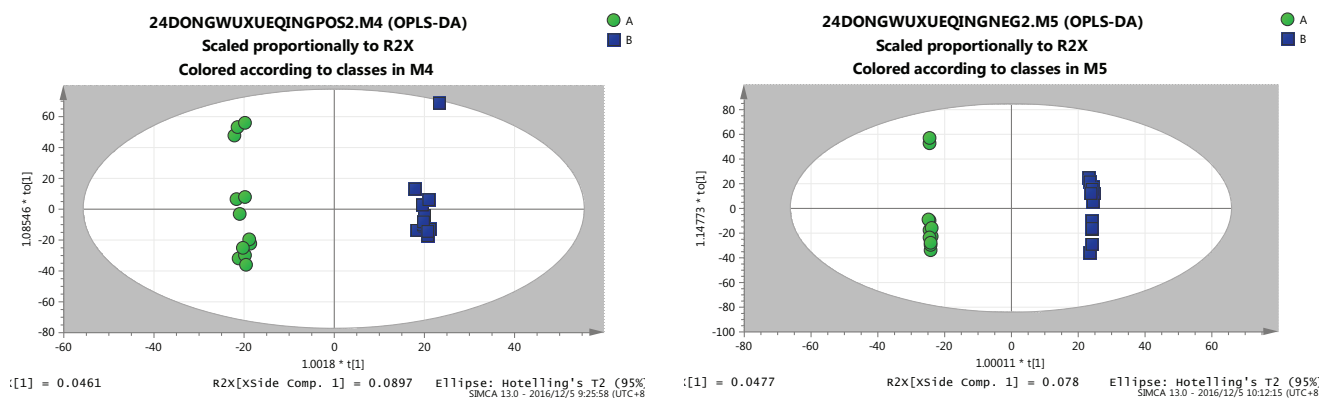


Fig. 1. OPLS-DA score plots of data from Groups A and B. (A) Data from positive mode (ESI+). (B) Data from negative mode (ESI−). Green circles, Group A (control) data; blue squares, Group B (pica) data.

obtained, where $R^2X = 0.352$, $R^2Y = 0.999$, and $Q^2 = 0.909$. All R^2 values generated were > 0.91 , indicating that the OPLS-DA analysis model is valid and can be used to compare differences between the two treatment groups (score chart, Fig. 1).

Identification and pathway analysis of metabolites differing between groups: In this study, we used the variable importance in the projection (VIP) value of the OPLS-DA model (threshold > 1), combined with p-values generated using the t-test ($P < 0.05$) to identify differentially expressed metabolites. A qualitative method for identification of differential metabolites involved searching the online database, Metlin (<http://metlin.scripps.edu/>), to compare the mass-to-charge ratio (m/z) or the exact molecular mass (Mass) of the mass spectra. Differential metabolite data are presented in Tables 1 and 2.

Effects of pica on carbohydrate metabolism: GDP-glucose is a substrate for UDP-glucose pyrophosphatase,

while UDP-glucose is a key intermediate in carbohydrate metabolism, used as a glucose donor for glycogen synthesis in animals. Decreased GDP-glucose concentration in the serum of pica calves will lead to reduced activity of UDP-glucose pyrophosphatase, thereby, downregulating the synthesis of UDP-glucose from glucose-1-phosphate, influencing glycogen synthesis, which is not conducive to glucose storage. Glucose is involved in the biosynthesis of ascorbic acid. After conversion from glucose to UDP-glucose, animals need to produce UDP-glucuronic acid esters by the action of dehydrogenase, which is involved in ascorbic acid biosynthesis. Ascorbic acid participates in physiological redox equilibrium and has an antioxidant role (Figueroa-Méndez and Selva 2015).

The concentrations of UDP- and GDP-glucose were decreased in the pica group, indicating that antioxidation function was decreased in this group, influencing the mutual transformation of pentose and glucuronic acid. Valine is an

Table 1. Differential metabolites in the Groups A and B in positive mode

Name	VIP	RT	Mass	t-test	Fold-change (B/A)	BMDB ID	KEGG
UDP-glucose	2.06	1.17	567.0219	0.03	0.42	BMDB00286	C00029
GDP-glucose	1.91	0.89	606.1175	0.05	0.38	BMDB03351	C00394
Glyceric acid	1.97	1.24	129.0202	0.04	< 0.001	BMDB00139	C00258
Valine	1.98	0.94	118.0806	0.04	2.54	BMDB00883	C00183
Creatine	1.91	0.93	132.0675	0.05	2.02	BMDB00064	C00300
Alanine	2.13	0.90	90.0912	0.03	1.96	BMDB00161	C00041
Spermidine	1.90	18.14	146.1537	0.05	1.84	BMDB01257	C00315
Spermine	1.99	3.37	203.2967	0.04	0.57	BMDB01256	C00750
Serine	2.04	9.54	106.0743	0.03	0.80	BMDB03406	C00065
Proline betaine	2.05	3.66	144.1077	0.03	0.17	BMDB04827	C10172
Proline	2.50	0.88	116.0009	0.01	0.36	BMDB00162	C00148
Methionine	2.14	14.45	150.0272	0.02	0.54	BMDB00696	C00073
Kynurenine	1.99	2.34	209.0723	0.04	0.25	BMDB00684	C00328
Glutamine	2.29	9.53	147.0648	0.01	0.74	BMDB00641	C00064
Cysteine	2.61	1.84	122.0364	< 0.001	0.36	BMDB00574	C00097
Citrulline	2.57	1.16	176.1898	0.01	0.65	BMDB00904	C00327

Name, name of the differential metabolite; VIP, contribution rate of different substances to the OPLS-DA model; Mass, charge-to-mass ratio of the substance; RT, retention time; t-test, T test to determine the significance value for differences; fold-change (B/A), ratio of the mean value for Group B to that for Group A.

Table 2. Differential metabolites in the Groups A and B in negative mode

Name	VIP	RT	Mass	t-test	Fold-change (B/A)	BMDB ID	KEGG
Arginine	2.09	5.04	209.0922	0.02	5.56	BMDB03148	C00062
PIP2(16: 0/16: 1(9Z))	1.98	0.88	967.4263	0.04	7.10	BMDB10033	C00626
Thyroxine	1.86	14.70	775.5998	0.05	1.46	BMDB00248	C01829
Urea	2.28	0.97	94.9945	0.01	0.62	BMDB00294	C00086
Acetylcarnitine	2.10	6.36	203.1384	0.02	0.49	BMDB00201	C02571
Xanthurenic acid	1.97	6.37	204.0397	0.04	0.13	BMDB00881	C02470
Xanthine	2.27	1.32	151.0348	0.01	0.03	BMDB00292	C00385
Carnitine	2.24	1.29	160.0839	0.03	0.16	BMDB00062	C00318
Hexadecanedioic acid	2.02	10.08	285.2068	0.02	0.39	BMDB00672	C19615

Name, name of the differential metabolite; VIP, contribution rate of different substances to the OPLS-DA model; Mass, charge-to-mass ratio of the substance; RT, retention time; t-test, T test to determine the significance value for differences; fold-change (B/A), ratio of the mean value for Group B to that for Group A.

essential amino acid and its metabolism is related to carbohydrates. Valine is associated with insulin resistance, as high valine levels are detected in blood from diabetic mice, rats, and humans (Lynch and Adams 2014). In this study, calves in the pica group had high levels of valine, due to disordered carbohydrate metabolism.

Effects of pica on amino acid metabolism: Creatine is produced by the decomposition of creatine phosphate, which functions as a form of stored energy in skeletal muscle that releases ATP when there is high physiological demand for energy. The increase of creatine concentration in the pica calf group indicates that the release of phosphocreatine in muscle tissue is involved in energy supply. Arginine is a precursor of urea and its accumulation in tissue occurs during hyperproteinaemia. Hyperargininaemia, an inborn error of the urea cycle, is caused by severe deficiency of liver arginase, resulting in elevated tissue levels of arginine and other guanidino compounds. Affected patients present with a neurological syndrome manifesting as a variable degree of mental retardation. In the pica group, we detected decreased glutamine, citrulline, and urea concentration, while levels of alanine and arginine were increased.

Methionine is an essential amino acid required for the normal growth and development of humans, other mammals, and avian species. It can reduce cholestasis in hepatocytes and enhances the trans-sulphur effect, thus enhancing the synthesis of cysteine, glutathione, and taurine in hepatocytes (Ball *et al.* 2006). Serine can be synthesised by glycerate, and serine synthesised by the glycerol pathway can produce cysteine via the homocysteine cycle. In the pica group, we detected decreased methionine, glyceric acid, serine, and cysteine concentrations.

Spermine is a biopolyamine formed by spermidine. It is reported partial deletion of the spermine synthase and *Pex* genes in hypophosphataemic mice. Further, it is reported that pica in dairy cows because of malnutrition and lack of various trace elements, such as phosphorus, in feed (Hanjuan 2016). In this study, spermidine was identified as significantly increased in the serum of pica calves, and spermine concentrations were significantly reduced.

Effects on lipid metabolism pathways: Carnitine is an important factor in mammalian fatty acid metabolism and its primary metabolic function is to transport fat to the mitochondria of muscle cells for oxidation. Consumption of antibiotics, malnutrition, and malabsorption may cause secondary carnitine deficiency (Stanley 2010). After calves are born, environmental, nutritional, and genetic factors can make them prone to licking and biting non-food items in their vicinity, resulting in indigestion, which can lead to calves suffering from the intractable eating disorder, pica. The researchers conducted a survey of a farmed cattle farmer in Bahrain Right Banner, Inner Mongolia. Through comparison of various symptoms, they found that cattle suffered from pica due to lack of nutrients. The low carnitine concentrations detected in serum from pica calves in this experiment may have been caused by a lack

of nutrients in the feed provided to the animals (Yun *et al.* 2017).

Phosphatidylcholine-sterol acyltransferase converts cholesterol and phosphatidylcholines (lecithins) to cholesteryl esters and lysophosphatidylcholines on the surface of high and low density lipoproteins (Piper *et al.* 2015). The decreased levels of cholesterol esters in the pica group reveal that lipoprotein metabolism was affected in these calves.

Effects of pica on antioxidant capacity and intestinal permeability of suckling calves: Cysteine has antioxidant properties and is an important source of sulphides in animal metabolism (Sekhar *et al.* 2011). Sulphides in iron-sulphur clusters and nitrogenases are extracted from cysteine and cysteine is converted to alanine during this process. Xanthurenic acid has antioxidant properties both *in vitro* and *in vivo*. It showed that calves with pica had weak antioxidant capacity (Dandan *et al.* 2018). In this study, the low cysteine concentration in serum from pica calves indicated low antioxidant capacity in this group.

Citrulline is a component of alanine, aspartic acid, arginine, and proline metabolism. It showed that serum citrulline reflects total small bowel enterocyte mass, since circulating citrulline is mainly produced by enterocytes (Semba *et al.* 2016). Citrulline is synthesised in enterocytes via the conversion of glutamine or arginine to ornithine (Marini 2012), followed by the conversion of ornithine by ornithine transcarbamylase. The association of both low serum citrulline and ornithine with increased gut permeability may reflect reduced small bowel enterocyte mass. Increased gut permeability can affect the absorption and metabolism of amino acids, proteins, lipids, carbohydrates, and other nutrients, leading to diarrhoea and other diseases (Keusch *et al.* 2014). In this study, the concentration of citrulline in serum was decreased in calves with pica, likely increasing the permeability of the intestinal mucosa and affecting the absorption and metabolism of amino acids, proteins, lipids, carbohydrates, and other nutrients in the pica calf group.

Kynurenine is a metabolite of tryptophan. Immune cells have important roles in the degradation of tryptophan to kynurenine during the regulation of immune responses to infection, inflammation, and pregnancy (Le Floch *et al.* 2011). In this study, we identified low concentrations of kynurenine in the serum of pica calves, demonstrating that pica reduces the immune function of suckling calves.

Thyroxine is released from thyroglobulin by protein hydrolysis and secreted into the blood and has a key role in mammalian development and metabolism. Thyroid hormone activity is regulated in a complex tissue-specific manner by three isoforms of deiodinase (Connor *et al.* 2015). Thyroid hormones are important for maintaining the excitability of the nervous system. When thyroid hormones are hyperactive, central nervous system excitability is elevated. Here, we found that serum thyroxine content was increased in pica calves, indicating increased excitability of the central nervous system in these animals.

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