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Response to weaning and dietary L-glutamine supplementation: metabolomic analysis in piglets by gas chromatography/mass spectrometry*

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Abstract: A novel metabolomic method based on gas chromatography/mass spectrometry (GC-MS) was applied to determine the metabolites in the serum of piglets in response to weaning and dietary L-glutamine (Gln) supplementation. Thirty-six 21-d-old piglets were randomly assigned into three groups. One group continued to suckle from the sows (suckling group), whereas the other two groups were weaned and their diets were supplemented with 1% (w/w) Gln or isonitrogenous L-alanine, respectively, representing Gln group or control group. Serum samples were collected to characterize metabolites after a 7-d treatment. Results showed that twenty metabolites were down-regulated significantly (P<0.05) in control piglets compared with suckling ones. These data demonstrated that early weaning causes a wide range of metabolic changes across arginine and proline metabolism, aminosugar and nucleotide metabolism, galactose metabolism, glycerophospholipid metabolism, biosynthesis of unsaturated fatty acid, and fatty acid metabolism. Dietary Gln supplementation increased the levels of creatinine, D-xylose, 2-hydroxybutyric acid, palmitelaidic acid, and α-L-galactofuranose (P<0.05) in early weaned piglets, and were involved in the arginine and proline metabolism, carbohydrate metabolism, and fatty acid metabolism. A leave-one-out cross-validation of random forest analysis indicated that creatinine was the most important metabolite among the three groups. Notably, the concentration of creatinine in control piglets was decreased (P=0.00001) compared to the suckling piglets, and increased (P=0.0003) in Gln-supplemented piglets. A correlation network for weaned and suckling piglets revealed that early weaning changed the metabolic pathways, leading to the abnormality of carbohydrate metabolism, amino acid metabolism, and lipid metabolism, which could be partially improved by dietary Gln supplementation. These findings provide fresh insight into the complex metabolic changes in response to early weaning and dietary Gln supplementation in piglets.

Key words: Piglet, Metabolomics, Weaning, Glutamine, Gas chromatography/mass spectrometry
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1 Introduction

Weaning is a critical but also inevitable stage in the postnatal growth and metabolism of mammals (Henning, 1981; Wang *et al*., 2008). Abrupt weaning is commonly associated with a period of low feed intake, decreased digestive capability, and increased occurrence of enteric diseases and diarrhea (Bruininx *et al*., 2001; Wijtten *et al*., 2011), generally leading to a net catabolic state (le Dividich and Sève, 2000). This suggests that weaning has adverse effects on

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mammals at the onset of segregation. L-Glutamine (Gln) serves as a major energy substrate for rapidly dividing cells (Marc Rhoads and Wu, 2009) and as a building block for polypeptides and proteins (Curi *et al*., 2005), but also as an essential precursor of bioactive molecules, such as glutathione, amino sugars, purines, and pyrimidines (Boza *et al*., 2000; Wu, 2009). The importance of Gln under hypermetabolic states, including those which occur during weaning, is now firmly established (Wang *et al*., 2008; Marc Rhoads and Wu, 2009). Dietary Gln supplementation prevents intestinal atrophy, and improves nutrient digestion and utilization in early weaning animals (Young and Ajami, 2001; Chamorro *et al*., 2010). Intestinal microbiota and blood amino acid concentrations were modified by Gln treatment in post-weaned animals (Wu *et al*., 1996; Chamorro *et al*., 2010). However, the interaction between metabolism and weaning or dietary Gln treatment, performed as a network, is a complex process involving multi-organ physiology and different levels of regulation, from genes to proteins and metabolites (Marc Rhoads and Wu, 2009). There is no information available on the effects of weaning and dietary Gln supplementation on the systemic metabolism of piglets.

Metabolomics, an important branch of systems biology, is defined as the quantitative measurement of small molecular metabolites in a biological sample (Mao *et al*., 2008; Nicholson and Lindon, 2008). It has offered novel insights into dietary effects by measuring and mathematically modeling changes in metabolic products (He *et al*., 2009). Metabolic fingerprints can be obtained using mass spectrometry (MS) or nuclear magnetic resonance (NMR) (Nicholson *et al*., 2004). Up to now, a comprehensive analysis of metabolites as potential indicators for metabolic alterations in weaning and Gln treatment has not been performed, to the best of our knowledge. The serum metabolites could be used to develop biomarkers to analyze metabolic changes and to provide ideas to relieve weaning stress.

The aim of our present study is to profile the serum of suckling piglets and their corresponding weaning counterparts to determine the different metabolites using gas chromatography/mass spectrometry (GC-MS), and thereby study the effects of dietary Gln supplementation on these different metabolites.

2 Materials and methods

2.1 Animals, diets, housing, and experimental design

Twelve sows were selected and each sow freely nursed nine piglets before weaning. Thirty-six 21-d-old healthy female piglets (Duroc×Landrace×Large Yorkshire strain) with similar body weights from 12 litters (three piglets per litter) were randomly assigned into three groups based on their litter origins (n=12), representing suckling, control, and Gln groups. Piglets in the suckling group continued to be nursed by sows, whereas the piglets in the control group and Gln group were weaned and supplemented with 1.22% (w/w) L-alanine (isonitrogenous control) or 1% (w/w) Gln to the basal diet (Wang *et al*., 2008), respectively. Piglets from the control and Gln groups were housed in pens (two piglets per pen) in the same animal facility with free access to a corn and soybean meal-based diet and drinking water. The basal diet without antibiotics was formulated to meet the nutrients recommended by the National Research Council. L-Alanine and Gln were added to the basal diet at the expense of corn starch. Environment temperature was maintained at (25±1) °C with continuous lighting. All procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University, China.

The ingredients of the basal diet are shown in Table 1. Procedures of Official Methods of Analysis (AOAC, 2000) were used to analyze the basal diet. Amino acids were determined following acid hydrolysis using a Hitachi L-8900 amino acid analyzer (Hitachi, Japan). The contents (g/kg, unless otherwise specified, as-fed basis) were as follows: dry matter, 887.4; crude protein, 214.8; crude ash, 52.2; total calcium, 9.1; total phosphorus, 6.1; total energy (kJ/g), 14.12; arginine, 11.68; alanine, 9.68; aspartate+asparagine, 18.25; cysteine, 3.10; glutamate+Gln, 39.66; glycine, 8.52; histidine, 4.86; isoleucine, 7.97; leucine, 17.14; lysine, 12.03; methionine, 3.41; phenylalanine, 9.10; proline, 8.91; serine, 9.05; threonine, 8.54; tyrosine, 5.11; and valine, 8.24. The amount of supplemental Gln and the reason for the choice of alanine as the isonitrogenous control were based on the researches of Wu *et al*. (1996) and Wang *et al*. (2008).

Table 1 Composition of the experimental diets

Ingredient	Content (g/kg)
Corn	565.0
Soybean meal	219.3
Extruded soybean	50.0
Corn starch	52.2
Fishmeal	40.0
Soybean oil	25.0
Whey powder	25.0
Calcium hydrogen phosphate	9.0
Limestone	4.5
Vitamins and minerals[a]	10.0

[a] Mineral and vitamin composition (mg/kg of feed, unless otherwise specified): Cu, 200 as copper sulfate; Fe, 240 as ferrous sulfate monohydrate; Mn, 40 as manganese sulfate; Zn, 1000 as zinc oxide; Co, 0.5 as cobalt sulfate; I, 0.4 as potassium iodine; Se, 0.35 as sodium selenite anhydrous; vitamin A, 17, 500 IU; vitamin D_3, 385 IU; vitamin E, 70 IU; vitamin K, 3.36; vitamin B_1, 3.43; vitamin B_2, 8.75; vitamin B_6, 5.15; vitamin B_{12}, 0.04; Ca-D-pantothenate, 17.15; niacin, 36; antioxidant, 0.42; folic acid, 1.70; biotin, 0.26

2.2 Serum collection and storage

On Day 28, piglet weight was determined, and blood samples (4 ml) were collected by venipuncture of the precaval vein between 07:00 and 08:00 a.m. according to the methods of Wang *et al.* (2008) and He *et al.* (2009). Serum was separated from whole blood by centrifugation at 1000×*g* and 4 °C for 10 min. The serum samples were then pooled in groups of two piglets from the same treatment and stored at −80 °C until GC-MS analysis.

2.3 Specimen processing

The serum samples were thawed at room temperature, vortex-mixed for 15 s, and then 100 μl of the sera were transferred into a tube. A total of 75 μl 2-chloro-L-phenylalanine (0.1 mg/ml) as internal standards and 825 μl of a mixture of methanol-deionized water (8:1, v/v) were added to each sample to precipitate the proteins. The mixtures were ultrasonicated at ambient temperature (25–28 °C) for 10 min. The samples were subsequently centrifuged at 12000×*g* for 10 min at 4 °C, and then 0.8 ml of the supernatant was collected into the test tubes. The collected supernatant was then evaporated to complete dryness under a stream of nitrogen gas. The dried samples were subsequently derivatized with the addition of 100 μl bis(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane into each GC vial. The mixture was left to react for 1 h in a microwave oven at 90 °C. After incubation, the samples were again vortex-mixed for 1 min and prepared for GC-MS analysis (Xue *et al*., 2008).

2.4 GC-MS analysis

Analysis was performed on an Agilent 6980 GC system equipped with a 30.0 m×0.25 mm i.d. fused-silica capillary column with a 0.25-μm HP-5MS stationary phase (Agilent, Shanghai, China). Helium was used as the carrier gas at a constant flow rate of 1 ml/min through the column. An injection volume of 1 μl was run under a splitless mode. The injector temperature was maintained at 250 °C. The column temperature was initially kept at 60 °C for 3 min and then increased to 140 °C at a rate of 7 °C/min, where it was held for 4 min, and further increased to 300 °C at a rate of 5 °C/min and maintained for 1 min. The column effluent was introduced into the ion source of an Agilent 5973 mass selective detector (Agilent Technologies). The MS quadrupole temperature was set to 150 °C and the ion source temperature to 230 °C. The mass spectrometer was operated in electron impact (EI) mode (70 eV) and mass was acquired from *m*/*z* 45 to *m*/*z* 500 at a mass accuracy of 0.1 atomic mass units. The acceleration voltage was turned on after a solvent delay of 6 min (Xue *et al*., 2008).

2.5 Biochemical measurements by detection kits

Serum metabolites measured included glucose, high density lipoprotein (HDL), triglycerides, total cholesterol, total amino acid (TAA), albumin, total protein, and urea nitrogen. All assays were evaluated with corresponding detection kits provided by the Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions.

2.6 Data processing and pattern recognition

AMDIS software (the National Institute of Standards and Technology, Gaitherburg, MD) was used for peak deconvolution, and NIST mass spectral library (Version 2.0d) for peak identification. The integrated peak areas of multiple derivative peaks belonging to the same compound were summed and considered as a single compound. A data matrix was then created for profiling the biochemical pattern of each sample using the relative area values of the peaks. The ratio of the

peak area of each compound to the corresponding internal standard was calculated as the response.

A two-sample *t*-test with an acceptable significance level of $P<0.05$ was used for the comparison of metabolite levels to determine the differences between the groups suckling vs. control, and Gln vs. control. False discovery rates (FDRs) were determined from the *P*-value using the Benjamini-Hochberg (BH) method. First, the peaks from the suckling piglets were compared with those of the control to determine the different metabolites. Then, the different metabolite peaks from the Gln group were compared with the peaks from the control group. The profiles of different metabolites were exhibited in the *z*-score plot (Qiao *et al.*, 2011). The *z*-score is expressed as: $z=(x-m)/\sigma$, where x represents a raw score to be standardized, and m and σ represent the mean and standard deviation (SD) of the reference population, respectively. Principal component analysis (PCA), a data visualization method, was used to differentiate the spectroscopic serum metabolite profiles of the three groups (Trygg *et al.*, 2007). Kruskal-Wallis tests were used for three-way comparisons among all three groups. These significant metabolites among three groups were added to random forest classifiers. The permutation-based mean decrease in accuracy was used to measure the importance of each metabolite to the classification (Breiman, 2001). Data analyses were performed using Matlab (v7.8; the Mathworks, Natick, MA, USA).

The levels of serum metabolites evaluated by detection kits were statistically analyzed by one-way analysis of variance (ANOVA) of Statistical Product and Service Solutions (v16.0; SPSS, Chicago, IL, USA) as appropriate. The results are presented as mean±standard error of the mean (SEM). Effects were considered significant at $P<0.05$.

3 Results

3.1 Growth performance

Body weight and average daily gain between Days 21 and 28 were reduced ($P<0.05$) by 16.53% and 127.98%, respectively, in control piglets compared with suckling ones (Table 2). Supplementing 1.0% Gln to the diet of the weaned piglets did not affect body weight in the 28-d-old piglets but elevated average daily gain between Days 21 and 28 by 29.99% ($P=0.032$) compared with the control group.

Table 2 Body weight and average daily gain of piglets

Group	BW (kg)		Day 21–Day 28 ADG (g/d)
	Day 21	Day 28	
Suckling	5.29	7.12[a]	260.56[a]
Control	5.31	6.11[b]	114.29[c]
Gln	5.30	6.34[b]	148.57[b]
SEM	0.39	0.54	12.57

BW: body weight; ADG: average daily gain; SEM: standard error of the mean; suckling: piglets suckling from the sows; control: weaned pigs receiving dietary supplementation with 1.22% (w/w) L-alanine; Gln: weaned pigs receiving dietary supplementation with 1% (w/w) L-glutamine. Values (mean, $n=12$) in a column not sharing the same superscripts are significantly different ($P<0.05$)

3.2 Method validation of GC-MS analysis

Four injections of the same serum sample were consecutively injected into the GC-MS system to test the stability of sample injection, and the data showed stable retention time with no drift in any of the peaks. The stability of the retention time ensured the accuracy of the extraction and matching of the co-eluting peaks. Relative standard deviation (RSD) values of the main peak area were less than 15%. In addition, the technical replicates were performed thrice. Almost all the corresponding peaks were shown at the same retention time, and RSD values of the main peak area were less than 20%.

3.3 Metabolomic profiles of samples

Representative GC-MS total ion current (TIC) chromatograms of samples from the suckling, control, and Gln groups are presented in Fig. 1. In the GC-MS TIC chromatograms of serum samples from the three groups, 68 peaks were identified as endogenous metabolites based on the NIST mass spectra library. These metabolites are known to be involved in multiple biochemical processes, especially in carbohydrate metabolism, aminosugar and nucleotide metabolism, protein metabolism, amino acid metabolism, and lipid metabolism. The identified peaks are listed in Table 3.

3.4 Weaning-associated changes in metabolites

A total of 20 altered metabolites were identified between suckling and control piglets (Table 4). These decreased metabolites include carbohydrates, lipids, 2-hydroxybutyric acid, 4-hydroxypentenoic acid,

creatinine, glycerol-3-phosphate, and phosphoric acid. The relative levels of the 20 metabolites are displayed in a *z*-score plot (control pigs as the reference) (Fig. 2). The plot revealed the robust metabolic alterations in the suckling pigs (*z*-score range: −0.63 to 35.57) compared to fewer changes in the control pigs (*z*-score range: −1.59 to 1.93). Notably, creatinine was presented in relatively high concentration in suckling piglets.

3.5 Influence of Gln treatment on weaning-associated changes in metabolites

The two-sample *t*-test analysis ($P<0.05$) confirmed 5 significantly elevated metabolites in the Gln group compared with the control group in terms of the 20 identified different metabolites (Table 5). Up-regulated metabolites were 2-hydroxybutyric acid, creatinine, D-xylose, palmitelaidic acid, and α-L-galacto-furanose, and could potentially serve as the main metabolic difference between the control and Gln-treated piglets. The *z*-score plot (control pigs as the reference) of the 20 identified different metabolites (Fig. 3) revealed the distribution resulted from Gln supplementation. Some metabolites, such as creatinine, D-xylose, and palmitelaidic acid, exhibited more distribution difference for the control and Gln-treated piglets. Notably, creatinine was greatly increased ($P=0.0003$) in Gln-treated piglets compared with control piglets.

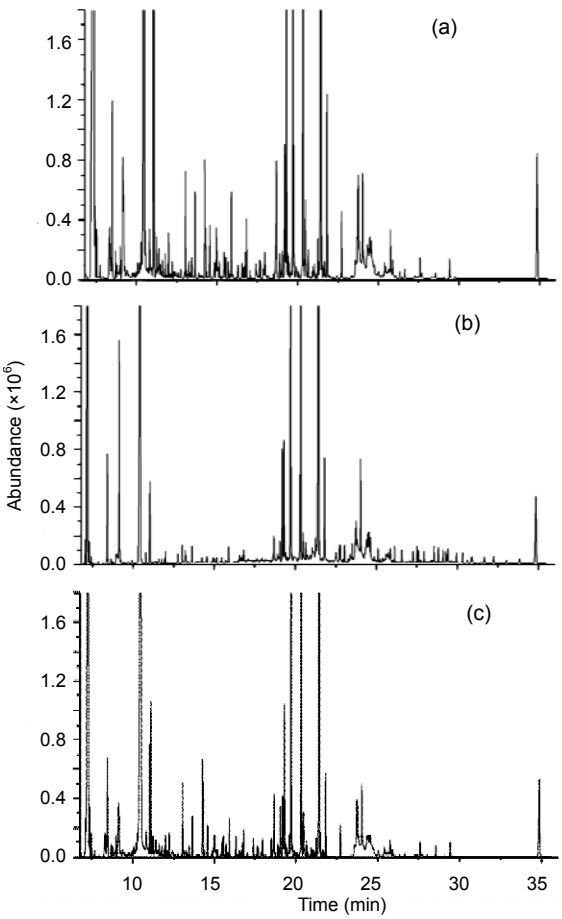

Fig. 1 Representative GC-MS TIC chromatograms of serum samples from suckling (a), control (b), and Gln (c) piglets

Table 3 Metabolites identified in serum samples of piglets using GC-MS

Group	Metabolite
Carbohydrates and polyols	1,3-Butylene glycol, D-fructose, diethylene glycol, D-mannitol, D-ribopyranose, D-xylose, erythritol, erythrose, galactopyranose, galactose, glucofuranose, glucose, glycerol, L-altrose, *myo*-inositol, propylenglycol, sorbose, sucrose, xylitol, α-D-galactofuranose, α-L-galactofuranose, β-D-galactofuranose, β-L-mannofuranose
Lipids	1-Monopalmitin, arachidonic acid, cholesterol, glyceryl monostearate, hexadecanoic acid, linoleic acid, octadecanoic acid, oleic acid, palmitelaidic acid
Amino acids	Glycine, glutamine, homocysteine, isoleucine, L-alanine, leucine, L-proline, L-tyrosine, L-valine, threonine, tryptophan
Metabolic intermediates	Acetic acid, glycerol-3-phosphate, malic acid, succinic acid, urea
Other metabolites	2-Hydroxybutyric acid, 2,2-bis(hydroxymethyl)propionic acid, 2,3-hydroxy acrylic acid, 3-methylindole, 3-deoxy-D-erythro-pentonic acid-γ-lactone, 3,4-dihydroxybutanoic acid, 4-hydroxypentenoic acid, acrylic acid, aminomalonic acid, creatinine, dimethylmalonic acid, erythronic acid, lactate, lactone pentonic acid, mannonic acid-lactone, *N*-acetylaspartic acid, phosphoric acid, retinoic acid, ribonic acid, β-idofuranuronic acid

A total of 68 peaks were identified as endogenous metabolites based on the NIST mass spectra library. Of these 68 metabolites, 23 were carbohydrates or polyols, 11 were amino acids, 9 were lipids, 5 were metabolic intermediates, and an additional 20 represented diverse metabolites

Table 4 Different metabolites between the control and suckling piglets

No.	Metabolite	Weaning	*P*-value	Adjusted *P*-value	No.	Metabolite	Weaning	*P*-value	Adjusted *P*-value
1	1-Monopalmitin	↓	0.0045	0.0070	11	Hexadecanoic acid	↓	0.0011	0.0037
2	2-Hydroxybutyric acid	↓	0.0039	0.0070	12	Linoleic acid	↓	0.0000	0.0000
3	4-Hydroxypentenoic acid	↓	0.0028	0.0061	13	Mannonic acid-lactone	↓	0.0082	0.0102
4	Cholesterol	↓	0.0030	0.0061	14	Oleic acid	↓	0.0045	0.0070
5	Creatinine	↓	0.0000	0.0000	15	Palmitelaidic acid	↓	0.0000	0.0000
6	D-Fructose	↓	0.0092	0.0103	16	Phosphoric acid	↓	0.0001	0.0004
7	D-Xylose	↓	0.0064	0.0092	17	Sorbose	↓	0.0436	0.0436
8	Galactose	↓	0.0079	0.0102	18	α-D-Galactofuranose	↓	0.0015	0.0038
9	Glycerol-3-phosphate	↓	0.0015	0.0038	19	α-L-Galactofuranose	↓	0.0001	0.0004
10	Glyceryl monostearate	↓	0.0096	0.0103	20	β-L-Mannofuranose	↓	0.0097	0.0103

"↓" indicates a down-regulated metabolite concentration in the control piglets compared with the suckling ones. *P*-value and adjusted *P*-value were the results of two-sample *t*-test and Benjamini-Hochberg tests performed using Matlab software, respectively

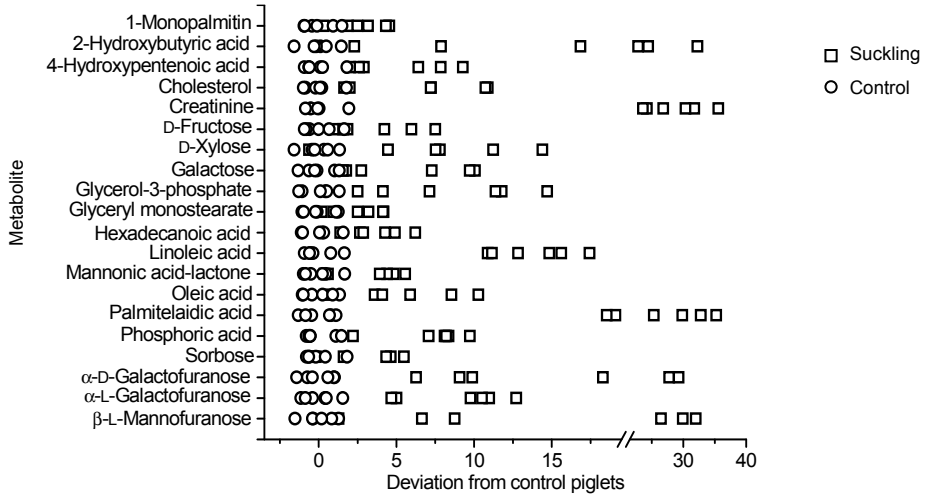

Fig. 2 *z*-score plot of the suckling piglets normalized to the mean of the control ones

Each point represents one sample in each group. *z*-score plots for the data centered to the means of control piglet samples and scaled by the standard deviation

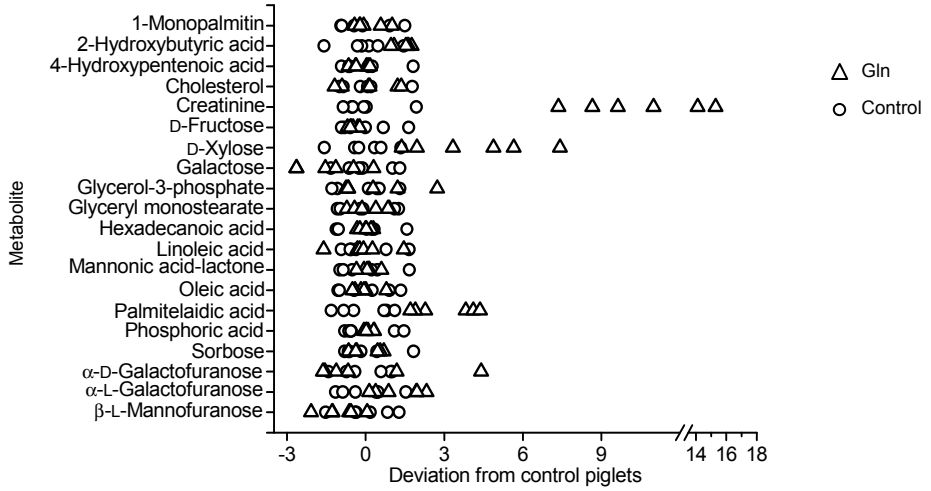

Fig. 3 *z*-score plot of the Gln-supplemented piglets normalized to the mean of the control ones

Each point represents one sample in each group. *z*-score plots for the data centered to the means of control piglet samples and scaled by the standard deviation

Table 5 Different metabolites between the control and Gln-supplemented piglets based on the 20 identified different metabolites

No.	Metabolite	Gln treatment	*P*-value	Adjusted *P*-value
1	2-Hydroxybutyric acid	↑	0.0077	0.0383
2	Creatinine	↑	0.0000	0.0003
3	D-Xylose	↑	0.0025	0.0168
4	Palmitelaidic acid	↑	0.0008	0.0078
5	α-L-Galactofuranose	↑	0.0455	0.1819

"↑" indicates an up-regulated metabolite concentration in the Gln-supplemented piglets compared with the control ones. *P*-value and adjusted *P*-value were the results of two-sample *t*-test and Benjamini-Hochberg tests performed using Matlab software, respectively

3.6 Comparison among the suckling, control, and Gln groups based on weaning-associated metabolite profile

A principle component analysis was conducted to compare the profiles of the suckling, control, and Gln groups based on the 20 different metabolites (Fig. 4). The score plot for the piglet sera, in accordance with their first two principal components (PCs 1 and 2), shows that the suckling samples were clustered from the Gln and control samples. However, it was observed that the samples from the Gln-treated piglets and control piglets were not separated into obviously different clusters. PCs 1 and 2 accounted for 89.87% of the variances. The results with Kruskal-Wallis tests for three-way comparisons among all three groups (Table 6) indicated that the *P*-values of 1-monopalmitin, D-xylose, glyceryl monostearate, mannonic acid-lactone, and sorbose were >0.01, which were excluded from the subsequent random forest analysis. Random forest was run on all 18 samples using a leave-one-out cross-validation (LOOCV). The mean percent decrease in accuracy is shown on Fig. 5. It can be seen that creatinine appears to be the most important followed by palmitelaidic acid, with an 8.51% and 7.83% decrease in classification accuracy when corresponding information is removed, respectively. The serum levels of creatinine in the suckling, control, and Gln groups are shown in Fig. 6.

Table 6 Kruskal-Wallis tests for three-way comparisons among the suckling, control, and Gln groups

No.	Metabolite	*P*-value	Adjusted *P*-value
1	1-Monopalmitin	0.0102	0.0128
2	2-Hydroxybutyric acid	0.0008	0.0051
3	4-Hydroxypentenoic acid	0.0034	0.0068
4	Cholesterol	0.0046	0.0071
5	Creatinine	0.0005	0.0051
6	D-Fructose	0.0060	0.0079
7	D-Xylose	0.0115	0.0128
8	Galactose	0.0017	0.0056
9	Glycerol-3-phosphate	0.0045	0.0071
10	Glyceryl monostearate	0.0250	0.0263
11	Hexadecanoic acid	0.0043	0.0071
12	Linoleic acid	0.0026	0.0068
13	Mannonic acid-lactone	0.0111	0.0128
14	Oleic acid	0.0057	0.0079
15	Palmitelaidic acid	0.0005	0.0051
16	Phosphoric acid	0.0033	0.0068
17	Sorbose	0.0691	0.0691
18	α-D-Galactofuranose	0.0032	0.0068
19	α-L-Galactofuranose	0.0017	0.0056
20	β-L-Mannofuranose	0.0017	0.0056

The *P*-values of 1-monopalmitin, D-xylose, glyceryl monostearate, mannonic acid-lactone, and sorbose were >0.01 by Kruskal-Wallis tests for three-way comparisons among all three groups, which were not used for leave-one-out cross-validation of random forest analysis. Adjusted *P*-values were the results of Benjamini-Hochberg tests performed using Matlab software

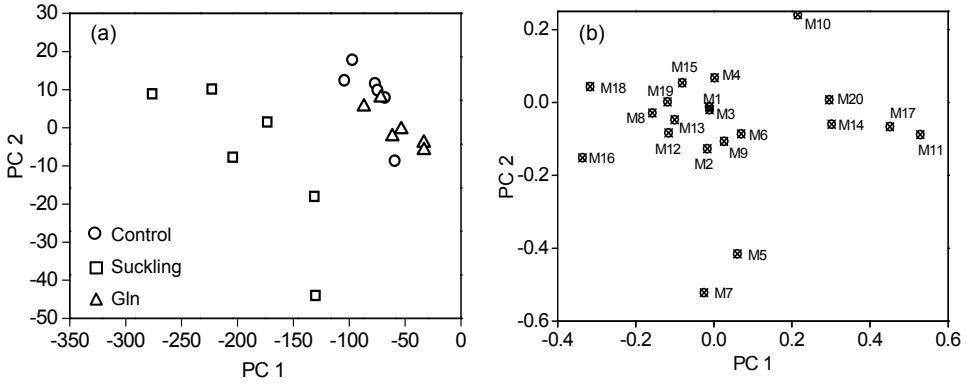

Fig. 4 PCA score plot (a) comparing the suckling, control, and Gln groups and the corresponding loading plot (b)
PC 1 and PC 2 accounted for 89.87% of the variances among the 20 different metabolites. Figures and M in (b) represent the number of the 20 different identified metabolites (shown in Table 4) between the control and suckling piglets

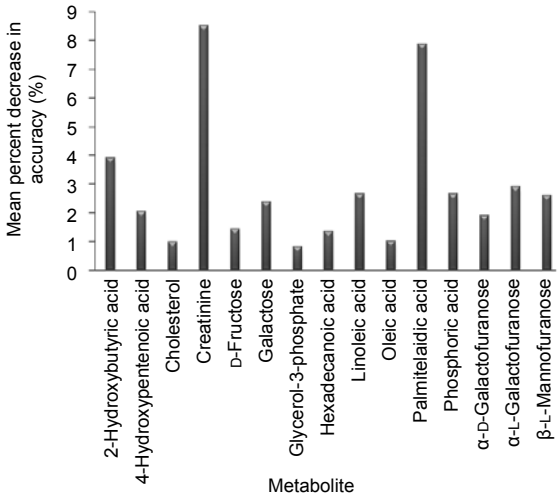

Fig. 5 Mean percent decrease in accuracy by a leave-one-out cross-validation of random forest analysis among the suckling, control, and Gln groups
The value of each metabolite reflects its weightiness with a decrease in classification accuracy

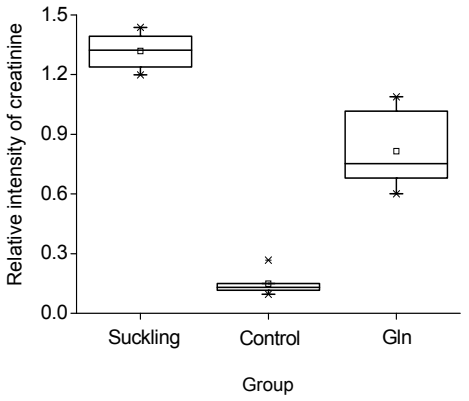

Fig. 6 Box and whisker plot of relative serum creatinine concentration (normalized to internal standard) among the suckling, control, and Gln groups (n=6)
A leave-one-out cross-validation of random forest analysis indicated that creatinine was the most important metabolite among the three groups in terms of the 20 identified different metabolites

3.7 Serum metabolites measured by detection kits

Serum metabolites in suckling, control, and Gln-supplemented piglets are shown in Table 7. Concentration of glucose was not altered (P=0.36), whereas total cholesterol was reduced by 32.80% (P=0.021) and 25.48% (P=0.038) in control and Gln-supplemented piglets, respectively, consistent with the relative intensities of glucose and cholesterol in GC-MS data. Serum levels of HDL and triglycerides were decreased by 24.14% (P=0.034) and 62.50% (P=0.009) in control piglets compared with suckling ones, but increased by 18.97% (P=0.089) and 27.08% (P=0.037) in Gln-supplemented piglets compared with control piglets, respectively. Concentration of urea was increased (P<0.05) in both control and Gln-supplemented piglets compared to suckling ones.

4 Discussion

Metabolomics is a useful system approach in the understanding of global changes of metabolites in animals in response to alterations in nutrition, genetics, environment, and gut microbiota (Nicholson *et al.*, 2004; Mao *et al*., 2008). However, it has not been extensively used in the field of swine study. Therefore, we used GC-MS-based metabolomics to investigate the metabolic characteristics of suckling, weaning, and Gln-treated piglets' sera. Twenty metabolites were identified to be significantly changed in response to weaning. These different metabolites were potentially involved in several Kyoto encyclopedia of genes and genomes (KEGG) metabolic pathways (Panagiotou *et al*., 2007; Antonov *et al*., 2008). Wiring diagrams were manually linked together to form

Table 7 Serum metabolites of piglets analyzed using conventional methods

Group	Glucose (mmol/L)	HDL (mmol/L)	Triglycerides (mmol/L)	Total cholesterol (mmol/L)	TAA (mmol/L)	Albumin (g/L)	Total protein (g/L)	Urea nitrogen (mmol/L)
Suckling	7.31	0.72^{a}	0.78^{a}	3.14^{a}	3.96	42.70	47.30	3.29^{b}
Control	7.05	0.58^{b}	0.48^{c}	2.11^{b}	3.61	38.58	48.26	4.23^{a}
Gln	6.96	0.69^{b}	0.61^{b}	2.34^{b}	3.91	37.32	47.07	3.96^{a}
SEM	0.35	0.06	0.11	0.26	0.14	1.72	1.29	0.20

HDL: high density lipoprotein; TAA: total amino acid; SEM: standard error of the mean. Suckling: piglets suckling from the sows; Control: weaned pigs receiving dietary supplementation with 1.22% (w/w) L-alanine; Gln: weaned pigs receiving dietary supplementation with 1% (w/w) L-glutamine. Values (mean, n=6) in a column not sharing the same superscripts are significantly different (P<0.05)

a metabolic network that involved in seven canonical KEGG pathways (Fig. 7), namely 'aminosugar and nucleotide metabolism', 'galactose metabolism', 'arginine and proline metabolism', 'biosynthesis of unsaturated fatty acid', 'glycerophospholipid metabolism', 'linoleic acid metabolism', and 'fatty acid metabolism'. It may be more informative to consider the global metabolic network for the interpretation of the metabolites.

Weaning is commonly related to profound social and environmental stresses, as well as acute alterations in both the pattern of intake and the type of diet (le Dividich and Sève, 2000). Large amounts of lactose, free and protein-bound amino acids, saturated fatty acids, and unsaturated fatty acids are present in sow's milk (Wu and Knabe, 1994; Mountzouris *et al*., 1999; van den Borne *et al*., 2007). After weaning, the piglets ingested complete feed rich in nitrogen compounds with small amounts of fat and carbohydrates (Gu and Li, 2003; Petrovič *et al*., 2009). The piglets also undergo tremendous changes in intestinal structure and in the ability to absorb nutrients after segregation from the sow (Miller *et al*., 1986; Gu *et al*., 2002). In the present study, we found that the serum concentrations of palmitelaidic acid, linoleic acid, hexadecanoic acid, oleic acid, 1-monopalmitin, glyceryl monostearate, and cholesterol were significantly depleted post-weaning. The changes in serum lipids and related metabolites may be the result of a lower ability to digest fat (Cera *et al*., 1990) and an increase in lipid β-oxidization for energy (Bazinet *et al*., 2003). The small intestinal alkaline phosphatase, crucial for fat absorption, has been shown to be reduced in early weaned pigs (Lackeyram *et al*., 2010). The energy supply of early-weaned pigs is very scant for low feed intake and digestibility (Petrovič *et al*., 2009). Fat provides much more energy than carbohydrates and protein for the maintenance or growth in the body during weaning (Gu and Li, 2003). In support of this notion, serum levels of triglycerides and total cholesterol were decreased in control piglets in the present data. Among these decreased fatty acids, linoleic acid (18:3ω6) is an important precursor of longer-chain polyunsaturated fatty acids (PUFAs), such as arachidonic acid (20:4ω6), eicosapentaenoic acid (20:5ω3), and docosahexaenoic acid (22:6ω3), which are essential for the normal growth and development of infants (Riediger *et al*., 2009). Additionally, oleic acid can interact with insulin and insulin-like growth factor-1 (IGF-1), which play important roles in normal growth (Tichelaar, 2000). The reduction of linoleic acid and oleic acid may help explain the poor body weight gain of the weaned piglets compared with the suckling ones.

Galactofuranose is present in various microbes but its existence has not been reported in mammals (Pedersen and Turco, 2003). The result of the present study shows that serum concentrations of α-L-galactofuranose and β-L-galactofuranose decreased in the weaned piglets, implying a change in the intestinal

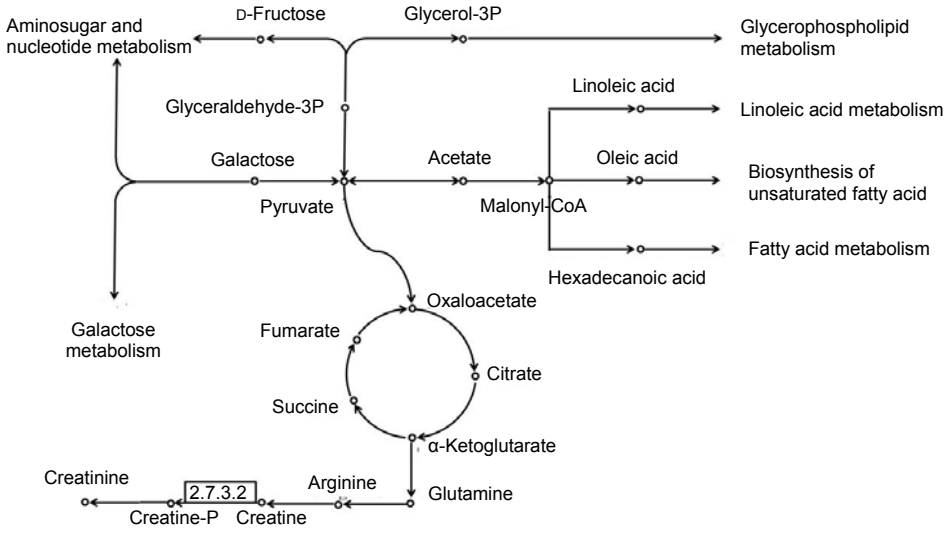

Fig. 7 KEGG pathways linked together manually

The creatine kinase (EC=2.7.3.2) involves in creatine metabolism in the pathway of arginine and proline metabolism. These pathways are based on the http://www.kegg.jp

bacterial population. This finding is consistent with those of a previous report indicating that microorganism communities undergo dramatic changes because of weaning (Janczyk *et al*., 2007). D-Xylose has been widely used to determine absorptive capacity (Hammon and Blum, 1997). Serum D-xylose concentration decreased, indicating that weaning reduced the intestinal absorptive capacity, as reported in other studies (van Beers-Schreurs *et al*., 1998; Gu *et al*., 2002). Galactose is a unique dietary sugar in that its exclusive source is milk lactose, and it provides approximately 20% of the total caloric intake in exclusively breastfed infants (Coss-Bu *et al*., 2009). We speculate that the depleted serum concentrations of galactose, D-fructose, and β-L-mannofuranose in weaned piglets could be caused by decreased absorptive ability, increased oxidation for energy, or by their increased contribution to glycogen synthesis.

Recent years have witnessed Gln playing an essential role in early weaned animals (Wu *et al*., 1996; Wang *et al*., 2008; Chamorro *et al*., 2010). In the current study, dietary Gln supplementation increased the serum concentrations of creatinine, palmitelaidic acid, D-xylose, α-L-galactofuranose, and 2-hydroxybutanoic acid. Based on the present knowledge of the regulatory role of Gln in physiology and nutrition of mammals (Stumvoll *et al*., 1999; Wu *et al*., 2007; Marc Rhoads and Wu, 2009), changes in these serum metabolites were not expected. Gln is a conditionally essential amino acid and a major energy substrate for rapidly dividing cells in mammals (Marc Rhoads and Wu, 2009). Previous studies have shown that dietary supplementation with Gln increases the weight of the small intestine as well as jejunal villus height (Wu *et al*., 1996), indicating its important role in improving nutrient absorption. Therefore, from the results obtained, the enhanced serum D-xylose and palmitelaidic acid concentrations may be due to improved nutrient absorption (Wang *et al*., 2008). The altered α-L-galactofuranose serum level in Gln-supplemented piglets is a novel finding, which implies that Gln favorably modulates the ecology of the gut microbiota. Arginine and proline can be generated through Gln metabolism (Stumvoll *et al*., 1999), and arginine regulates intestinal microbial metabolism, as has been recently reported (He *et al*., 2009).

A new and intriguing observation from the current study is that the serum concentration of creatinine was greatly reduced in control piglets compared with the suckling ones, whereas it was increased in Gln-supplemented piglets. Creatinine is produced from creatine metabolism and is related to arginine and proline metabolism (Deminice *et al*., 2009). An amount of creatine and amino acids are provided by sow milk as piglets suckle from the sow (Brosnan *et al*., 2009), which suggests that more creatine is ingested in suckling piglets and more creatinine is converted from the creatine (Wyss and Kaddurah-Daouk, 2000). The increasing creatinine concentration in Gln-supplemented piglets may be from the nonenzymatic degradation of creatine and ingested creatinine, due to the fact that Gln can afford the substrates for creatine synthesis indirectly (Brosnan *et al*., 2009; Deminice *et al*., 2009) and improve the absorption of dietary nutrients (Wang *et al*., 2008). These novel data from the metabolomic study provide crucial insight into cellular metabolic alterations of creatine and creatinine in response to weaning and dietary Gln supplementation.

5 Conclusions

A novel metabolomic approach based on GC-MS was employed to comprehensively investigate the changes of serum metabolites in suckling, weanling and dietary Gln-supplemented piglets. Serum metabolite profiles from early weaned piglets were significantly different from those of the suckling piglets, with regard to a range of important metabolic pathway intermediates and the relative amounts of creatinine, D-fructose, D-xylose, galactose metabolism, linoleic acid metabolism, biosynthesis of unsaturated fatty acid, glycerophospholipid metabolism, and fatty acid metabolism intermediates. The findings indicate that serum metabolic profiles have great potential in investigating the response to early weaning in piglets. Dietary Gln supplementation increases the serum levels of 2-hydroxybutyric acid, creatinine, D-xylose, palmitelaidic acid, and α-L-galactofuranose, which were reduced in early weaned piglets. Notably, the concentration of creatinine was greater in suckling piglets and Gln-supplemented piglets compared to the control piglets. Further research is necessary to clarify the reasons for the changes of these metabolites to develop a comprehensive view of the response to weaning and dietary Gln supplementation.

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