



Hydroxytyrosol Benefits Boar Semen Quality via Improving Gut Microbiota and Blood Metabolome

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Semen quality is one of the most important factors for the success of artificial insemination which has been widely applied in swine industry to take the advantages of the superior genetic background and higher fertility capability of boars. Hydroxytyrosol (HT), a polyphenol, has attracted broad interest due to its strong antioxidant, anti-inflammatory, and antibacterial activities. Sperm plasma membrane contains a large proportion of polyunsaturated fatty acids which is easily impaired by oxidative stress and thus to diminish semen quality. In current investigation, we aimed to explore the effects of dietary supplementation of HT on boar semen quality and the underlying mechanisms. Dietary supplementation of HT tended to increase sperm motility and semen volume/ejaculation. And the follow-up 2 months (without HT, just basal diet), the semen volume was significantly more while the abnormal sperm was less in HT group than that in control group. HT increased the “beneficial microbes” *Bifidobacterium*, *Lactobacillus*, *Eubacterium*, *Intestinimonas*, *Coprococcus*, and *Butyricoccus*, however, decreased the relative abundance of “harmful microbes” *Streptococcus*, *Oscillibacter*, *Clostridium_sensu_stricto*, *Escherichia*, *Phascolarctobacterium*, and *Barnesiella*. Furthermore, HT increased plasma steroid hormones such as testosterone and its derivatives, and antioxidant molecules while decreased bile acids and the derivatives. All the data suggest that HT improves gut microbiota to benefit plasma metabolites then to enhance spermatogenesis and semen quality. HT may be used as dietary additive to enhance boar semen quality in swine industry.

Keywords: hydroxytyrosol, boar, semen quality, gut microbiota, blood metabolome

INTRODUCTION

Artificial insemination (AI) has been extensively used in swine industry in order to take the advantages of the superior genetic background and higher fertility capability of boars and sows (1–4). The application of AI is restricted to the semen quality, which can be influenced by multiple factors including the boar itself (e.g., age) and the environment factors (e.g., temperature and light) (1–4). Sperm plasma membrane contains a large proportion of polyunsaturated fatty acids (PUFA), which is easily impaired by oxidative stress and thus to diminish semen quality (1, 2). Increasing evidence suggests the potential to use nutritional interventions to enhance antioxidant capacity for

improving boar semen quality (3, 4). *In vitro* studies found that supplementation with exogenous antioxidants, such as rosmarinic acid, polysaccharide, and skim milk could improve boar sperm quality by alleviating oxidative stress during the cryopreservation (5–7). Moreover, a lot of evidence from *in vivo* studies also illustrated that dietary supplementation with antioxidants improved the boar semen quality. For example, dietary supplementation of L-arginine and lysine improved boar semen quality by increasing antioxidant capacity (3, 4).

Hydroxytyrosol (HT), a polyphenol, is extracted from olive leaves and oil. HT has attracted broad interest due to its strong antioxidant, anti-inflammatory, and antibacterial activities (8, 9). HT exhibited antioxidant capacity by scavenging oxidant chemical species and promoting the expressions of antioxidant enzymes via activating nuclear factor E2-related factor 2 (Nrf2) signaling (8). Furthermore, Wei et al. reported that HT increased bacterial membrane permeabilization and then interacted with DNA, which collectively inhibited bacterial growth (10). Our recent study also showed that olive fruit extracts enriching HT exhibited strong antioxidant effects by benefiting gut microbiota in mice (11). Moreover, our previous study found that improved gut microbiota was able to ameliorate sperm quality by modulating the plasma metabolomes and small intestinal function in mice (12, 13). To search the effective strategies to improve sperm quality and enhance the production of swine production, current investigation was designed to explore the effects of dietary supplementation of HT on the boar semen quality and the underlying mechanisms.

MATERIALS AND METHODS

Boars and Experimental Design

All animal procedures were approved by the Animal Care and Use Committee of the Institute of Animal Sciences of Chinese Academy of Agricultural Sciences (IAS2021-67). A total of 20 healthy boars (Duroc) aged from 31 to 33 months were selected in this study at the artificial insemination center of Yangxiang Joint Stock Company (Guangxi, China) (1). Boar feeding conditions have been previously reported (2). All boars were randomly divided into 2 groups ($n = 10$ per group). Boars in the control group (Con) were fed with a commercially prepared corn and soybean meal-based diet (Table 1), and boars in the HT supplement group (HT) were fed with a basal diet supplemented with 20 mg/kg body weight of HT. The dose of HT was adopted according to our previous mice experiment (11). The boars received two meals at 11:00 and 17:00 and the total feed intake amounts were 2.5 kg (1.25 kg per meal) every day. HT was mixed with the diet when the boars received the first meal and HT was taken almost completely by the boars each day. HT (purity > 99%) was kindly donated by Viablife Biotech Co., Ltd. (Hangzhou, China). The boars were housed in individual crates and HT was supplied for 60 days (Figure 1A).

Semen samples were collected by gloved-hand techniques. After collection, four semen parameters were assessed: semen volume, sperm concentration, sperm motility, and abnormal sperm rate, according to the reported methods (1, 14). Blood samples were collected into ethylenediamine tetraacetic acid

TABLE 1 | Composition and nutrient analysis of basal diet.

Ingredient	Content, %
Corn	35.15
Barley	24.83
wheat	15.82
Rice bran meal	9.40
Soybean meal	7.90
Soybean oil	2.00
L-lysine	0.40
Methionine	0.14
Threonine	0.24
Ground limestone	1.44
Monocalcium phosphate	1.21
Sodium chloride	0.48
Premix*	1.00
Total	100
Nutrient, %	
Calculated NE, kcal/kg	2.24
Crude protein, %	14.50
Crude fat, %	3.22
Crude ash, %	6.18
Crude fiber, %	4.15

*Premix provided the following minerals per kilogram: 17 mg Cu, 160 mg Fe, 140 mg Zn, 50 mg Mn, 0.50 mg I, 0.50 mg Se, and 0.22 mg Cr.

(EDTA) plasma tubes by venipuncture from the hindlimb vein of boars during ejaculations. Each blood sample was then centrifuged at $3,000 \times g$ for 10 min at 4°C to obtain a plasma sample and subsequently stored at -80°C until analysis. Each boar's rectum was massaged to stimulate defecation, and then fresh feces were collected and stored at -80°C for subsequent microbiota analysis (1).

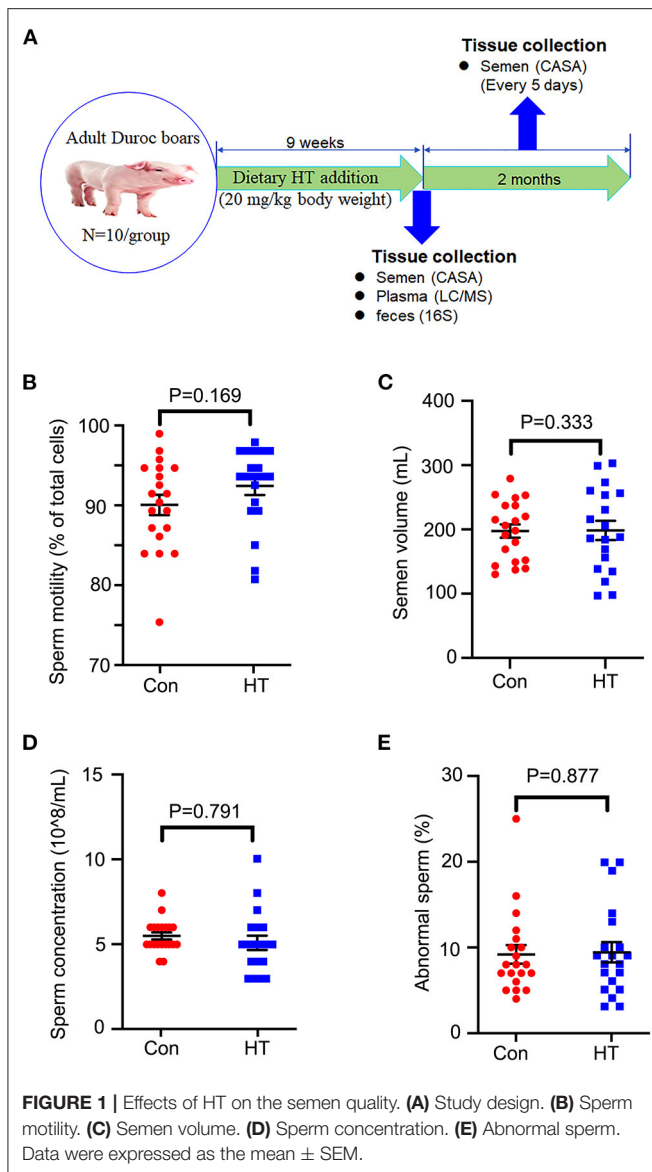
The follow-up long term beneficial effects of HT on boar semen quality were determined. After HT supplementation, all the boars were fed with basal diet (without HT supplementation). The semen was collected every 5 days and the semen quality was analyzed. The long-term analysis was for 2 months (Figure 1A).

Evaluation of Spermatozoa Motility Using a Computer-Assisted Sperm Analysis System

Spermatozoa motility and concentration, and abnormal sperm rate were determined by the computer-assisted sperm assay (CASA) method according to World Health Organization guidelines and our previous reports (ML-210JZ, Nanning SongJing TianLun Biological Technology Co., LTD, Nanning, China) (15–18).

Morphological Observations of Spermatozoa

Boar sperm was stained with Eosin Y (1%) (15–17). Spermatozoa abnormalities were then viewed using an optical microscope and were classified into head or tail morphological abnormalities: two



heads, two tails, blunt hooks, and short tails. The examinations were repeated three times, and 500 spermatozoa per animal were scored.

Boar Feces Microbiota Sequencing

Total genome DNA from feces was extracted using E.Z.N.A.R Stool DNA Kit (Omega Biotek Inc, USA). The V3-V4 region of the bacterial 16S rRNA gene was amplified using a specific primer (338F, 5'-ACTCCTACGGGAGGCAGCAG-3'; 806R, 5'-GGACTACHVGGGTWTCTAAT-3'). Then the library was sequenced on the Illumina HiSeq 2500 platform and 300 bp paired-end reads were generated at the Novo gene. The sequences were analyzed and assigned to operational taxonomic units (OTUs; 97% identity). OUT abundance information was normalized using a standard of sequence number corresponding to the sample with the least sequences (12, 13).

Plasma Metabolites Determined by LC-MS/MS

The metabolites were detected as reported in our early study (19). Briefly, boar plasma was collected and maintained at -80°C . LC-MS/MS analysis with ACQUITY UPLC and AB Sciex Triple TOF 5600 (LC/MS) was applied (19). Before LC-MS/MS analysis, the serum samples (100 μl) were precipitated with 10 μl methanol (0.3 mg/ml L-2-Chlorophe) on ice and processed to remove proteins. All samples were then centrifuged at 13,000 g for 10 min at 4°C and the supernatants were injected onto a ACQUITY UPLC HSS T3 column, which was at a flow rate of 0.35 ml/min with 2 μl mobile phase. MS analyses were conducted using electrospray ionization in the positive and negative ion models. Using full scan analysis. Progenesis QI v. 2.3 (Nonlinear Dynamics, Newcastle, UK) was applied to normalize the peaks. Human Metabolome Database (HMDB), Lipidmaps (v. 2.3), and METLIN software were applied to qualify the data. Furthermore, the data were analyzed with SIMCA software (v. 14.0, Umetrics, Umeå, Sweden) and KEGG database (<https://www.kegg.jp/>) was applied for the pathway enrichment analysis (19).

Detection of Protein Levels and Location in Spermatozoa Using Immunofluorescence Staining

Microscope slides loaded with boar sperm cells were incubated with 2% Triton X-100 for 1 h at room temperature. Then the samples were blocked for non-specific binding by incubating in 1% BSA with 1% goat serum for 30 min at room temperature. After washing, the cells were incubated with appropriate diluted primary antibodies (anti-Catsper, 1:100; anti-p-ERK, 1:100; anti-PKA, 1:100; anti-ZAG, 1:100) at 4°C overnight and then stained with secondary antibodies (Alexa Flour 546 goat-anti-rabbit IgG) at 1:200 dilution in PBS for 30 min at room temperature. Cell nuclei were counterstained with DAPI. All the samples were observed at room temperature using Leica Laser Scanning Confocal Microscope (LEICA TCS SP μ II, Germany) (17).

Determination of Protein Levels by Western Blotting

Proteins was isolated from sperm cells using RIPA buffer containing protease and phosphate inhibitors (Sangong Biotech, Ltd, Shanghai, China). Total protein concentration was measured using BCA kit (Beyotime Institute of Biotechnology, Shanghai, Chain) according to the manufacture's instruction. Fifty microgram protein was loaded onto 10% polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) PVDF membrane. The membranes were blocked for 1 h at room temperature in TBST buffer containing 5% non-fat milk. Then the membranes were incubated overnight at 4°C with the primary antibodies (Novex $^{\text{®}}$ by life technologies, USA) at a dilution of 1:500 prepares in blocking solution. The control samples were incubated with actin antibody. After washed 3 times, the membranes were incubated with secondary donkey anti-goat IgG-HRP (Beyotime Institute of Biotechnology, Shanghai, P.R. China) or goat anti-rabbit IgG-HRP (Novex $^{\text{®}}$ by

life technologies, USA) for 1 h at room temperature. finally, the blots were imaged following three washes (12, 13, 17).

Statistical Analysis

All statistical analyses were performed by using the student's *t*-test (SPSS 21 software). Spearman correlation analysis between the relative abundance of gut microbiota and plasma metabolites using GraphPad Prism 7.0. Data are expressed as the mean \pm SEM. *P*-value <0.05 was considered significant.

Data Availability

The microbiota raw sequencing data generated in this study has been uploaded to the NCBI SRA database with the accession number PRJNA779574.

RESULTS

Effects of HT on Semen Quality

The semen quality was analyzed by using the semen obtained from the last two collections before the end of the experiment.

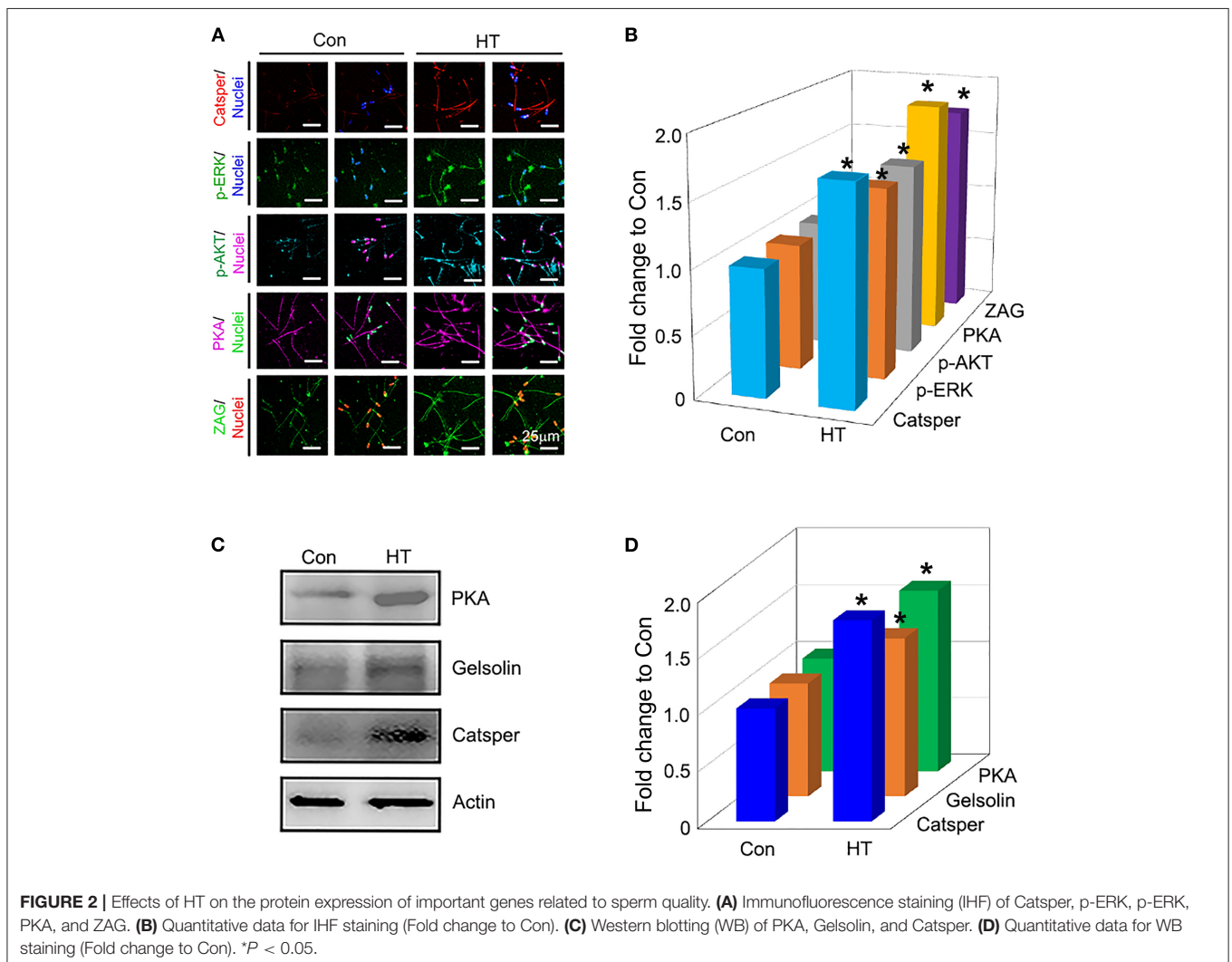
Compared to control group, dietary supplementation of HT tended to increase sperm motility and semen volume/ejaculation even though not significantly (Figures 1B,C). However, HT did not alter sperm concentration or abnormal rate (Figures 1D,E).

Effects of HT on the Protein Expression of Important Genes Related to Sperm Quality

Compared to control group, dietary supplementation of HT increased the protein levels of important genes related to sperm quality such as *Catsper*, *p-ERK*, *PKA*, *p-AKT*, and *ZAG* by IHF staining analysis (Figures 2A,B; *P* < 0.05). The data were confirmed by the WB analysis of *Catsper*, *Gelsolin*, and *PKA* (Figures 2C,D; *P* < 0.05) which suggested that HT improved sperm quality by enhancing the protein expression of important genes related to sperm quality.

Improvement of HT on Fecal Microbiota

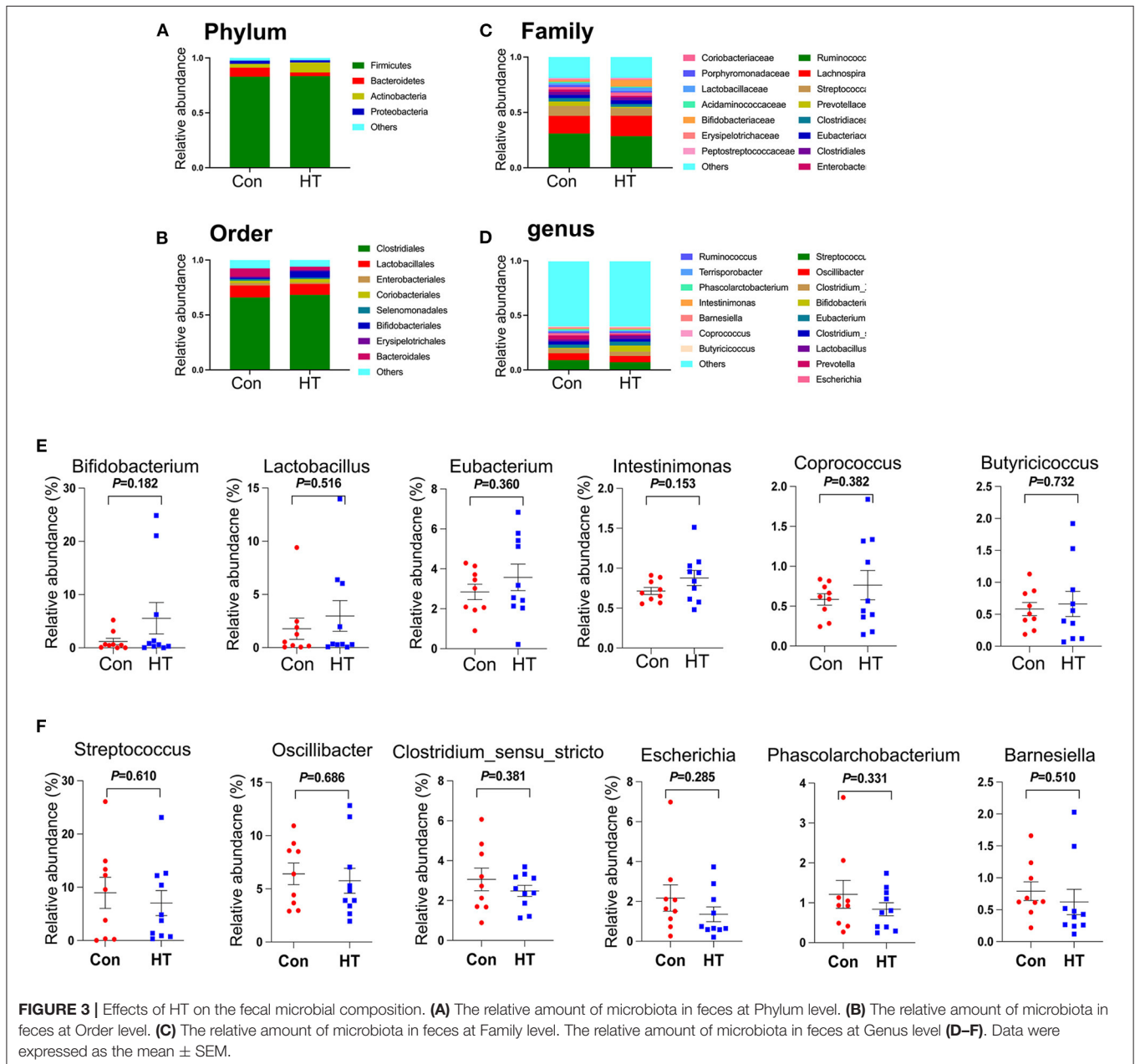
The results showed that dietary supplementation of HT had no significant effects on the α -diversity of fecal microbiota,



which was characterized by ACE, Chao1, Simpson, and Shannon indexes (**Supplementary Figure 1A**). There were 1,109 common OTUs for both Con and HT groups (**Supplementary Figure 1B**). Moreover, the Con group and HT group contained specific 93 and 77 OTUs, respectively (**Supplementary Figure 1B**).

The microbial structure was different between HT group and Con group by PCoA analysis (**Supplementary Figure 1C**). At the phylum level, HT tended to reduce the relative abundance of *Bacteroidetes* and to increase the relative abundance of *Actinobacteria* (**Figure 3A**; **Supplementary Figure 1E**). At the order level, HT tended to decrease the level of *Bacteroidales* and to increase the relative abundance of *Bifidobacteriales* (**Figure 3B**; **Supplementary Figure 1F**). Moreover, at the family

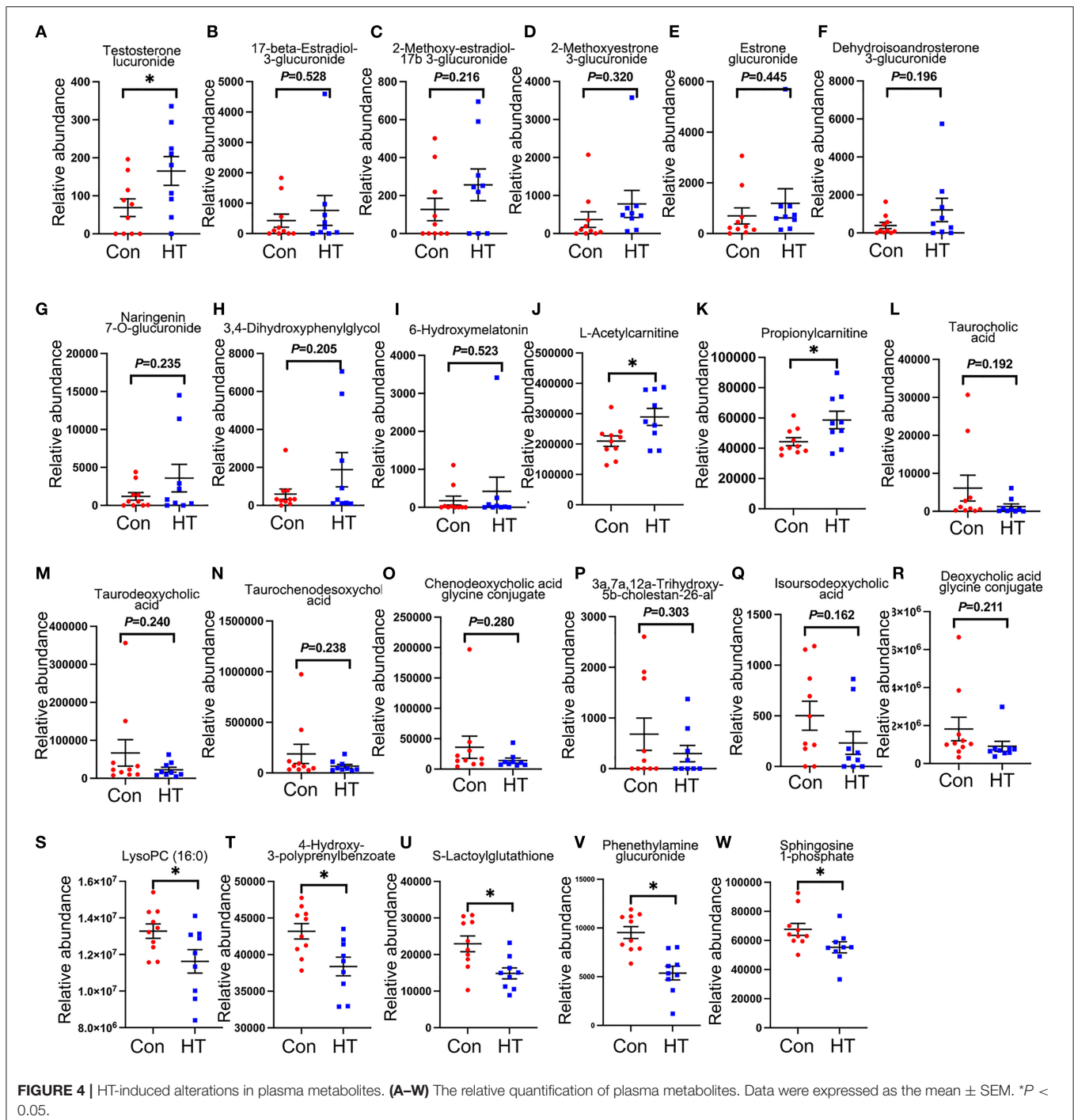
level, HT had the tendency to decrease the relative abundance of *Prevotellaceae* (**Figure 3C**; **Supplementary Figure 1G**). At the genus level, HT tended to increase the relative abundance of “beneficial microbes” *Bifidobacterium*, *Lactobacillus*, *Eubacterium*, *Intestinimonas*, *Coprococcus*, and *Butyricoccus* (**Figures 3D,E**; **Supplementary Figure 1H**), however, to decrease the relative abundance of “harmful microbes” *Streptococcus*, *Oscillibacter*, *Clostridium_sensu_stricto*, *Escherichia*, *Phascolarctobacterium*, and *Barnesiella* (**Figures 3D,F**; **Supplementary Figure 1H**) (12). LDA effect size (LEfSe) analysis of the taxonomic alterations revealed that the content of some microbiota was different between HT group and control group (**Supplementary Figure 1D**).



Effects of HT Supplement on the Plasma Metabolites

Compared to control group, HT significantly changed boar plasma metabolites indicated by the plot from the partial least squares discriminant analysis (PLS-DA) (**Supplementary Figure 2A**). There were 16 significantly changed metabolites between the HT and Con groups (**Supplementary Figure 2B**). The potential metabolic pathways of the changed metabolites were determined by KEGG

pathway analysis. The results showed that the changed metabolites were involved in sphingolipid metabolism, Fc gamma R-mediated phagocytosis, apelin signaling pathway, choline metabolism in cancer, calcium signaling pathway, phospholipase D signaling pathway, tuberculosis, sphingolipid signaling pathway, and insulin resistance signaling pathways (**Supplementary Figure 2C**). It is very interesting to notice that HT increased plasma level of steroid hormones and their derivatives (**Figures 4A–F**), especially testosterone



glucuronide (Figure 4A). And HT tended to increase the plasma levels of flavonoids: Naringenin 7-O-glucuronide, and 3,4-Dihydroxyphenylglycol (Figures 4G,H). Moreover, it is very interesting that melatonin metabolite 6-hydroxymelatonin, L-Acetylcarnitine and Propionylcarnitine were also elevated by HT in boar plasma although not significantly (Figures 4I–K). However, HT decreased plasma levels of bile acids: taurocholic, taurodeoxycholic acid, taurochenodesoxycholic acid, chenodeoxycholic acid glycine conjugate, 3a,7a,12a-Trihydroxy-5b-cholestan-26-al, isoursodeoxycholic acid, and deoxycholic acid glycine conjugate (Figures 4L–R). Furthermore, HT tended to decrease plasma LysoPC (16:0), 4-Hydroxy-3-polyphenylbenzoate, Phenethylamine glucuronide and Sphingosine 1-phosphate (Figures 4S–W).

Correlation of Fecal Microbiota, Plasma Metabolites, and/or Sperm Quality

There was very good correlation between fecal microbiota and plasma metabolites, between plasma metabolites and semen parameters, and between fecal microbiota and semen parameters (the average of semen quality of each boar obtained from the last two collections before the end of the experiment) by the spearman correlation analysis (Figure 5). As shown in Figure 5A, the fecal *Lactobacillus* and *Bifidobacterium* were positively correlated with the plasma naringenin 7-O-glucuronide; while *Streptococcus* and *Escherichia* were negatively correlated with the plasma 3,4-dihydroxyphenylglycol and naringenin 7-O-glucuronide; *Coprococcus* was negatively correlated with plasma taurocholic, taurodeoxycholic acid, taurochenodesoxycholic acid, chenodeoxycholic acid glycine conjugate. Plasma LysoPC (16:0), S-lactoylglutathione, phenethylamine glucuronide, 4-hydroxy-3-polyphenylbenzoate, and ethyl glucuronide were negatively correlated with sperm motility (Figure 5B); while plasma gamma-glutamyl leucine, lithocholate 3-O-glucuronide, estrone glucuronide, and gamma-glutamylleucine were positively correlated with sperm motility (Figure 5B). Moreover, there was a significantly positive association between the sperm motility and the fecal *Ruminococcus* and a significantly negative association between the sperm volume and the fecal *Streptococcus* and *Escherichia* (Figure 5C).

Long-Term Beneficial Effects of HT on Semen Quality

HT had a long-term beneficial effect on boar semen quality by the increase in the semen volume and the decrease in the percentage of abnormal sperm in the follow-up 2 months analysis (without HT supplementation) (Figures 6A,B). However, HT did not change sperm concentration and motility in the follow-up determination (Figures 6C,D).

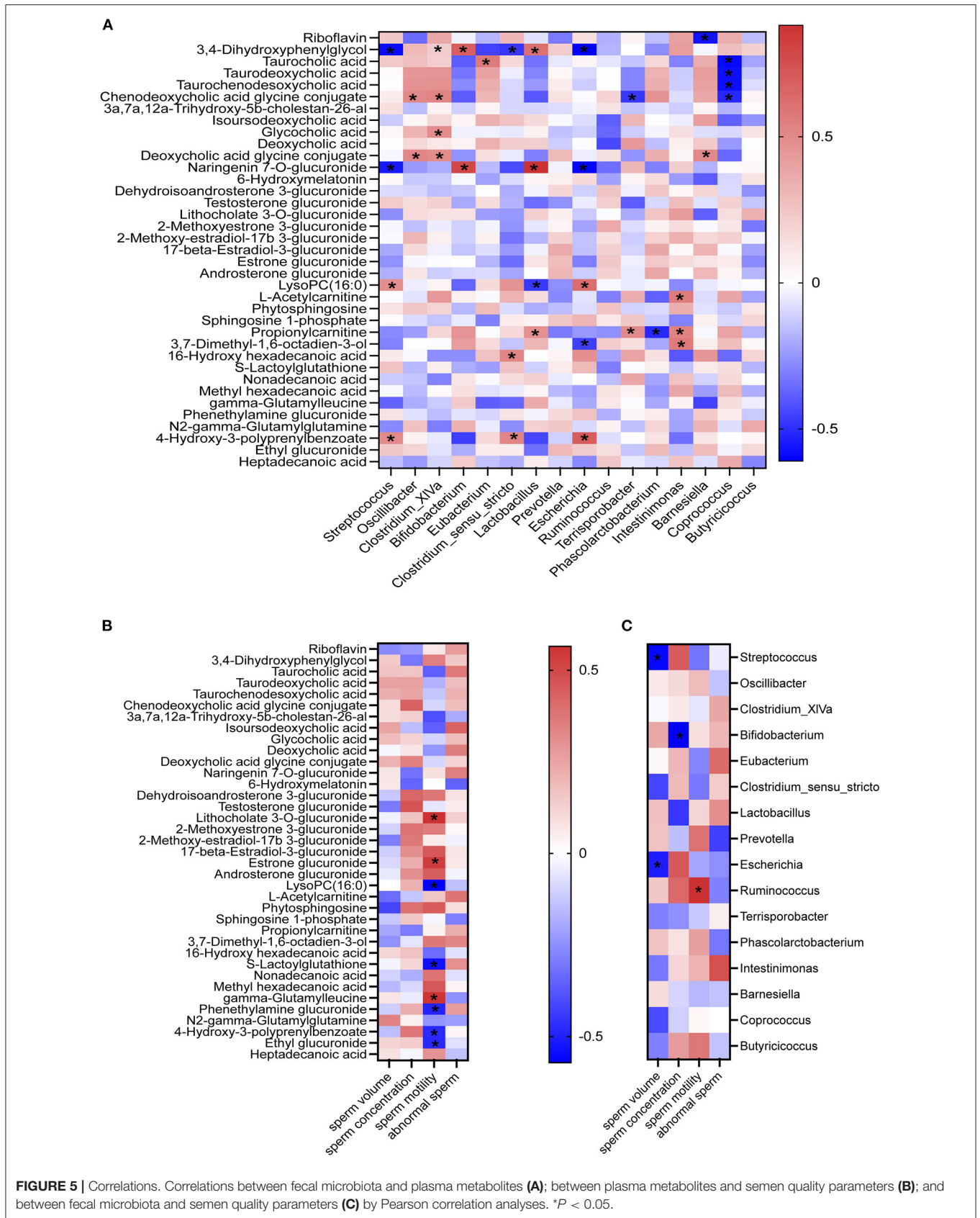
DISCUSSION

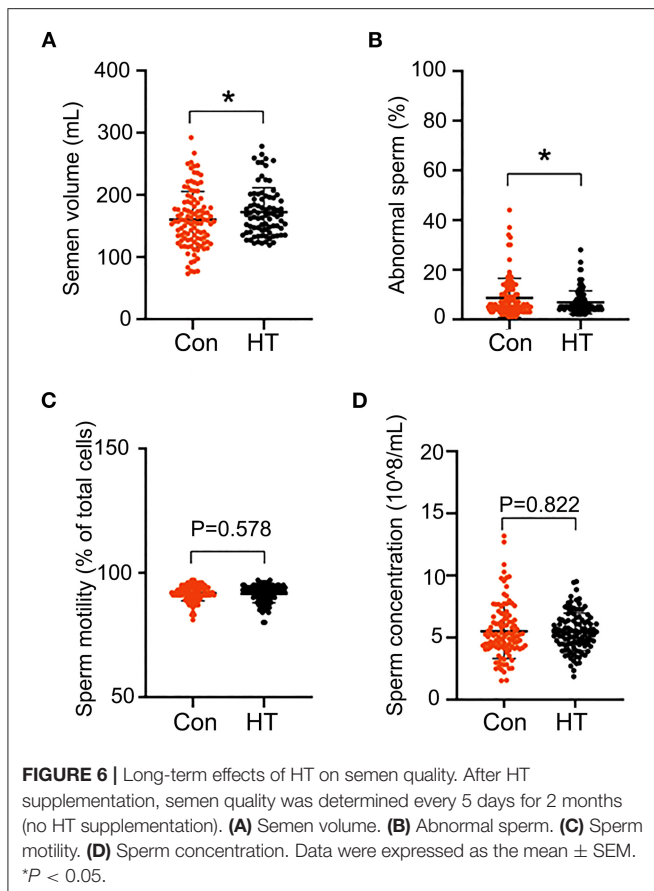
Previous studies have reported that dietary supplementation of antioxidants, such as lysine and L-arginine could improve sperm quality in boars (3, 4). Our recent study found that HT had antioxidant effects via modulating gut microbiota and enhancing

the expressions of antioxidant enzymes in mice (11). In current investigation, we found that dietary supplementation of HT tended to increase the semen quality, which may be associated with the alterations in gut microbiota and plasma metabolites.

Accumulating evidence suggests that gut microbiota involves in various perspectives of host health (20, 21). Moreover, previous studies have well-established the causal relationship between the gut microbiota and sperm quality in both animal models and humans (1, 12, 13, 22, 23). Polyphenols, including HT, can be metabolized by gut microbiota in the colon and on the other hand HT can modify gut microbiota (24, 25). HT enhanced the relative abundance of *Actinobacteria*, *Prevotellaceae_UCG-001*, and *Lactobacillus johnsonii* in mice (26, 27). Similarly, in current study, we found that HT tended to increase the relative abundance of *Lactobacillus*, which has been shown to have the ability to improve sperm motility in zebrafish (28, 29). Moreover, it has been shown that the increase in the relative abundance of *Bacteroides* and *Prevotella* was associated with higher circulating endotoxin and decreased spermatogenesis (22). HT decreased the relative amount of *Prevotella* and *Bacteroides* in current study. We previously found that alginate oligosaccharides (AOS) could enhance the sperm motility and concentration by the increase in the relative abundance of *Bifidobacteriales* in mice (12). In current study, we found that HT tended to increase the relative abundance of *Bifidobacteriales*. Collectively, our data suggested that HT had the potential to improve spermatogenesis and sperm motility by benefiting gut microbiota.

Our previous study has shown that improved gut microbiota can enhance sperm quality by altering circulating metabolome (12). In current investigation, dietary supplementation of HT altered plasma metabolites by the increase in steroid hormones, antioxidant molecules, and many others. Testosterone and its derivatives play vital roles in spermatogenesis which were increased by HT in boar plasma. L-carnitine plays an important role in improving sperm motility and has been used to treat infertility due to its strong anti-oxidant and anti-inflammatory effects in men and animal models (30–33). It has been shown that epididymis has high level of L-carnitine (32). Interestingly, in current investigation, HT enhanced the plasma level of propionyl-L-carnitine, which is a derivative of L-carnitine and can enhance cellular content of L-carnitine (34). Additionally, in this study, we found that HT enhanced the plasma levels of flavonoids and riboflavin. It has been shown that flavonoids and flavanone metabolites, such as naringenin 7-O-glucuronide and 3,4-dihydroxyphenylglycol, had powerful antioxidant ability, thus they may contribute to improved sperm quality and function (35–38). 6-Hydroxymelatonin (increased by HT) is one of the metabolites of melatonin and has been shown to have strong antioxidant activity (39). Thus, we speculate that HT might improve sperm quality by enhancing the antioxidant capacity of boars. Furthermore, various studies have reported that bile acids can cause oxidative stress by promoting the production of oxygen free radicals from mitochondria (40). Moreover, bile acids contribute to infertility by activating farnesoid X receptor and G-protein-coupled bile acid receptor expressed in sperm, which then influence glucose and lipid metabolism and lead to abnormal sperm (41, 42). In current study, plasma levels of several bile acids and their derivatives were decreased by HT.





Furthermore, there was a positive correlation between the plasma level of propionyl-L-carnitine and the relative abundance of *Lactobacillus*. Gut microbiota, including *Lactobacillus*, regulates the L-carnitine metabolism (43, 44). Collectively, these data demonstrated that HT might have the potential to improve plasma metabolome to benefit spermatogenesis.

Indeed, HT improved spermatogenesis by the increase in the protein levels of important genes (*PKA*, *p-AKT*, *p-ERK*, *Catsper*, *ZAG*, and *Gelsolin*) for spermatogenesis and sperm quality. Cation channel of sperm (*Catsper*) is the primary spermatozoan calcium ion channel and plays an essential role in male fertility via modulating sperm tail calcium entry and sperm hyperactivated motility (45). It has been reported that the cyclic adenosine monophosphate (cAMP) dependent protein kinase (protein kinase A, PKA), AKT, and ERK signaling are involved in modulating the sperm maturation, capacitation, and motility (46, 47). Zn-alpha2-glycoprotein (*ZAG*) can promote sperm motility via cAMP/PKA signaling pathway (48). Notably, in current study, HT increased the protein levels of these genes which suggested HT enhancing spermatogenesis.

The underlying mechanisms of HT improving semen quality is that it benefits gut microbiota to improve plasma metabolites then to enhance spermatogenesis and semen quality. The data were confirmed by the follow-up long-term analysis. After the feeding period, all the boars were fed a basal diet (without

HT supplementation) and semen samples were collected and analyzed every 5 days for 2 months. HT increased semen volume while decreased the percentage of abnormal sperm in the follow-up 2 months. In summary, HT improves boar semen quality via the benefiting on gut microbiota and plasma metabolome. HT may be applied as dietary additive to improve boar semen quality in swine industry.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of the Institute of Animal Sciences of Chinese Academy of Agricultural Sciences (IAS2021-67).

AUTHOR CONTRIBUTIONS

YZha and HZ designed the experiment. HH, RZ, YZho, BX, LC, LL, HS, JT, and FT conducted the experiment and analyzed the data. HZ and YZha wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnut.2021.815922/full#supplementary-material>

Supplementary Figure 1 | Effects of HT on the fecal microbial diversity. **(A)** α -diversity with chao1, ACE, Shannon, and Simpson indexes. **(B)** Venn diagram of OUT. **(C)** PCoA of OUT. **(D)** Phylum level. **(E)** Order level. **(F)** Family level; **(G)** Genus level.

Supplementary Figure 2 | Effects of HT on plasma metabolites. **(A)** OPLS-DA. **(B)** Heatmap of altered serum metabolites. **(C)** KEGG enriched pathways of altered plasma metabolites.

Supplementary Table 1 | Primary antibody information.

Supplementary Data Set 1 | Plasma metabolites.

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Conflict of Interest: HS and JT were employed by company YangXiang Joint Stock Company. FT was employed by company Hangzhou Viablife Biotech Co., Ltd.

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