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Article

Metabolomic Profiling Reveals the Distinct Nutritional Properties of Breed and Feed on the Muscles in Chinese Taihe Black-bone Silky Fowl (*Gallus gallus domesticus Brisson*)

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Abstract Chinese Taihe Black-bone silky fowl (TBsf) is the homology of medicine and food and has high nutritional and medical value all over the world. However, the nutritional compositions and specific metabolite advantages of Taihe silky fowl muscle are still poorly understood. In this study, we investigated the differences of nutritional components between TBsf and another similar breed (Black Feathered chicken and laid green-shelled eggs, BF-gsc). Meanwhile, we also explored the divergences in muscle characteristics of Taihe silky fowl fed with two different diets, that is normal chicken feed (TBsf-ncf) and *Broussonetia papyrifera*-fermented feed (TBsf-bpf). Firstly, the growth performance and biochemical index of Taihe silky fowl was significantly different compared with black-feathered chicken. Secondly, we identified the metabolic alterations in Taihe silky fowl by performing an un-targeted UHPLC-Q-TOF-MS/MS analysis. Our results suggested that the whole metabonomic characteristics had obvious separation between TBsf-ncf, TBsf-bpf and BF-gsc groups both in the positive and negative ion mode by PCA analysis. Next, OPLS-DA multivariate analysis revealed that 57 metabolites (in positive mode) and 49 metabolites (in negative mode) were identified as differential metabolites between TBsf-ncf and BF-gsc group. These differential metabolites were mainly enriched to ABC transporters, biosynthesis of amino acids and aminoacyl-tRNA biosynthesis. Besides, there were 47 metabolites (in positive) and 13 metabolites (in negative) were differentially regulated between TBsf-ncf and TBsf-bpf group, which were majorly involved in histidine metabolism and linoleic metabolism. Furthermore, the integrated network analysis suggested that DL-arginine, DL-isoleucine, linoleoylcarnitine, stearoylcarnitine (positive) and ricionleic acid, D-proline, uric acid (negative) were the significantly metabolic biomarkers in Taihe silky fowl. Moreover, the metabolites of primaquine, ticlpoidine, riboflavin, acetylcarnitine (positive) and salicylic acid, acetaminophen sulfate, glutamic acid (negative) were markedly changed in the Taihe silky fowl fed with BP-fermented feed. In summary, a global survey of the nutritional components and metabolite differences were performed in muscle tissues of Taihe silky fowl between various breeds and feeds. The comprehensive expression profiles of the metabolites in Taihe silky fowl affected by genetic and environmental factors were acquired. This study provided valuable evidence to breed and feed-induced putative biomarkers as well as improved the economic value of Taihe silky fowl through targeted metabolite regulation.

Keywords: Taihe silky fowl; metabolic components; un-targeted metabolome; breed and feed; biosynthesis of amino acids

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1. Introduction

Black-bone silky fowl (*Gallus gallus domesticus Brisson*), is a chicken originating from Taihe County, east of Wushan Mountain in Jiangxi Province of China, which has been raised for more than 2000 years (Eda, 2021; Zhang et al., 2016). The distinct features of Taihe black-bone silky fowl (TBsf) is the snow-white feathers but presence of melanin in various organs such as skin, meat and bones when compared with the other common chickens (Mi et al., 2018; Tu et al., 2009). TBsf has the highly nutritive value and pharmaceutical action which is known as the marvel of traditional Chinese medicine for various aliments (Kriangwanich et al., 2021; Zhu et al., 2014). According to the previous reports, TBsf has certain medicinal value to cure headache, hepatitis, asthma and other heart diseases (Jian et al., 2021; Liu et al., 2013). At the molecular level, TBsf contains the higher levels of carnosine in the mixed meat and breast meat than other chickens (O'Neill et al., 1999; Tian et al., 2007). Besides, natural melanin is considered as one of the most important components in TBsf, which has wide range of biochemical activities such as anti-oxidation, free radical-scavenging and immunomodulatory effects (Chen et al., 2008; Nganvongpanit et al., 2020). However, the overall metabolite profile of Chinese Taihe black-bone silky fowl and its metabolic differences compared with other chickens has not yet been fully investigated up to now.

In order to fully utilize the economic value of black-bone silky fowl, previous studies have conducted many explorations from the perspective of genetics and molecular biology (Guo et al., 2017). For example, whole genome resequencing revealed that the EDN3 gene might interact with the ncRNA to generate melanin in Xichuan black-bone chicken (Li et al., 2020). The Chinese indigenous chickens had great phenotypic variation and possessed more genetic diversity than that reported in many other countries (Ling et al., 2011; Qu et al., 2006). In addition, environmental factors such as nutrition and stress may also have important role in modifying muscle traits (Zampiga et al., 2021; Zhang et al., 2020). For example, feeding chickens with the olive oil-supplemented diet could increase the expression of avian uncoupling protein in chicken muscle (Mujahid et al., 2009). Meanwhile, *Broussonetia papyrifera* (BP) belongs to the family of Moraceae, which is usually used as high-quality feed ingredients for livestock animals due to the high nutritional value, content of crude protein, lysine and methionine (Si et al., 2018; Sun et al., 2012). Addition of BP-fermented feed could significantly improve growth performance and meat quality in sheep (Su et al., 2020). BP-fermented feed can significantly increase the WPS-2 and actinobacteria, and result in changes in the intestinal microbes of laying hens (Zhu et al., 2022). From all above studies, it is suggested that different breeds and feeds may lead to differences in muscle nutrients of chickens, but the change characteristics of these metabolites affected by breed and feed in Taihe black-bone silky fowl have largely unknown.

Metabolomics is the comprehensive study and describes the whole of endogenous metabolites in various organisms or tissues, which is widely used in a variety of fields including biomedicine and food science to identify novel metabolic biomarkers (Fu et al., 2019; Gika et al., 2014). Metabolome and other "omics" such as proteomics, transcriptomics and genomics, which frequently represents the global assessment of metabolite level in a biological sample (Frueh and Burczynski, 2021; Yizhak et al., 2010). Metabolome can be divided into non-targeted and targeted metabolome, and non-targeted metabolome is generally based on liquid chromatography (LC) and mass spectrometry (MS) technology to conduct qualitative and quantitative analysis of metabolites in the samples at the same time (Bajoub et al., 2016; Naz et al., 2014). Non-targeted metabolomics can reflect the overall metabolite levels and have been widely used to reveal the dynamic characteristics of metabolites under different conditions (Mizuno et al., 2017; Ribbenstedt et al., 2018). At present, UHPLC-Q-TOF-MS/MS technique has been widely used to determine the levels of metabolites in animals (Chen et al., 2019; Yin et al., 2019). Several previous studies have been reported concerning on the metabolite changes in chicken eggs by using un-targeted LC-MS analysis (Chang et al., 2021; Goto et al., 2019). However, the muscle metabolites of

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Taihe silky fowl influenced by the breed and feed traits have not been explored at the metabolome level.

Taihe black-bone silky fowl has many unique properties compared with other chickens. In this study, we systematically analyzed the metabolite differences in muscle tissues from three different breed and feed conditions (TBsf-ncf, TBsf-bpf and BF-gsc). We mainly used the UHPLC-Q-TOF-MS/MS technique to identify the differentially metabolites responsible for its unique phenotype in Taihe black-bone silky fowl. In addition, many metabolites were identified and related enriched signaling pathways were determined in Taihe silky fowl. For the first time, we demonstrated that both the genetic and environmental factors are critical to determining muscle composition in Taihe silky fowl, which should be considered in the efforts to meet consumer needs and develop nutritionally functional chickens in the near future.

2. Results

2.1. Growth performance and biochemical components of Chinese Taihe Silky Fowl

In order to investigate the effects of breed and feed on meat quality in Chinese Taihe silky fowl, we used three kinds of chickens: Taihe Black-bone silky fowl fed with normal chicken feed (TBsf-ncf), Taihe Black-bone silky fowl fed with *Broussonetia papyrifera*-fermented feed (TBsf-bpf) and Black-feathered chicken with green-shelled eggs (BF-gsc). The morphological characteristics, egg diversity and muscle color in each breed of chicken was shown in Fig. 1A. Firstly, we evaluated the growth and development status of each chicken and our results suggested that the body weight of Taihe silky fowl (TBsf-ncf and TBsf-bpf) was significantly lower than that of black-feathered chicken (BF-gsc) (Fig. 1B). Meanwhile, the egg weight of Taihe silky fowl was also greatly reduced compared with black-feathered chicken, but there was no significant difference between TBsf-ncf and TBsf-bpf (Fig. 1C). Furthermore, we further detected the biochemical parameters in muscle tissues of these chickens, the results revealed that the content of triglyceride (TG) of Taihe silky fowl was also sharply decreased than that of black-feathered chicken (Fig. 1D). Taken together, these results demonstrated that the growth performance and biochemical components of Taihe silky fowl were significantly different from black-feathered chicken.

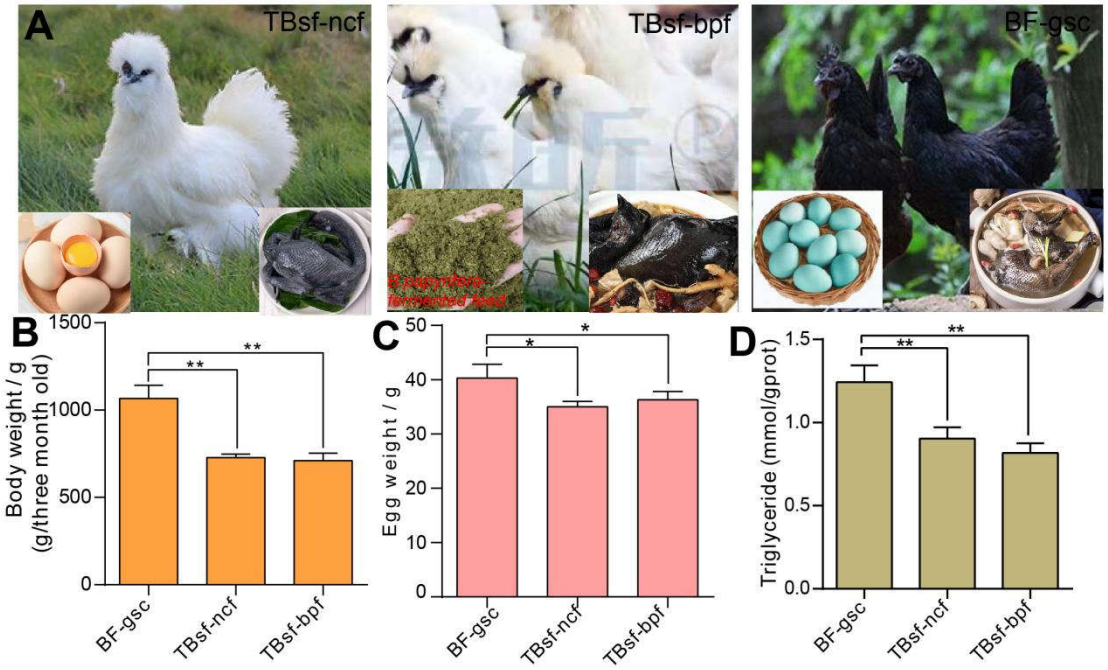

Figure 1. The growth performance and biochemical components of Taihe silky fowl was significantly different from Black-feathered chicken. (A) The morphological characteristics of each chicken in TBsf-ncf, TBsf-bpf and BF-gsc, respectively. The egg was shown in the lower left corner and meat quality was shown in the lower right corner, and the *Broussonetia papyrifera*-fermented feed was also

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shown in the TBsf-bpf group. (B) The body weight of three kinds of chickens at three month old was measured in each group (n=12). (C) The egg weight of three kinds of chickens was calculated in each group (n=12). (D) The contents of triglyceride (TG) in three kinds of chickens were detected in muscle tissues (four biological replicates per group). For all experiments, the value was represented as mean ± S.D. * $p < 0.05$, ** $p < 0.01$. Abbreviations: Taihe black-bone silky fowl fed with normal chicken feed (TBsf-ncf); Taihe black-bone silky fowl fed with *Broussonetia papyrifera*-fermented feed (TBsf-bpf); Black-feathered chicken and laid green-shelled eggs (BF-gsc).

2.2. Multivariate statistical analysis of the untargeted metabolomics data

For the non-targeted metabolomics analysis, the processed data with removing low-quality values were normalized by the log transformation and Pareto scaling. The molecular features extracted from all experimental samples and quality control (QC) samples were analyzed by the principal component analysis (PCA). Our results showed that three QC samples were tightly clustered in the PCA space for the electrospray ionization (ESI) both in the positive and negative mode, respectively (Fig. 2A and Fig. 2B). The consistently repeated QC injections indicated the excellent reliability and stability of the experimental procedure in metabolomics analysis. Moreover, in order to evaluate the global expression levels of muscle metabolites in three kinds of black-bone chicken samples, the unsupervised PCA method was applied to detect the degree of dispersion between the two groups of samples. The results demonstrated that there were significant differences compared with TBsf-ncf, TBsf-bpf and BF-gsc groups in the 2D PCA plots (Supplementary material Fig. S1). It is worth mentioning that the variation degree of four replicates samples in TBsf-bpf group was greater than that in TBsf-ncf group, which suggested that *Broussonetia papyrifera*-fermented feed may greatly affect the metabolite difference of muscle tissue in Taihe silky fowl.

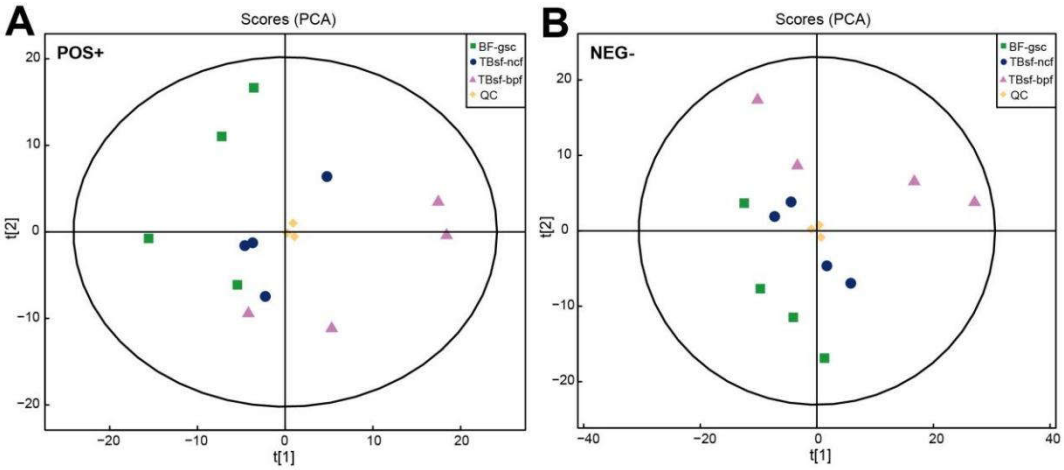

Figure 2. Quality assessment of UHPLC-Q-TOF-MS/MS metabolomic data in Taihe silky fowl. (A) The PCA scores of the chicken muscle samples in each group with the ESI positive ion mode. (B) The PCA scores of the chicken muscle samples in each group with the ESI negative ion mode**.** T[1] represents principal component 1 and T[2] represents principal component 2, which the aggregation degree of QC samples reflects the repeatability of the experimental data. Abbreviations: green dots indicate the BF-gsc samples, blue dots indicate the TBsf-ncf samples, purple dots indicate the TBsf-bpf samples and yellow dots indicate the QC samples.

Orthogonal partial least-squares discrimination analysis (OPLS-DA) maximizes the difference between groups in t [1], while the orthogonal principal component to [1] reflects the variation within the group, which has good sample classification and reliable predictive ability. As shown in Fig.3 A-B, there was a clear separation between TBsf-ncf group and BF-gsc group based on the OPLS-DA plot both in the positive ion mode (R^2Y=0.995; Q^2= 0.735) and negative ion mode (R^2Y=0.999, Q^2= 0.751). Similarly, there was a significant difference between TBsf-bpf group and BF-gsc group both in the positive ion mode (R^2Y=0.999; Q^2= 0.739) and negative ion mode (R^2Y=0.997, Q^2= 0.732) (Fig.3 C-D). Besides,

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OPLS-DA score maps between TBsf-ncf group and TBsf-bpf group were presented both in the positive ion mode (R^2Y=0.995; Q^2= 0.735) and negative ion mode (R^2Y=0.997, Q^2= 0.694), which suggested that different feeds affected the metabolite components in Taihe silky fowl (Fig.3 E-F). in sum, the above results indicated that there were obvious separation characteristics between Taihe silky fowl and black-feathered chicken, which further demonstrated that the metabolites of Taihe silky fowl exhibited a certain degree of alterations in muscle tissues compared with other chickens.

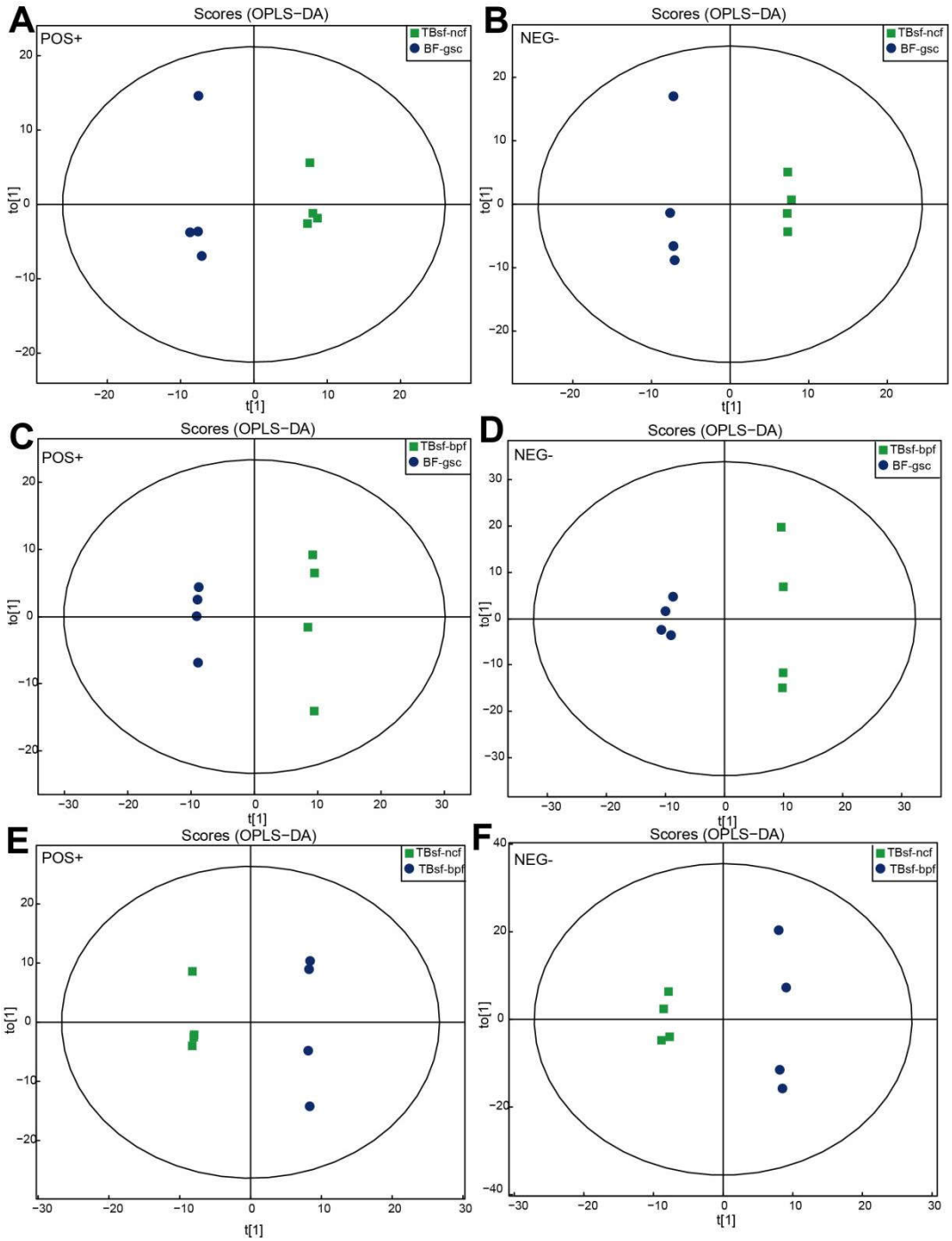

Figure 3. OPLS-DA plots derived from UHPLC-Q-TOF-MS/MS spectra inTaihe silky fowl. (A-B) The OPLS-DA score map between TBsf-ncf (green dots) and BF-gsc group (blue dots) both in the positive and negative ion mode, respectively. (C-D) The OPLS-DA score map between TBsf-bpf (green dots) and BF-gsc group (blue dots) both in the positive and negative ion mode, respectively. (E-F) The OPLS-DA score map between TBsf-ncf (green dots) and TBsf-bpf group (blue dots) both in the positive and negative ion mode, respectively.

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2.3. Identification of differential metabolites in muscle tissues of Taihe silky fowl

The metabolites in three kinds of chicken samples were identified by matching with the retention time, molecular weight, secondary fragmentation and other information in our local database. A total of 14,410 molecular features were extracted from HILIC column, in which 1, 022 metabolites were identified, including 533 in positive ion mode and 489 in negative ion mode (Fig. 4A). The detailed results of metabolite identification were shown in Supplemental Table S1. All metabolites identified in this experiment were classified and counted according to their chemical taxonomy, and the proportion of various metabolites was shown in Fig. 4B. Our results suggested that the top three main metabolite molecules in chicken muscle samples were lipids and lipid-like molecules (26.12%, 267), organic acids and derivatives (22.21%, 227) and organoheterocyclic compounds (12.82%, 131).

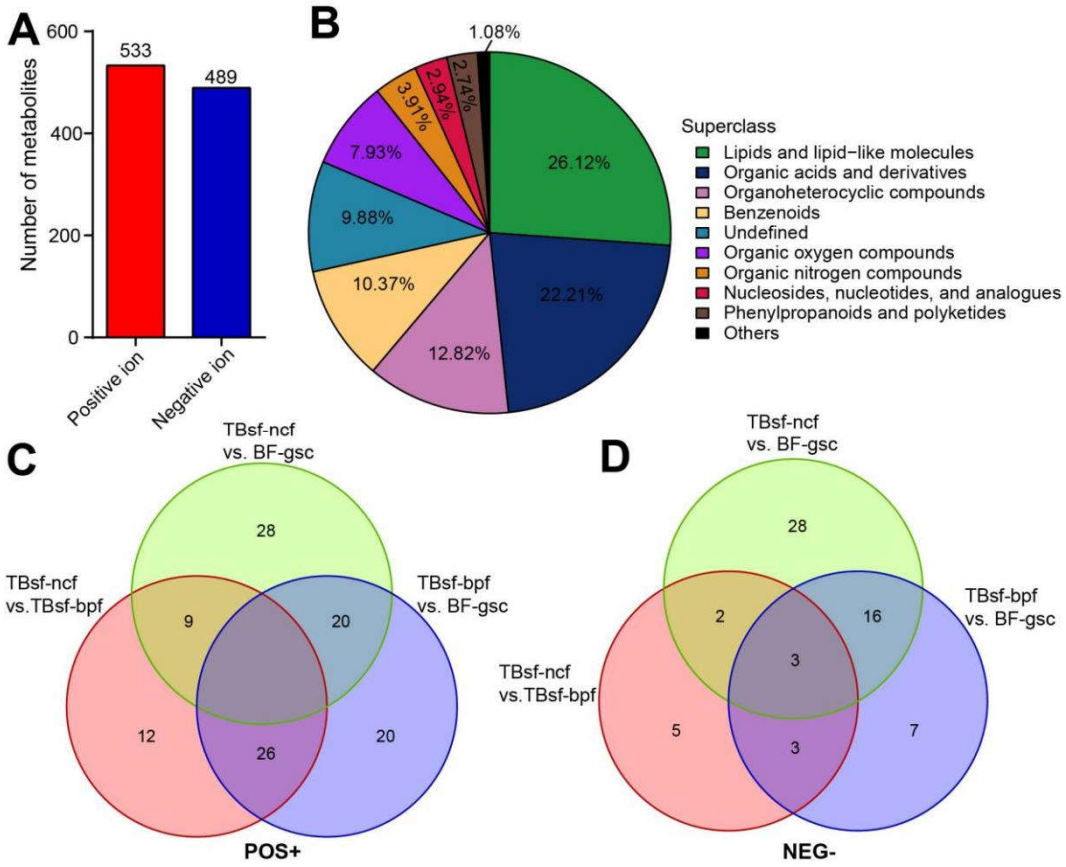

Figure 4. The differential metabolites were identified in Taihe silky fowl by metabolomic analysis. (A) The number of metabolites extracted from positive and negative ion peaks in HILIC column were presented. (B) The proportion of identified metabolites in each chemical classification. The specific chemical categories of each metabolite can be found in the legend, and the proportion of each superclass was presented in the corresponding pie chart. (C) Venn diagram showing the shared and unique differential metabolites between Taihe silky fowl and black-feathered chicken in positive ion mode. (D) Venn diagram showing the shared and unique differential metabolites between Taihe silky fowl and black-feathered chicken in negative ion mode. The overlapping regions represent metabolites that are concomitantly regulated in two or three samples.

The variables importance for the projection (VIP) obtained from OPLS-DA model can be used to filter metabolites with little change in each group, which could screen the differential metabolic molecules with biological significance. In this metabolomics analysis, we used the strict screening criteria (OPLS-DA VIP > 1 and *P* value < 0.05) as the metabolites with significant differences. Based on the statistical analysis and the VIP value in the OPLS-DA model, 57 metabolites (in positive mode) and 49 metabolites (in negative mode) were identified as significantly differential biomarkers in TBsf-ncf compared with BF-gsc

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group. Furthermore, 66 metabolites (positive) and 49 metabolites (negative) compared TBsf-bpf with BF-gsc group, and 47 metabolites (positive) and 13 metabolites (negative) compared TBsf-ncf with TBsf-bpf group were also identified. The detailed names and related information of these differential metabolites between the two groups were described in Supplemental Table S2. Besides, the relative expression level of differential metabolites were also displayed in the hierarchical clustering analysis, which suggested that the number of the up-regulated metabolites accounted for the majority in Taihe silky fowl than black-feathered chicken (Supplemental Fig. S2).

Next, the overlapping relationship of different metabolites in three kinds of chicken samples was further analyzed by Venn diagram. These results suggested that the differential metabolites in the positive ion mode had a higher overlapping proportion than that in the negative ion mode (Fig. 4C-D). There were 20 metabolites in positive ion mode and 16 metabolites in negative ion mode were shared between TBsf-ncf vs. BF-gsc and TBsf-bpf vs. BF-gsc, respectively. Interestingly, the number of common metabolites shared in all the three comparison groups was none in the positive and only 3 in the negative ion mode, which further indicating that Taihe silky fowl showed greater metabolites differences than black-feathered chicken in muscles.

2.4. Functional enrichment analysis of differential metabolites in Taihe silky fowl

To further evaluate the molecular function of these differential metabolites in Taihe silky fowl, all the differential components in each group were mapping into the KEGG database. KEGG enrichment analysis suggested that these differential metabolites were mainly enriched in the following metabolic pathways (TBsf-ncf vs. BF-gsc): ABC transporters, biosynthesis of amino acids aminoacyl-tRNA biosynthesis, valine & leucine and isoleucine biosynthesis, glycine & serine and threonine metabolism, pyrimidine metabolism (Fig. 5A). Bioinformatics analysis revealed that these abnormal metabolites were majorly involved in protein transmembrane transport, translation and amino acid metabolism.

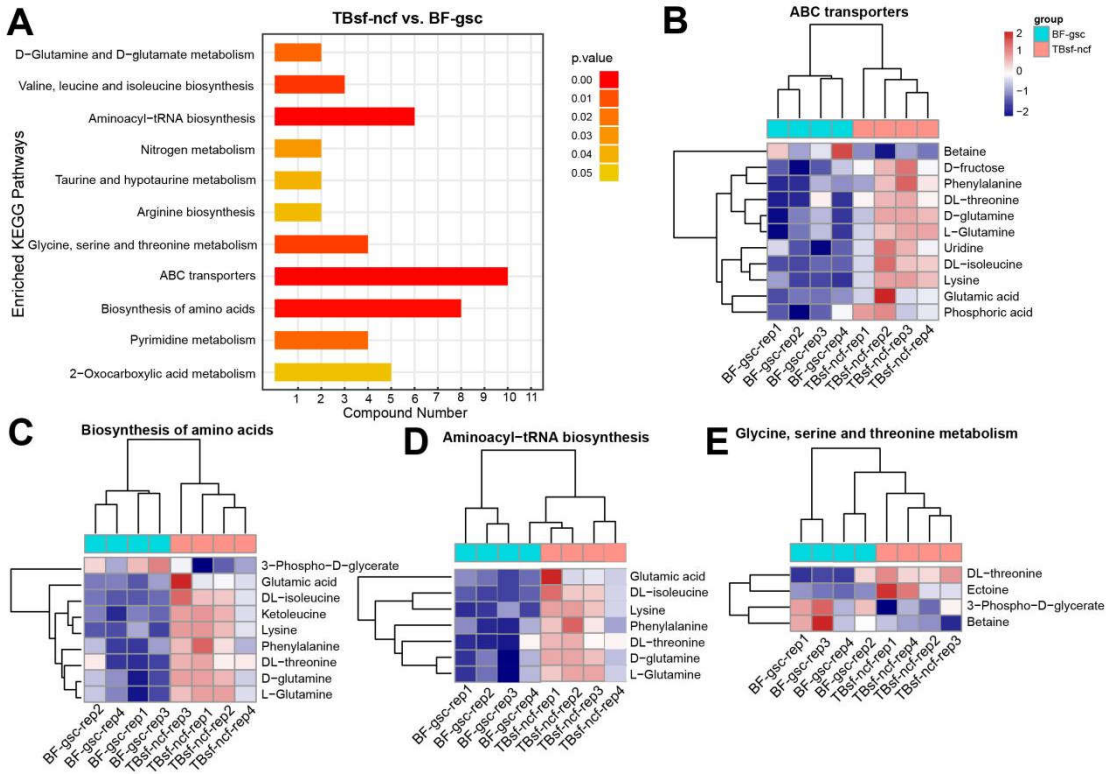

Figure 5. The KEGG enrichment analysis revealed the pathways of protein synthesis and transport were mainly activated in Taihe silky fowl. (A) The KEGG pathway enrichment analysis of differential metabolites in Taihe silky fowl compared with the black-feathered chicken. (B-E) Hierarchical

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clustering analysis of differential metabolites in ABC transporters, biosynthesis of amino acids, aminoacyl-tRNA biosynthesis, and glycine, serine, and threonine metabolism, respectively.

The hierarchical clustering analysis of these representative metabolites in ABC transporters were shown in Fig. 5B. These results suggested that most metabolite components such as (D- and L-) glutamine, uridine, glutamic acid, D-fructose, phenylalanine in ABC transporters were significantly up-regulated in TBsf-ncf groups. However, betaine is an amino acid that has potential benefits for helping promote muscle gain and fat loss, and the expression levels of which was significantly decreased in the TBsf-ncf samples. Amino acids are the precursors for the synthesis of many metabolites and our results revealed that the majority of metabolites in biosynthesis of amino acids were increased in the TBsf-ncf samples, which suggested that the ability of Taihe silky fowl to synthesize protein and other substances is stronger than black-feathered chicken (Fig. 5C). Similarly, aminoacyl-tRNAs are the substrates for translation and all the components in this pathway were activated in the TBsf-ncf group compared with the BF-gsc group (Fig. 5D). Meanwhile, threonine is an essential amino acid, which animals cannot synthesize. Here, we reported that the metabolites of DL-threonine and ectoine in glycine, serine and threonine catabolic pathway were up-regulated in muscle tissues while the metabolites of 3-phospho-D-glycerate were down-regulated in the TBsf-ncf group (Fig. 5E). In summary, these results further demonstrated that Taihe silky fowl had significant improvement in protein synthesis and amino acid transport compared with black-feathered chicken.

2.5. Integrated regulatory networks of differential metabolites in Taihe silky fowl

The interaction between differential metabolites can be shown by integrated regulatory network diagram using the Cytoscape software. Our results suggested that the substrates for protein synthesis such as DL-arginine and DL-isoleucine were significantly enriched in the positive mode in the TBsf-ncf samples compared with BF-gsc group (Fig. 6A). Besides, stearoylcarnitine is a fatty ester lipid molecule and acts as a metabolomics biomarker for preeclampsia, and stearoylcarnitine was also obviously enriched in the positive mode. On the other hand, ricionleic acid, proline, uric acid and 6-hydroxyhexadecanoic acid were significantly enriched in the negative ion mode in the Taihe silky fowl (Fig. 6B). These results further demonstrated that lipids and organic acids were greatly differential regulated in the muscle tissues of Taihe silky fowl.

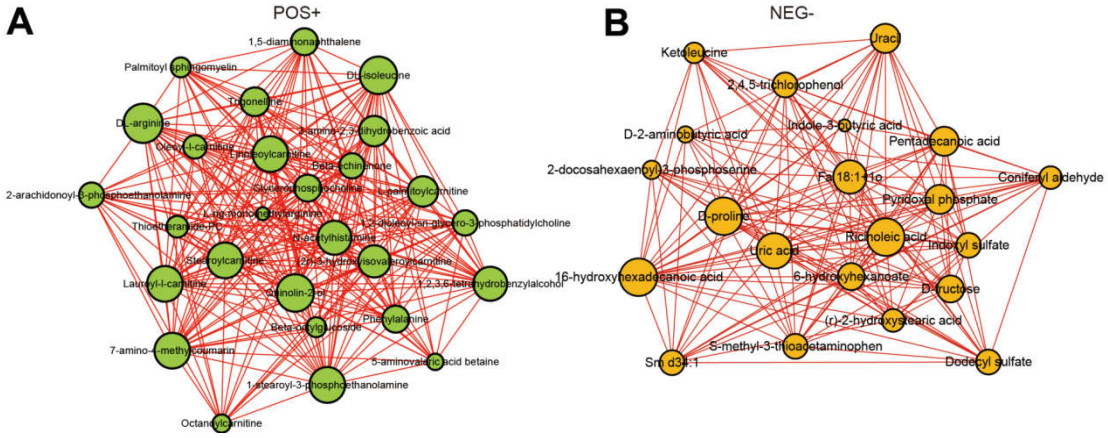

Figure 6. The integrated networks showed the correlation in various differential metabolites of Taihe silky fowl. (A) Network data integration and visualization of differential metabolites of Taihe silky fowl in the positive ion mode. (B) Network data integration and visualization of differential metabolites of Taihe silky fowl in the negative ion mode. The circle represents significant difference metabolites. The size of the circle is related to the degree of connectivity. The larger the degree, the larger the circle.

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2.6. BP-fermented feed induced the differential metabolites in Taihe silky fowl

Restrictive feeding influences systemic metabolism of nutrients in animals, while this effect has not been evaluated in chickens. Therefore, we investigated the effect of *BP-fermented* feed (TBsf-bpf group) compared with normal chicken feed (TBsf-ncf group) in Taihe silky fowl. KEGG enrichment analysis suggested that histidine metabolism, linoleic acid metabolism and beta-alanine metabolism were significantly enriched in TBsf-bpf sample compared with the TBsf-ncf sample (Fig. 7A). Moreover, the cluster analysis revealed that linoleic acid, histidine, 2,4,5-trichlorophenol were significantly down-regulated while glutamic acid, L-pyroglutamic acid and brassicasterol were significantly up-regulated in the TBsf-bpf group (Fig. 7B). Besides, functional network analysis suggested that primaquine, riboflavin, acetylcamitine and ticlpoidine were significantly enriched in the positive ion mode (Fig. 7C). Meanwhile, salicylic acid, acetaminophen sulfate, and 3-phosphoethanolamine were significantly enriched in the negative ion mode (Fig. 7D). From above these results, it is suggested that *Broussonetia papyrifera-fermented* feed can significantly change the metabolite composition in Taihe silky fowl.

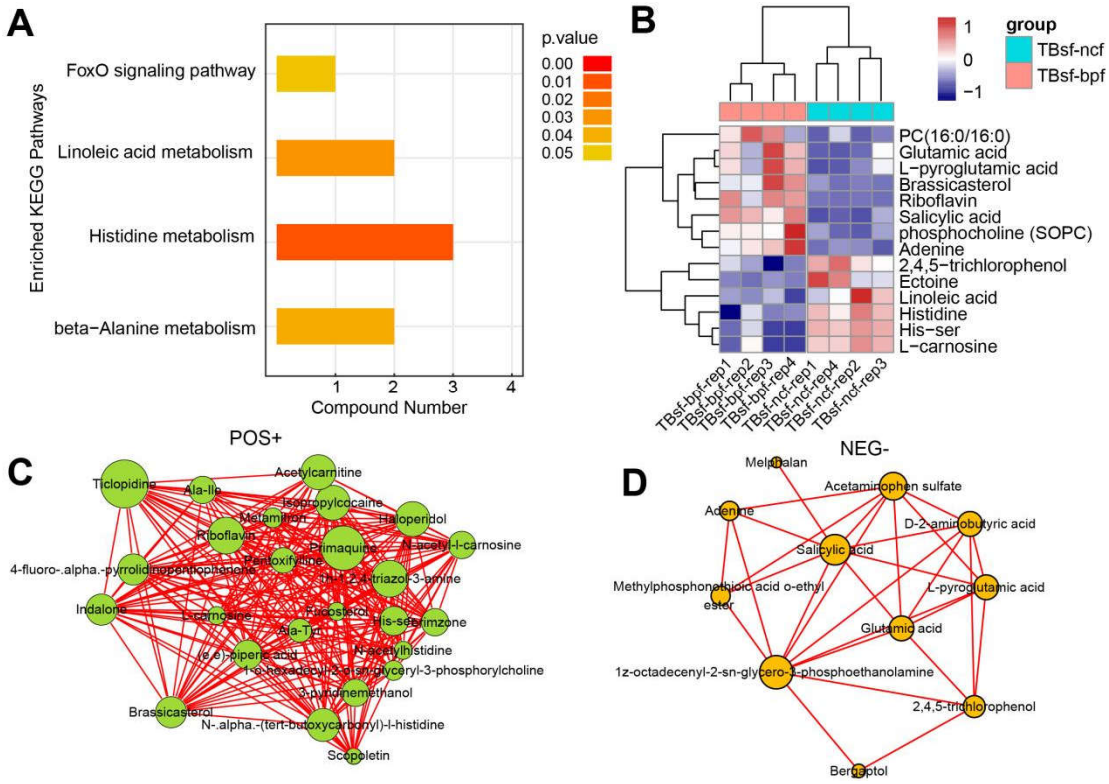

Figure 7. The functional analysis of differential metabolites in Taihe silky fowl fed by BP-fermented feed. (A) The KEGG pathway enrichment analysis of differential metabolites in Taihe silky fowl fed with *Broussonetia papyrifera*-fermented feed compared with normal chicken feed. (B) The hierarchical clustering analysis of differential metabolites between TBsf-ncf and TBsf-bpf groups. (C) The integrated networks of enriched differential metabolites in the positive ion mode between TBsf-ncf and TBsf-bpf groups.. (D) The integrated networks of enriched differential metabolites in the negative ion mode between TBsf-ncf and TBsf-bpf groups.

3. Conclusions

In this study, we explored the breed and feed effects on the nutritional components in Chinese Taihe silky fowl. Our studies found that Taihe silky fowl had significant differences in protein synthesis and amino acid transport compared with black-feathered chicken. In addition, different diets may also significantly change the muscle composition in Taihe silky fowl. In summary, these metabolomics data provides the basic information of muscle metabolites in Taihe black-bone silky fowl, which is helpful to improve the potential economic value of Taihe silky fowl in the future.

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4. Discussion

Taihe silky fowl is a National Protection of Geographical Indications that received numerous international prizes, which has many nutritional and medicinal benefits (Wang, 2014; Xiong et al., 2015). This study was aimed to investigate the effects of breed and feed on two kinds of Taihe silky fowl and one kind of black-feathered chicken. Our results have showed that there were significant difference such as body weight, egg weight and eggshell color between Taihe silky fowl and black-feathered chicken. Meanwhile, the contents of triglyceride in Taihe silky fowl (TBsf-ncf and TBsf-bpf) were also significantly lower than that in black-feathered chicken. Interesting, there was no significant difference in growth and development characteristics in Taihe black chicken fed with normal feed and BP-fermented feed, respectively. Previous studies have suggested that BP-fermented feed had no significant impact on improved egg production and egg quality (Zhu et al., 2022). In addition, other studies have showed that *Broussonetia papyrifera* fermented feed could decrease the mRNA expression levels of pro-inflammatory cytokines, affect the intestinal antioxidant capacity and microbiota in grass carp (Tang et al., 2021). Therefore, whether genetic and environmental factors including different feed conditions, can change the muscle quality of Taihe black chicken is a hot issue worthy of our study.

With the development of high-throughput sequencing technologies, high-resolution mass spectrometry plays an important role in metabolite detection due to its higher resolution and sensitivity (Liang et al., 2018). Additionally, the rapid UHPLC-Q-TOF-MS/MS metabolome technique was successfully employed to elaborate multiple metabolites and metabolic profile in Taihe silky fowl. PCA method can reflect the overall distribution of metabolic profiles and variability between and within the sample groups (Kayano and Kida, 2015). Our results suggested despite the four biological repeats in each group are relatively discrete, they are basically clustered in one region in the PCA plots. OPLS-DA is a supervised statistical method that establishes the relationship model between metabolite expression and classification of samples, which can remove the irrelevant variations contained within the mass spectrum and improve effectiveness of the model (Qiu et al., 2009; Santos et al., 2018). Thus, OPLS-DA was conducted to confirm the separation and identify the potential biomarkers between Taihe silky fowl and black-feathered chicken. Our results suggested that the metabolites were significantly regulated by both genetic and environmental factors.

Metabolomics analysis identified 57 differential metabolites in the positive mode and 49 differential metabolites in the negative mode between TBsf-ncf and BF-gsc groups. These differential metabolites were majorly related to protein anabolism and fatty acid oxidation. The KEGG pathway analysis revealed that the ABC transporters, biosynthesis of amino acids and aminoacyl-tRNA biosynthesis were significantly enriched in the Taihe silky fowl compared with black-feathered chicken. Meanwhile, the majority of substrates in protein synthesis such as glutamic acid, threonine and glutamine were significantly upregulated in the TBsf-ncf group. Betaine is a nutrient contained in many foods and can prevent fatty liver, cancer and hypertension, however, the expression levels of which was clearly decreased in the Taihe silky fowl. On the other hand, 47 metabolites (positive) and 13 metabolites (negative) were identified as differentially expressed between in the TBsf-bpf and TBsf-ncf group. Functional analysis suggested that histidine metabolism and linoleic acid metabolism were significantly enriched in TBsf-bpf compared with the TBsf-ncf group. Linoleic acid is a long-chain polyunsaturated fatty acid (PUFA) and an essential nutrient in wide range of animals including chickens, which plays an important role in immunity and reproduction (Fritsche, 2014; Malcicka et al., 2018). Our results revealed that the levels of linoleic acid in BP-fermented feed chickens were significantly higher than that in Taihe silky fowl fed by normal diets. These results further demonstrated that different genetics and diets may alter muscle metabolite status in Taihe silky fowl.

The metabolic proxies between significantly different metabolites and the regulatory relationships between metabolites in the biological process can be evaluated by correlation analysis (El-Hawary et al., 2021; George et al., 2014). In this study, we found that

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many amino acids were significantly enriched in the positive mode and some lipid molecules were significantly enriched in the Taihe silky fowl. Meanwhile, BP-fermented feed can significantly induce the change of primaquine and acetylcamitine (in positive) and salicylic acid and acetaminophen (in negative) in Taihe silky fowl. In the modern chicken industry, hybrid chickens rather than pure breeds are often used for meat production. This study indicates that the combination of breed and feed should be considered to modulate metabolite levels in muscles. In conclusion, we found significant effects of breed and feed on body performance and metabolite components in the Taihe silky fowl. We first demonstrated that both genetic and environmental factors were critical to determining muscle composition, which should be considered to meet consumer needs and develop nutritional and functional chickens.

5. Materials and methods

5.1. Laboratory animals and growth performance analysis

In this experiment, the chickens of TBsf-ncf and TBsf-bpf were obtained from Wangbeitu Taihe silky fowl Development Co., Ltd. and Aoxin Taihe silky fowl Development Co., Ltd, respectively. Besides, the chickens of BF-gsc were acquired from Ji'an local farm. They were introduced into the experimental farm of the institute of modern agricultural development in Jinggangshan University at 12 weeks of age. After introduction, all chickens were reared in individual cages under a photoperiod cycle of 16 h L/ 8h D, which could free access to diet and water. Approximately at 24 weeks of age, the growth indexes including body weight and egg weight were measured and calculated in each group, respectively. After that, all chickens were immediately killed and the chest muscles were cut down and used for subsequent analysis. Meanwhile, the biochemical parameters such as the contents of triglyceride were measured using the corresponding detection kit according to the instructions of the manufacturer (CAS No. F001-1-1, Nanjing Jiancheng Bioengineering Institute, China). Furthermore, the animal management and experimental procedure were strictly carried out following the guidelines approved by the Animal Welfare Committee of Jinggangshan University.

5.2. Collection and preparation of chicken samples

To investigate the effects of breed and feed on metabolome, we compared the metabolites differences in two breeds (Taihe silky fowl and Black feather chicken) and two feeds (Normal chicken feed and BP-fermented feed). In order to identify the nutritional components in different breeds of chickens, we collected TBsf-ncf (n=4) chickens, TBsf-bpf (n=4) chickens and BF-gsc (n=4) chickens and chest muscles were randomly sheared in each group. After that, the samples were frozen by liquid nitrogen and kept at −80°C until for further analysis.

Next, the chicken samples were slowly thawed on ice. And about 50 mg muscle tissues was homogenated with three volumes of cold methanol/acetonitrile/H_2O (2:2:1) to precipitate proteins. The mixtures were adequately vortexed and subjected to ultrasound for 30min, and incubated at −20 °C for 10 min. After that, the samples were centrifuged and re-dissolved in 100 μL of acetonitrile/water (1:1, v/v) for mass spectrometry loading analysis. On the other hand, the pooled QC sample was also used for control analysis. We employed QC samples to evaluate the system stability and performance before sample loading and during the whole experimental process.

5.3. UHPLC-Q-TOF-MS /MS analysis

The metabolites of chicken samples in each group were separated by using UHPLC system with HILIC column, which is consisted of solvent A (water + 25 mM ammonium acetate + 25 mM ammonia) and solvent B (acetonitrile). The gradient elution began at 95% B from 0-0.5 min, and B linearly decreased to 65% from 0.5-7min, and B linearly reduced to 40% from 7-8 min, and B maintained at 40% from 8-9 min. Next, B linearly rised from 40% to 95% at 9-9.1 min, and B maintained at 95% from 9.1-12 min. During the whole

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process, the samples were placed at the 4℃ automatic sampler. Meanwhile, the QC samples were used to evaluate the reliability of experimental data.

The mass spectrometry conditions of Q-TOF were as follows: the samples were analyzed by the AB Triple TOF 6600 system (AB SCIEX, MA, USA), and positive and negative ion mode were detected by the electrospray ionization (ESI). The setting parameters of ESI source were the following: auxiliary heating gas 1 (gas1), 60 psi; auxiliary heating gas 2 (gas2), 60 psi; Collision energy (CE) was set at 35±15 eV and the declustering potential (DP) was set at ±60 V. The secondary mass spectrum was obtained by information-dependent acquisition (IDA) mode and screened by peak intensity value.

5.4. Quality assessment and differential metabolites identification

The raw data with an instrument specific format (.wiff) were converted into the standard format (.mzXML) by ProteoWizard MS converter tool (Chambers et al., 2012). Next, peak alignment and area extraction were performed by using XCMS online tools (Domingo-Almenara and Siuzdak, 2020). After that, structural identification of metabolite was performed based on the accuracy of m/z value (<10 ppm) and MS/MS data that were matched to our self-built database. Furthermore, MetaboAnalyst 5.0 was employed for further statistical analysis (Pang et al., 2021).

After normalization, the processed data wad used for multivariate analysis such as PCA and OPLS-DA. The evaluation parameters of the model (R^2Y, Q^2) were obtained through 7-fold cross-validation, as well as the permutation test was used to test the effectiveness of the model. The variable importance in the projection (VIP) value in the OPLS-DA model and Student's *t*-test were applied to evaluate the significance of differential metabolites. We defined the metabolites with statistically significant difference was the VIP value >1 and *P* value <0.05.

5.5. Functional enrichment and correlation analysis of differential metabolites

Based on the differential metabolites identified in various comparing conditions, KEGG pathway analysis was conducted to investigate the putative metabolomics pathways affected by the effects of breed and feed in Taihe silky fowl. Hierarchical clustering of differential metabolites in mainly enriched pathways was performed by using heatmap.2 function within gplots package in R environment. Pearson's correlation coefficient was used to evaluate the association between breed and feed in differential metabolites. A correlation coefficient $|r| \geq 0.8$ and $p \leq 0.05$ was considered to reflect a high correlation. To make a visual representation, the metabolites were selected and constructed the regulatory enrichment network by using Cytoscape software.

5.6. Statistical analysis

SPSS v.20.0 (Chicago, IL, USA) and GraphPad v.7.0 were used for all statistical analysis and figure drawing, respectively. An independent sample T-test or one-way analysis of variance (ANOVA) followed by a Dunnett's post-hoc test was used to evaluate the mean differences between taihe silky fowl and black feather chickens. All the experiments contained at least three biological repeats and the results were expressed as means ± standard deviation (SD). Statistical significance was considered as $^*P < 0.05$ and $^{**}P < 0.01$.

Author Contributions: G. X. and X. L. designed the experiments; G.X., J.W.,F. L. and X.L. executed the experiments; G.X. and H.L. analyzed the data; G. X. and X. L. prepared the manuscript.

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