

Comparative metabolomic analysis of caecal digesta between Jinhua pig and Landrace pig

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Abstract: The metabolic difference in caecal digesta between Jinhua pig and Landrace pig was compared. Twenty weaned piglets at 28 days of age, including ten Landrace pigs (a Western pig breed) and ten Jinhua pigs (a Chinese native pig breed), were randomly selected and allocated into two groups. The pigs were fed the same corn-soybean diet on the same pig farm. At the age of 240 days, all pigs of each group were slaughtered, the digesta in the caecum of the twenty pigs were collected for metabolomic analysis and determination of short-chain fatty acids (SCFAs). The results showed that a total of 56 different metabolites (22 metabolites named and 34 metabolites without identification) were detected in caecal digesta using a gas chromatography time-of-flight/mass spectrometry (GC-TOF-MS)-based metabolomic approach. Forty-six of the 56 metabolites were upregulated significantly ($P < 0.05$) in Landrace group compared with Jinhua group. The metabolic pathways with different impact value in which different metabolites were mainly involved were tyrosine metabolism, citrate cycle and steroid biosynthesis. In addition, we found that Landrace accumulated more SCFAs in caecal digesta, while the concentrations of acetic acid ($P < 0.01$) and butyric acid ($P < 0.05$) in caecal digesta of Jinhua pig were markedly lower than those of Landrace pig. Collectively, our study was the first to compare the metabolic difference in caecal digesta between Jinhua pig and Landrace pig using a metabolomics approach, which might be used as a potential metabolomics mechanism to research different breeds of pigs.

Keywords: caecal digesta; Jinhua pig; Landrace pig; metabolic difference; short-chain fatty acids

Western and indigenous Chinese pig breeds show marked differences in many aspects, such as muscle growth or meat quality (Guo et al. 2011). The Jinhua pigs, one of the most important local breeds in China, exhibit early sexual maturity, low

growth rate and low prolificacy. But the meat of Jinhua pigs is an excellent raw material for Jinhua ham (Miao et al. 2009). By contrast, Landrace pigs have been intensively selected over the past decades for faster growth rate and muscularity, which

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is considered to have resulted in a deterioration of meat quality (Lefaucheur et al. 2002; Shan et al. 2009). Hence, these two pig breeds can be used as an ideal comparison for studying the differences between Western and Chinese pig breeds (Wu et al. 2013). So far, most studies have focused on the porcine growth performance and meat quality, whereas the underlying metabolic mechanism has not been fully studied and published comprehensive studies on metabolites are few (Miao et al. 2009; Oresic 2009; Sun et al. 2017).

Metabolism plays an important role in biological systems. Identification and quantification of the compounds in the metabolome are defined as metabolic profiling, which could be used to compare the metabolic difference between two breeds (Dunn et al. 2011). Metabolomics, the study of the metabolome, is the latest “omics” approach that could provide a new insight into identification and quantitation of the compounds produced during the metabolic process in the endogenous and exogenous metabolomic characteristics of biological fluids (urine, plasma and caecal content) using high-throughput approaches (Ng et al. 2012). Gas chromatography time-of-flight mass spectrometry (GC-TOF-MS), a technique commonly used in metabolism analysis, is a sensitive and reproducible method to enable complex mixtures of metabolites to be resolved (Lindon et al. 2006; Oliver et al. 2011). Besides, the GC-TOF-MS method is suitable for an untargeted metabolic fingerprinting (Dettmer et al. 2007), because it combines the separation power of GC with structural information obtained by high resolution mass spectrometry.

In this study, two breeds of pigs with different growth rates were used as the model. We aimed to compare the metabolic difference between Jinhua and Landrace pigs and then detect the significantly different metabolites by studying the metabolite profiles of caecal digesta samples using a metabolomics approach based on GC-TOF-MS. Moreover, we also hope to pinpoint the pathways where the different metabolites are involved and clarify the metabolic mechanism which may explain the phenotypic differences between the two breeds.

MATERIAL AND METHODS

Animals, housing, and diets. All animal experiments and procedures were conducted in

accordance with guidelines established and approved by the Animal Science College of Zhejiang University. All of the pigs were raised on a commercial farm in Jinhua City, Zhejiang Province of China. A total of ten Landrace piglets and ten Jinhua piglets weaned at 28 days of age were used in this study ($n = 10$). All piglets, kept in a standard shared pen throughout the experiment, were provided food and water *ad libitum*. Piglets were fed the same corn-soybean diet formulated with trace minerals and vitamins to meet the requirements of the National Research Council for different ages. The experiment lasted 212 days. At the age of 240 days, 10 Jinhua pigs (body weight = 62.01 ± 2.65 kg) and 10 Landrace pigs (body weight = 124.52 ± 2.26 kg) were slaughtered. A total of 20 digesta samples sourced from the proximal colon at necropsy were collected aseptically and immediately frozen in liquid nitrogen. Subsequently, samples were stored at -80°C until further analysis (Ng et al. 2012; Nie et al. 2015).

Metabolite sample preparation. Totally 100 mg of caecal digesta were weighed in 2-ml EP tubes and 20 μl of L-2-chlorophenylalanine (1 mg/ml stock in dH_2O) were added to each sample as internal standard. Samples were extracted with 0.5 ml of methanol extraction liquid (V methanol : V chloroform = 3 : 1) and vortex-mixed for 30 s (Quifer-Rada et al. 2016). The mixture was homogenized in a ball mill for 4 min at 45 Hz and then ultrasound treated for 5 min (incubated in ice water). The samples were subsequently centrifuged at 13 000 g for 15 min at 4°C , and then 0.4 ml of the supernatant was transferred into a fresh 2 ml GC/MS glass vial. After the extracts were evaporated to complete dryness in an unheated vacuum concentrator, 80 μl of methoxyamine hydrochloride (20 mg/ml in pyridine) were added and mixed softly, the solution was then incubated for 30 min at 80°C . Then 100 μl of the BSTFA reagent (1% TMCS, v/v) was added to each sample aliquot, followed by incubation for 2 h at 70°C (Xiao et al. 2012; Sun et al. 2015). At last, the solution was again vortex-mixed for 1 min and prepared for the GC-TOF-MS analysis.

GC-TOF-MS analysis. The GC-TOF-MS analysis was performed using an Agilent 7890 gas chromatograph equipped with a Pegasus HT time-of-flight mass spectrometer (J&W Scientific, USA). A sample volume of 1 μl aliquot was injected by the autosampler into GC which was equipped with a DB-5MS capillary column (30 m \times 250 μm inner diameter) coated with 5% diphenyl cross-linked

with 95% dimethylpolysiloxane. Helium served as the carrier gas at a continuous flow rate of 1 ml/min through the column. The column temperature was initially kept at 50°C for 1 min and then increased to 310°C at a rate of 10°C/min, where it was held for 8 min. The temperature of injection was maintained at 280°C in the experimental process. The transfer line temperature was set to 270°C and the ion source temperature was 220°C. The mass spectrometer was operated in electron impact mode (70 eV). The MS data were acquired in full scan mode over the range between 50 and 500 m/z at a rate of 20 spectra/s. The acceleration voltage was switched on after a solvent delay of 5.2 min. In this process, the stability of the sample injection was tested with the retention time (RT) of an internal standard (L-2-chlorophenylalanine).

Data analysis. The Chroma TOF 4.3X software of LECO Corporation and LECO-Fiehn Rtx5 database were used to filter and calibrate the data baselines, to align the data base, to solve the deconvolution analysis, peak identification and integration of the peak area (Kind et al. 2009). The bulks of the peaks in the TIC chromatograms were identified by their retention time, mass spectra characteristics and the LECO/Fiehn Metabolomics Library (Yang et al. 2016). For all samples, a numerical simulation method with half of the minimum value was used to simulate the missing value of the original data. Accordingly, the noise removal was conducted using a quartile range to filter data, and data were standardized by means of the internal standard normalization method (Sun et al. 2015).

SIMCA 14.1 software package (V14.1, MKS Data Analytics Solutions, Sweden) was used for pattern recognition multivariate analysis. The resulting three-dimensional data consisting of arbitrary peak index (retention time), sample name (observations), and normalized peak area (variables) were entered into software for multivariate statistical analysis. Principal component analysis (PCA), exhibiting the similarity and difference of data, was used to assess the metabolomics data set. The results of PCA model could indicate the systematic trends in raw data, such as clustering trend, time trend. In order to acquire a higher level of group separation and get a better understanding of variables responsible for classification, supervised orthogonal partial least squares discriminant analysis (OPLS-DA) was performed. To validate models, the quality of the model was evaluated by three

parameters: R^2X , R^2Y and Q^2 . The goodness-of-fit was quantified by parameters R^2X and R^2Y , and the predictive ability was evaluated by parameter Q^2 . After 200 permutations, the R^2 and Q^2 intercept values were obtained. The low values of Q^2 intercept manifested the robustness of the models, and thus showed a low risk of overfitting and reliable. Besides, the partial least squares discriminant analysis (PLS-DA) was further implemented by 7-fold cross validation to estimate the robustness and the predictive ability of our model.

Identification of significantly different metabolites and pathways between Jinhua pig and Landrace pig. In further data processing, the identified metabolites were verified by comparison with the chromatographic, chemical or spectral characteristics of specific criteria. In further analysis, a variable importance in the projection (VIP) value of 1.0 was applied to the list of metabolites for the purpose of creating statistical models based on the significantly different metabolites between Jinhua and Landrace pigs. In order to find metabolites that led to the discrimination, the surplus variables were then assessed by Student's *t*-test with an acceptable significance level of *P*-value < 0.05 (Gu et al. 2015). Furthermore, online databases, including Kyoto Encyclopaedia of Genes and Genomes (KEGG), Chemical Abstracts Service (CAS), National Institute of Standards and Technology (NIST), were used for metabolite identification and pathway construction.

Analysis of SCFA concentrations in the caecal digesta of Jinhua pig and Landrace pig. The short-chain fatty acid (SCFA) concentrations in caecal digesta were analysed using capillary column gas chromatography. Each of the samples was mixed with 10 ml/g deionized water. Then the mixtures were centrifuged (10 000 rpm for 10 min) and 1000 µl of the supernatant were combined with 200 µl of crotonic acid (internal standard). Finally, the mixed solution was used to determine the concentration of SCFAs using a capillary gas chromatograph GC-2010 plus (Shimadzu, Japan) after filtering through a membrane filter. The experimental parameters were set according to the method described by Yang et al. (2014).

Statistics. All statistical analyses were performed using SPSS software Version 22.0. The differences between the two groups were examined for significance by an independent-sample *t*-test to conduct the analysis of variance. The results presented in

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this article are shown as the means \pm SEM and were considered significant at $P < 0.05$.

RESULTS

Metabolomic profiles of samples. The total ion current (TIC) chromatograms of Jinhua pig and Landrace pig produced by GC-TOF-MS analysis are shown in Figure 1. A total of 597 peaks were detected from all samples and 554 valid peaks of metabolites could be left through the interquartile range de-noising method. There were various differences in the shape and quantity of peaks between the two groups, with distinct peaks in each group. In addition, a significant decline in the abundance of typical peaks was especially observed in Landrace group (20–23 min) compared with Jinhua group

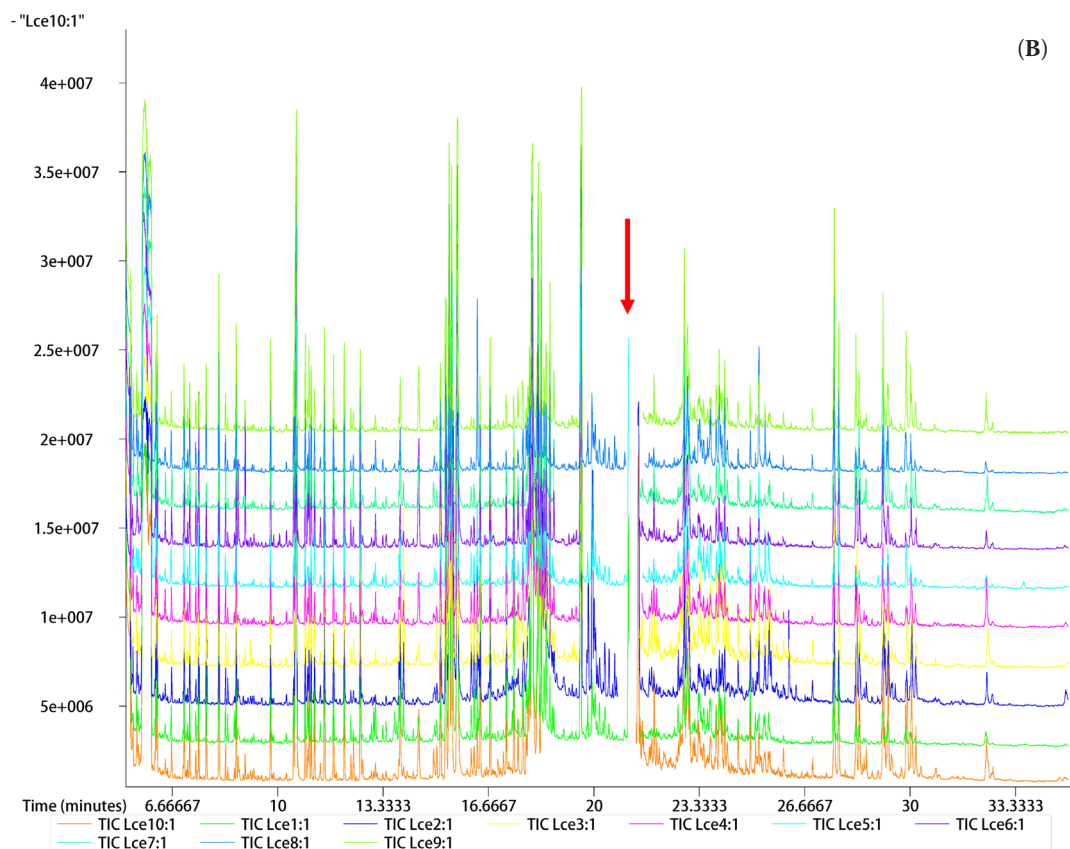
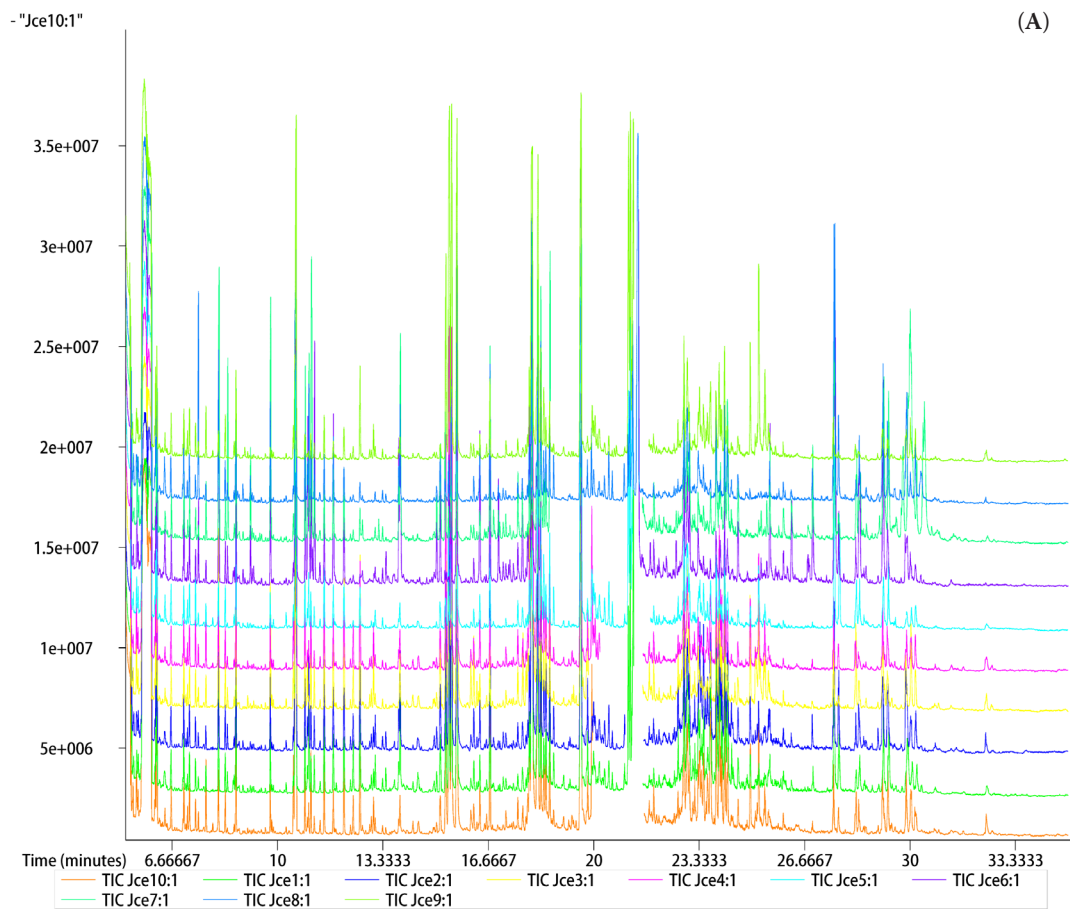
(Figure 1, highlighted by arrow). Therefore, total ion chromatograms from GC-TOF-MS analyses could show the difference directly in metabolite profiles between Jinhua and Landrace pigs.

Multivariate data analysis and metabolite quantification. Multivariate data analysis was used to assay the variation between Jinhua and Landrace pigs. PCA of GC-TOF-MS metabolic profiles of caecal digesta samples displayed significantly divided clusters between the two groups in the PCA scores plot (Figure 2A), indicating that their metabolic profiles differ vastly from each other. The R^2X values of the PCA model that represent explained variance were 0.533. Furthermore, none of the samples from either group fell outside Hotelling's T -squared ellipse with 99% confidence limit of the model, which suggested that no outlier was present among the samples analysed.

Table 1. Differentially expressed metabolites in caecal digesta in Jinhua pig and Landrace pig

Metabolite name	Similarity	RT	Mass	Mean Jinhua	Mean Landrace	VIP	P -value	FC
Lactic acid	954	7.50	117	0.21488	0.67252	1.22308	0.00692	0.31951
Fructose 1	934	17.76	103	0.35549	0.70910	2.07117	0.02695	0.50133
Succinic acid	930	11.16	147	0.54453	1.12967	1.03060	0.00755	0.48203
Thymine	891	12.31	255	0.07222	0.16808	2.22365	0.00093	0.42967
Methyl phosphate	799	9.20	241	0.02272	0.05405	1.63888	0.00654	0.42032
Tyramine	724	18.27	174	0.01839	0.34446	2.37778	0.00102	0.05338
Cholestan-3 β -ol	665	28.58	55	0.04300	0.02200	1.20670	0.04218	1.95479
Zymosterol 1	625	28.61	55	4.14E-08	0.04126	2.27565	0.00988	1.00E-06
1-Monopalmitin	613	24.14	215	0.37114	0.14893	1.77003	0.02355	2.49203
N-Acetyl-L-leucine 1	582	13.70	156	0.00671	0.01644	1.08400	0.01340	0.40787
Lyxonic acid, 1,4-lactone	552	16.38	217	0.11150	0.05157	2.23002	0.03243	2.16239
α -Ketoisocaproic acid 2	495	9.24	89	0.02092	0.05483	1.14053	0.00380	0.38150
α -Ecdysone 1	451	29.42	83	0.00896	0.03718	2.03917	0.00871	0.24095
Ergosterol	413	28.80	55	0.01019	0.02177	1.39548	0.00465	0.46830
Oxamic acid	394	10.40	101	0.00081	0.00907	2.37337	0.01194	0.08907
2-Ketoadipate 3	350	10.20	89	0.00578	0.01266	1.96882	0.03283	0.45641
Phytol	341	20.66	67	0.01298	0.12022	1.18830	0.02813	0.10794
N-Ethylglycine 1	322	12.43	174	0.00838	0.02373	1.65606	0.00982	0.35294
4-Hydroxybutyrate	320	10.02	103	0.00337	0.00850	1.24910	0.03732	0.39714
1-Indanone 2	262	12.40	156	0.00164	0.01664	2.50299	0.00028	0.09846
Galactonic acid	247	19.08	88	0.00349	0.02677	1.88993	0.01388	0.13052
9-Fluorenone 2	240	17.07	74	0.01012	0.04167	1.67821	0.00200	0.24279

RT = retention time, VIP = variable importance in the projection, FC = fold change, mean value of peak area obtained from Jinhua group/mean value of peak area obtained from Landrace group. FC value < 1 indicates that the metabolite in Jinhua pig is lower than that in Landrace pig



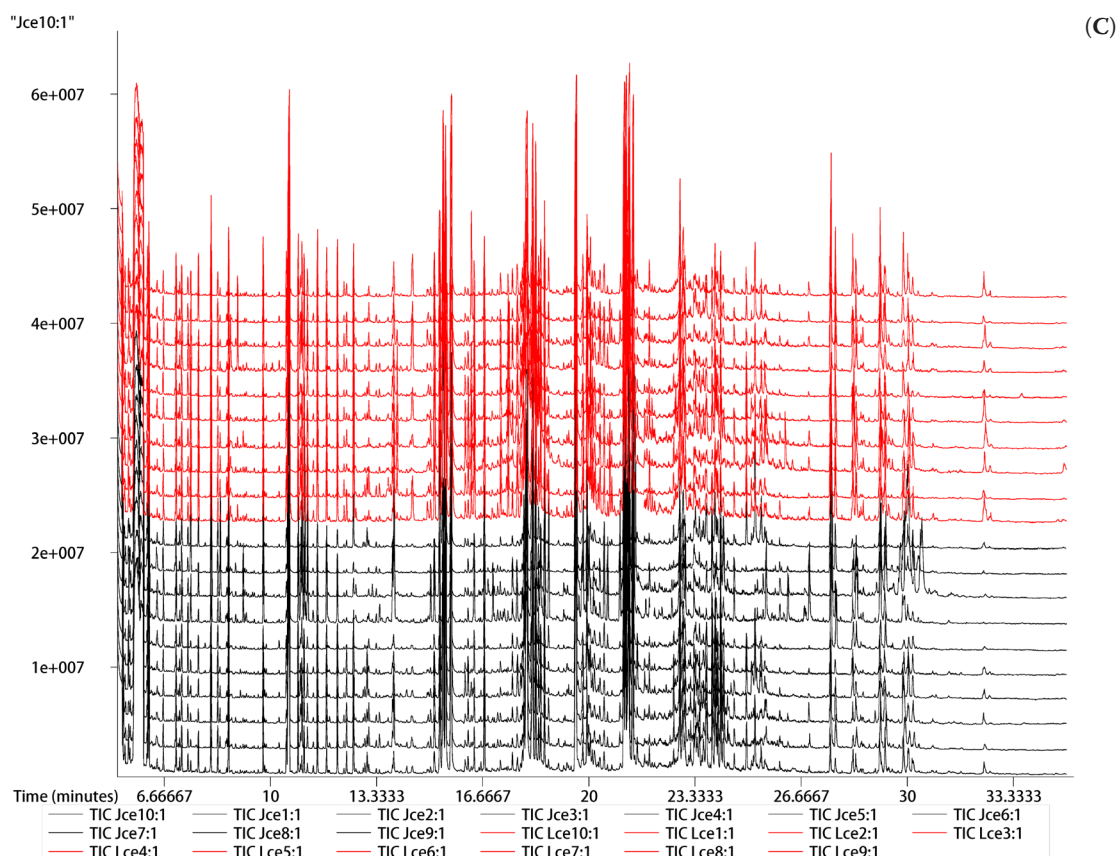


Figure 1. Typical GC-TOF-MS TIC chromatograms of cecal digesta samples obtained from (A) Jinhua pig, (B) Landrace pig (arrow indicates a significant decline in the abundance of typical peaks especially observed in Landrace group (20–23 min) compared with Jinhua group), and (C) all the samples

Figure 2B displays the OPLS-DA results of caecal digesta samples from the two experimental groups, with satisfactory modelling and predictive abilities ($R^2X = 0.246$, $R^2Y = 0.944$, $Q^2 = 0.629$). Clear separation and distinction were found between Jinhua group and Landrace group, suggesting that the OPLS-DA model can be applied to identify the differences between Jinhua and Landrace pigs.

The parameters for the assessment of the PLS-DA model quality in identifying the metabolic difference in caecal digesta between Jinhua and Landrace pigs could be represented by the validation plot, as shown in Figure 2C. The intercept (R^2 and Q^2 when the correlation coefficient is zero), correlated with the extent of overfitting, was rather small ($R^2 = 0.87$, $Q^2 = -0.58$), which indicated that the model was highly effective.

List of discriminant metabolites. In total, high-throughput profiling of the caecal digesta quantitatively detected 56 potential discriminant metabolites (VIP > 1, 22 metabolites named and 34 metabolites without identification) (Table 1).

All of these 56 metabolites achieved significance ($P < 0.05$) when their means were compared using Student's *t*-test, confirming their significant contributions as discriminant metabolites to separation between Jinhua group and Landrace group. These differentially expressed metabolites were represented as “volcano plot”, which provided quick dissemination and identification of significant features based on metabolite fold changes (ratio of metabolites between Jinhua group and Landrace group) and their *P*-value. As illustrated in Figure 3, the red dots indicate upregulated metabolites of Jinhua group when compared with Landrace group, such as 1-monopalmitin, lyxonic acid-1,4-lactone. The downregulated metabolites (lactic acid, succinic acid, oxamic acid, galactonic acid, etc.), are represented by the blue dots.

Metabolic pathways of different metabolites. Commercial databases, including KEGG (<http://www.genome.jp/kegg/>) and NIST (<http://www.nist.gov/index.html>), were utilized to cast about for the pathways involved in different metabolites.

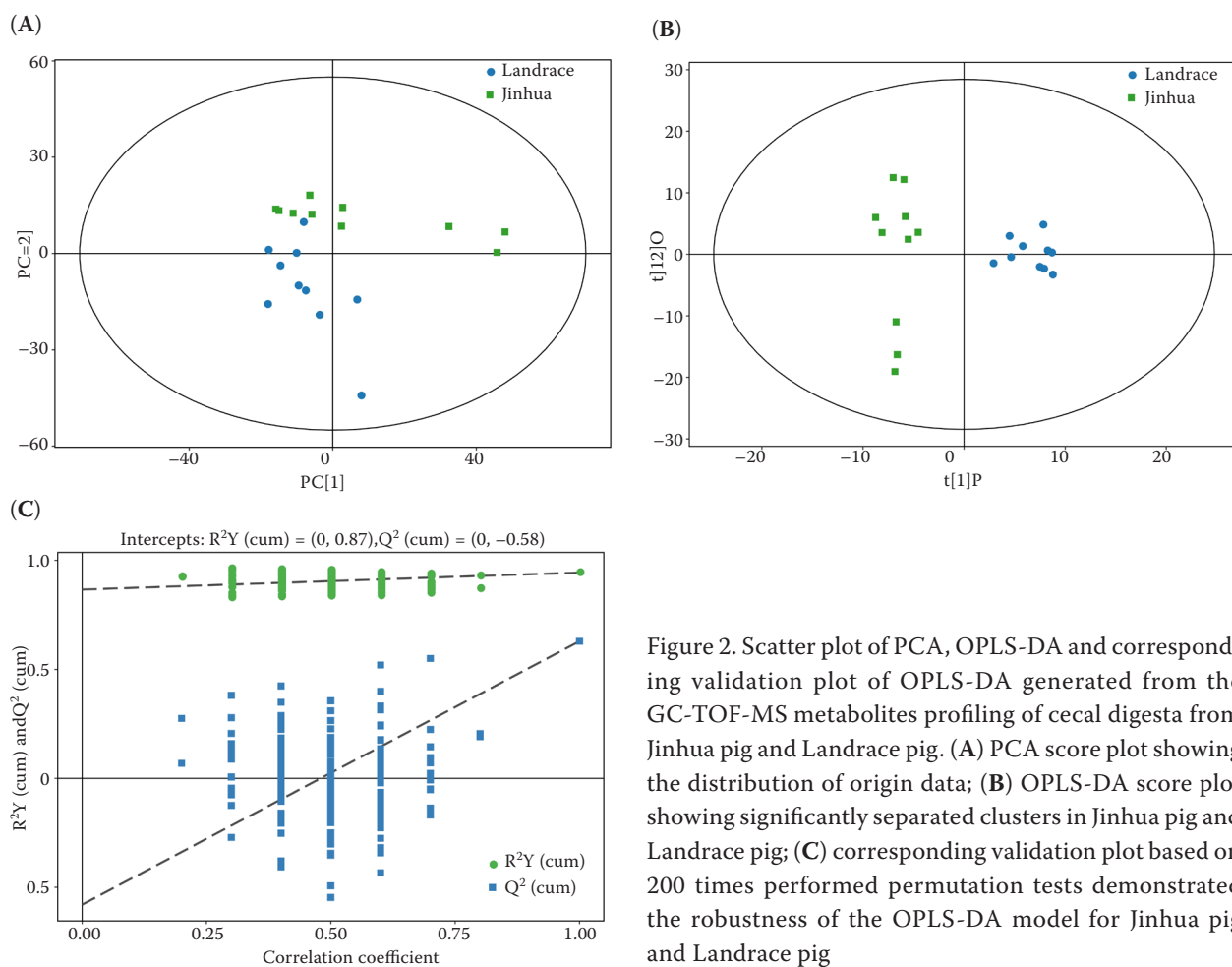


Figure 2. Scatter plot of PCA, OPLS-DA and corresponding validation plot of OPLS-DA generated from the GC-TOF-MS metabolites profiling of cecal digesta from Jinhua pig and Landrace pig. (A) PCA score plot showing the distribution of origin data; (B) OPLS-DA score plot showing significantly separated clusters in Jinhua pig and Landrace pig; (C) corresponding validation plot based on 200 times performed permutation tests demonstrated the robustness of the OPLS-DA model for Jinhua pig and Landrace pig

In addition, MetaboAnalyst was an indispensable web-based tool (<http://www.metaboanalyst.ca>), which used the high-quality KEGG metabolic pathway as the backend knowledgebase for metabolic pathway analysis. The metabolic pathway analysis

result of the 22 different metabolites between Jinhua and Landrace pigs was presented as bubble plot (Figure 4). Each bubble in the bubble plot represented a metabolic pathway. Thus it could be seen that the main enriched pathways for differ-

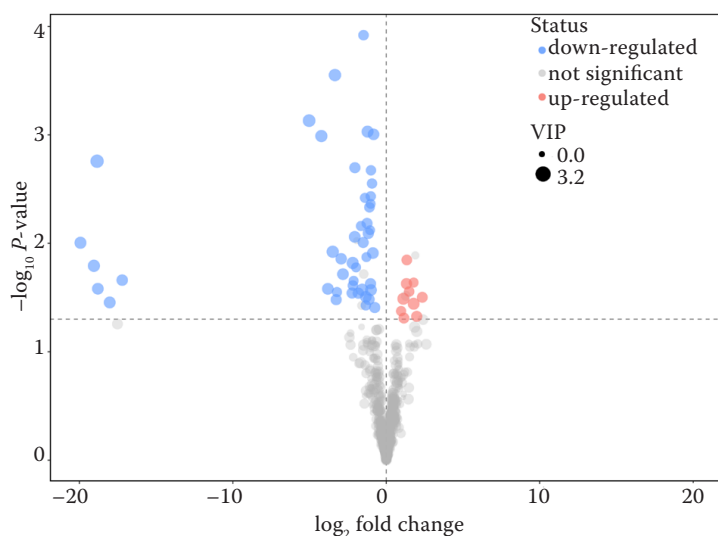


Figure 3. Volcano plot for Jinhua pigs and Landrace pigs. Each point represents a kind of metabolite, the scatter colour represents the final screening result. Significant up-regulated metabolites are indicated in red, significantly down-regulated metabolites are indicated in blue, and non-significant differences in metabolites are grey

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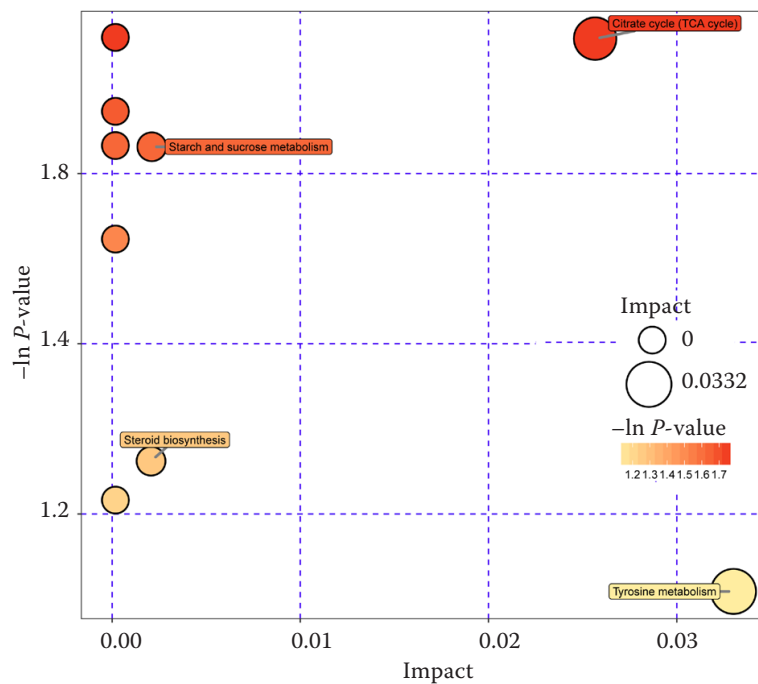


Figure 4. Metabolome view map of significantly different metabolites identified in cecal digesta between Jinhua pig and Landrace pig. x -Axis represents pathway impact, y -axis represents pathway enrichment. Larger sizes and darker colours represent higher pathway enrichment and higher pathway impact values, respectively

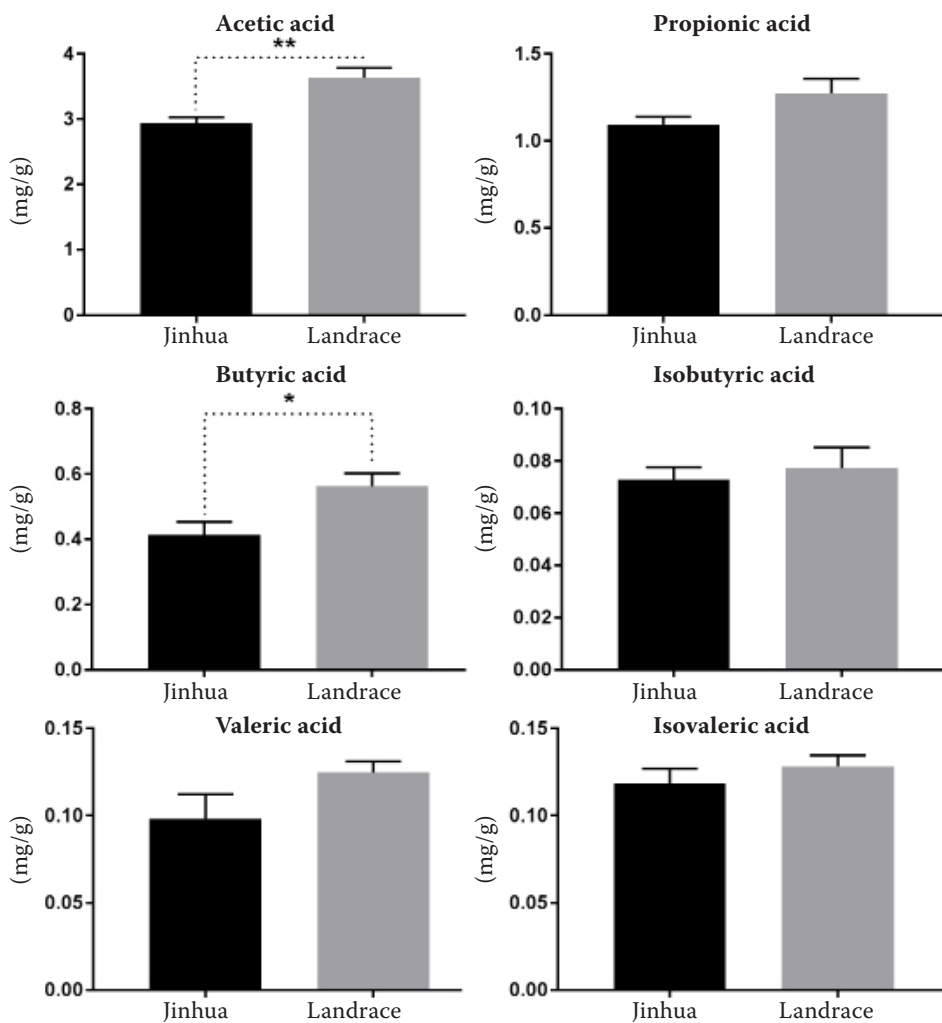


Figure 5. Concentrations of short-chain fatty acids (SCFAs) in the cecal digesta in Jinhua pig and Landrace pig. Error bars represent standard error of the mean

* $P < 0.05$, ** $P < 0.01$

ent metabolites were tyrosine metabolism, citrate cycle and steroid biosynthesis.

Concentrations of SCFAs in the caecal digesta of Jinhua pig and Landrace pig. We also measured the concentrations of SCFAs in the caecal digesta of Jinhua pig and Landrace pig (Figure 5). Interestingly, the most abundant SCFA in the caecal digesta of Jinhua and Landrace pigs was acetic acid. In addition, we found significantly higher concentrations of acetic acid ($P < 0.01$) and butyric acid ($P < 0.05$) in the caecal digesta of Landrace pig when compared with Jinhua pig. Moreover, there were slightly higher levels of propionic acid, isobutyric acid, valeric acid and isovaleric acid in the caecal digesta of Landrace relative to Jinhua pig.

DISCUSSION

The pig is one of the economically most important animals, and it is distributed throughout the world. Pigs of different breeds have phenotypically distinct genetic makeups that influence diverse physiological traits (Pajarillo et al. 2014). Jinhua pigs are a famous native breed to China with favourable meat flavour and early puberty, but they grow at a much slower rate than the Western commercial breeds (Yang et al. 2012; Xian et al. 2014). Landrace pigs, a commercial Danish breed, have been intensively selected for a thin subcutaneous fat layer and high muscularity in the carcass, which has led to a deterioration of meat quality (Zhang et al. 2015). Guo et al. (2011) compared the differences in pork quality traits, muscle fibre types, and metabolic enzyme activities between Jinhua and Landrace pigs; their results may provide valuable information for understanding the molecular mechanism responsible for breed specific differences in growth performance and meat quality. Wu et al. (2013) found the differentially expressed genes in the *longissimus dorsi* muscles between Jinhua and Landrace pigs. However, previous studies paid little attention to the metabolic mechanism of Jinhua and Landrace pigs, and little was known about the overall metabolic difference in caecal digesta of the two pig breeds. Therefore, Jinhua pigs (a fatty type) and Landrace pigs (a lean type) were chosen as comparison objects, we compared the metabolic difference in caecal digesta of the two pig breeds under the same feeding environment and age.

The metabolomic analysis using “omics” approaches will help identify the different metabolites and find out the pathways in which the different metabolites are involved. In our study, GC-TOF-MS-based metabolomics was utilized to compare the difference in metabolism and to detect differential metabolites between Jinhua and Landrace pigs. We found that the metabolome in caecal digesta was significantly different between Jinhua and Landrace pigs, breeds with a varied growth rate. The present results showed that a total of 56 differentially expressed metabolites were identified in caecal digesta samples, with at least 2.0-fold difference ($P < 0.05$) between the two groups. Among the 56 different metabolites, only 9 metabolites were highly expressed in Jinhua pigs. The rest of the different metabolites, including lactic acid, succinic acid, ergosterol, zymosterol, thymine and tyramine, were expressed at a higher level in Landrace pigs than in Jinhua pigs, which was consistent with the higher concentration of SCFAs in the caecal digesta determined by a capillary column gas chromatograph.

SCFAs, an important energy source, are produced by bacteria through the fermentation of low-digestible carbohydrates and proteins (Kong et al. 2014). It is well known that acetic acid, propionic acid and butyric acid are the major SCFAs produced in the caecum and colon, of which butyric acid is thought to be the most beneficial for human and animal health. Propionic acid, as a health-promoting metabolite being produced in the large intestine, has the potential to reduce cholesterol concentrations (Hosseini et al. 2011). Our results have shown that the concentrations of SCFAs, including acetic acid and butyric acid, were significantly higher in caecal digesta of Landrace pigs than in those of Jinhua pigs, which was similar to other studies (Yan et al. 2017). In addition, Jiang et al. (2016) reported that the concentrations of intestinal luminal SCFAs of Bama mini-pigs (a fatty-type Chinese local pig strain) were lower than those of Landrace pigs, which is also consistent with our results. Previous studies also indicated that there was a close correlation between the gut bacteria and obesity (Nadal et al. 2009). Hence, we could speculate that the levels of intestinal luminal SCFAs are possibly affected by fat-type and lean-type pig breeds. Considering the importance of gut microbes in production of SCFAs, the intestinal microbial compositions should be investigated to assess the metabolic difference between the two pig breeds in future studies.

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In the present study, more small-molecule metabolites, such as lactic acid, succinic acid, oxamic acid, galactonic acid, were higher in caecal digesta of Landrace pigs than in Jinhua pigs. These different metabolites were mainly produced by gut microbes, which partly indicated that the acid-producing bacteria in the intestines of Landrace pigs may be more abundant. It can be regarded as a potential reason to explain the higher growth rate of Landrace pigs than that of Jinhua pigs. It would be of great interest in future studies to determine how these metabolites contribute to the differences in growth rate between Jinhua and Landrace pigs. The present study not only detected the different metabolites between Jinhua and Landrace pigs, but also indicated the pathways where these metabolites were involved. According to the metabolic pathway analysis, tyrosine metabolism, citrate cycle and steroid biosynthesis were the pivotal different metabolic pathways identified between Jinhua and Landrace pigs, which might be the most crucial pathways on behalf of metabolic differences in caecal digesta between the two pig breeds (Sun et al. 2015). However, further researches from several aspects (metagenomics, transcriptomics and the like) are also necessary to explore the differences between Jinhua and Landrace pigs.

CONCLUSION

In the present study, we compared the metabolic difference in caecal digesta between Jinhua and Landrace pigs under the same feeding environment and age. We found that the metabolome in caecal digesta was significantly different between Jinhua and Landrace pigs. In addition, our results have also shown that concentrations of SCFAs were significantly higher in caecal digesta of Landrace pigs than in those of Jinhua pigs, which was probably related to different growth rates of the two breeds. It could be regarded as a potential reason to explain the higher growth rate of Landrace pigs than that of Jinhua pigs.

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