Received: 2 September 2022 | Accepted: 8 November 2022

DOI: 10.1111/1751-7915.14180

MICROBIAL BIOTECHNOLOGY Applied Microbiology International

RESEARCH ARTICLE

Symbiotic bacteria stabilize the intestinal environment by producing phenylpropanoids

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Funding information
Modern Agricultural Technology Industry System of Shandong province, Grant/Award Number: SDAIT-11-02; National Key Research and Development Program of China, Grant/Award Number: 2021YFD1300102; National Natural Science Foundation of China, Grant/Award Number: 31601980 and 31872343; Natural Science Foundation of Shandong Province, Grant/Award Number: ZR2018MC026

Abstract

Salmonella enterica serovar Enteritidis (*S.* Enteritidis) can colonize in the intestinal tract of chickens and transmit to humans. In order to decrypt the mechanism of avian resistance to *S.* Enteritidis, we utilized two China local chicken breeds to generate the reciprocal crosses (the Cross and the Reverse-cross). The two lines of hybrids were orally inoculated with *S.* Enteritidis at 2-day old and sampled at 3 days post-inoculation. Along the analysis direction of multi-omics, differential metabolites, functional pathways and correlated microbes, we found that 12 species of microbes thrived upon *S.* Enteritidis challenge and probably contributed to the intestinal stability in the Cross by enhancing the production of phenylpropanoids. Our findings can help to understand the symbiotic and resistant mechanisms derived from the intestinal microbiota.

INTRODUCTION

Salmonella enterica serovar Enteritidis (*S.* Enteritidis) has caused the biggest epidemiological change worldwide during the last 40 years for its infection of eggs and poultry meat (Barrow et al., 2012). As a non-host-specific serotype, *S.* Enteritidis can enter into the gastrointestinal tract of chickens and colonize for a period with little or no signs of disease (Kogut & Arsenault, 2017; Wigley, 2014). Due to this silent invasion, it is hard to isolate contaminated animals, then resulting in horizontal and vertical propagation in poultry (Beaumont et al., 2009).

Among the ~2600 serotypes identified, *S.* Enteritidis appears in the most common clinical isolates of human and poultry (Knodler & Elfenbein, 2019; Stevens et al., 2009). For health of animals and humans, it is necessary to further understand the colonization and pathogenesis of *S.* Enteritidis in host. Avian gastrointestinal tracts are highly populated with a diverse array of microorganisms that share a symbiotic relationship with their hosts and contribute to the overall health and disease state of the intestinal tract (Mon et al., 2015). It was reported that the newly hatched chickens inoculated with cecal microbiota of over 3-week-old chickens can be protected from the subsequent *S.* Enteritidis

challenge (Varmuzova et al., 2016), indicating a colonization resistance derived from microbiota.

Although the studies on colonization resistance are plentiful in respect of the competition for conditions (oxygen, nutrition and niches) and the bacterial antibiotics (e.g. bacitracin), due to the complexity of intestinal microbiota, our understanding of this ecosystem remains incompletely characterized especially in the context of infection (Eckburg et al., 2005; Hooper & Gordon, 2001; Hu, Liu, Miao, Zhao, Peng, et al., 2022). Anchored in the basic biology of *Salmonella* infection, new technologies combined with careful experimental design will accelerate the discovery pipeline to define the mechanisms of immunity (Barrow et al., 2012). Herein, we utilized two China local chickens (Guangxi Yao and Jining Bairi) to generate the reciprocal crosses. The two lines of hybrids have the similar genetic background. By comparing the differences in transcriptome, microbiome, and metabolome of reciprocal crosses in response to *S.* Enteritidis, we explored the symbiotic relationship between host and intestinal microbiota and focused on the colonization resistance to pathogenic bacteria derived from microbiota rather than that from hosts.

MATERIALS AND METHODS

Animals and experimental design

The chickens came from two populations of reciprocal crosses [the Cross (Guangxi Yao ♂×Jining Bairi ♀) and the Reverse-cross (Guangxi Yao ♀×Jining Bairi ♂)]. For each hybrid, 100 mixed gender, *Salmonella*-negative chickens were randomly divided into two groups (50 chickens per group) at the day of hatch and housed in two separate isolators (32–35°C, 50–60% RH, and 24 h light) with ad libitum access to water and antibiotic-free feed. The bedding material was single layer of plastic mesh with aperture of about 0.5 cm. The strain of *S.* Enteritidis (CVCC3377) was purchased from China Veterinary Culture Collection Center (Beijing, China). After overnight culture in nutrient broth (Hopebio, Qingdao, China) at 37°C with shaking of 220 rpm, the bacterial solution was centrifuged at 1531 *g* for 5 min and suspended with sterilized PBS to 1.0×10^8 cfu/ml. At 2-day old, one group was orally inoculated with 0.3 ml *S.* Enteritidis per chick as *Salmonella* treatment group, and the other group was mock inoculated with the same volume of PBS as control group. According to the three stages of chicken defence strategies to *Salmonella* infection (Kogut & Arsenault, 2017), the 2–4 days after infection is the period transforming from resistance to tolerance. Thus, we collected samples at 3 days post-inoculation (dpi). In each group, nine to 12 chickens were sacrificed by cervical dislocation. The cecum tissues of middle segment were collected aseptically for RNA sequencing, and the mixing of whole cecal contents were collected for 16S rRNA gene sequencing and metabolome determination. The experimental procedure was repeated on another hybrid. Thus, four groups were generated as Cross Control (CC, $n = 3$ for transcriptome, 11 for microbiome and four for metabolome), Cross Treatment (CT, $n = 3$, 11 and 7), Reverse-cross Control (RC, $n = 3$, 9 and 6) and Reverse-cross Treatment (RT, $n = 3$, 9 and 7). The uneven distribution of samples was mainly limited by the sample weight. The cecal contents were sent to Majorbio Biotech Co., Ltd. (Shanghai, China) for microbiome sequencing and metabolome determination. Due to the consumption of microbiome sequencing ($n = 40$), the number of samples whose weight met the needs of the latter was fewer ($n = 24$). The animal experiment was approved by the Laboratory Animal Management and Use Committee of Shandong Agricultural University (Permit Number: SDAUA-2018-058). We strive to reduce the suffering of animals.

Total RNA isolation, library preparation and sequencing

The details were described in our previous study (Hu, Liu, Miao, Zhao, & Li, 2022). In brief, total RNA was extracted from the cecum tissues with mirVana™ miRNA Isolation Kit (Ambion, AM1561). The quality of RNA samples were verified by 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA) and NanoDrop 2000 (Thermo Scientific, Wilmington, USA). The libraries were constructed using TruSeq Stranded Total RNA with Ribo-Zero Gold (Illumina, RS-122-2301) according to the manufacturer's instructions and then were sequenced on Illumina HiSeq X Ten platform. Average 98.57 M clean reads of each sample were retained, which were mapped to the chicken genome (GRCg7b) using HISAT2. FPKM of each gene was calculated using Cufflinks, and the read counts of each gene were obtained by HTSeq-count. Overall, Q30 base of RNA sequencing was 92.23–94.02%, GC content was 45.63–47.48% and the comparison rates between samples and genome was 93.51–94.45%. RNA sequencing and preliminary analysis were completed in Shanghai OE Biotech Co., Ltd. (Shanghai, China).

Bacterial DNA extraction and 16S rRNA gene sequencing

The details were described in our previous study (Hu, Liu, Miao, Zhao, Peng, et al., 2022). In brief, bacterial DNA was extracted from the samples of cecal contents using Soil DNA Kit (Omega, D5625-01) according to manufacturer's protocols. The quality of DNA samples were verified by NanoDrop 2000 and 1% agarose gel electrophoresis. The V3-V4 region of the bacteria 16S rRNA gene was amplified using the following primer

pair: 338F, ACTCCTACGGGAGGCAGCAG, and 806R, GGACTACHVGGGTWTCTAAT. The PCR products were paired-end sequenced (2×300) on the Illumina MiSeq platform (Illumina, San Diego, USA) according to standard protocols. After preliminary quality control and merger, operational taxonomic units (OTUs) were clustered with 99% similarity cutoff using UPARSE and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analysed by RDP Classifier algorithm against the Silva138 16S rRNA database using confidence threshold of 70%. All the samples were rarefied to 30,221 reads. The 16S rRNA gene sequencing and preliminary analysis were completed in Majorbio Biotech Co., Ltd. (Shanghai, China).

Liquid chromatography–tandem mass spectrometry (LC–MS) analysis

To 50 mg cecal contents of each sample, 400 μl methanol: water (4:1, v/v) solution was added. The mixture was treated by high-throughput tissue crusher Wonbio-96c (Wanbo, Shanghai, China) at –10°C for 6 min followed by ultrasound at 40 kHz for 30 min at 5°C. The samples were placed at –20°C for 30 min to precipitate proteins. After centrifugation at 13,000 *g* at 4°C for 15 min, the supernatant was carefully transferred to a sample vial for LC–MS analysis. The quality control samples were prepared by mixing aliquots and were analysed accordingly. They were injected at regular intervals throughout the analytical run to provide a set of data from which repeatability could be evaluated.

Chromatographic separation of the metabolites was performed on a Thermo UHPLC system equipped with an ACQUITY UPLC HSS T3 (100 mm×2.1 mm i.d., 1.8 μm; Waters, Milford, USA). The mobile phases consisted of 0.1% formic acid in water: acetonitrile (95:5, v/v) (solvent A) and 0.1% formic acid in acetonitrile:isopropanol:water (47.5:47.5:5, v/v) (solvent B). The solvent gradient changed according to the following conditions: from 0 to 0.1 min, 0% B to 5% B; from 0.1 to 2 min, 5% B to 25% B; from 2 to 9 min, 25% B to 100% B; from 9 to 13 min, 100% B to 100% B; from 13 to 13.1 min, 100% B to 0% B; from 13.1 to 16 min, 0% B to 0% B for equilibrating the systems. The sample injection volume was 2 μl and the flow rate was 0.4 ml/min. The column temperature was maintained at 40°C. During the period of analysis, all these samples were stored at 4°C. The mass spectrometric data were collected using a Thermo UHPLC-Q Exactive mass spectrometer equipped with an electrospray ionization source operating in either positive or negative ion mode. The optimal conditions were set as follows: heater temperature, 400°C; Capillary temperature, 320°C; sheath gas flow rate, 40 arb; Aux gas flow rate, 10 arb; ion-spray voltage floating, –2800 V in negative mode and 3500 V in positive mode respectively; Normalized collision energy, 20, 40, 60 eV rolling for MS/MS. Full MS resolution was 70,000, and MS/MS resolution was 17,500. Data acquisition was performed with the data-dependent acquisition mode. The detection was carried out over a mass range of 70–1050 *m/z*.

After UHPLC–MS/MS analysis, the raw data were imported into the Progenesis QI 2.3 (Nonlinear Dynamics, Waters, USA) for peak detection and alignment. The preprocessing results generated a data matrix that consisted of the retention time, mass-to-charge ratio (*m/z*) values and peak intensity. Metabolic features detected at least 80% in any set of samples were retained. After filtering, minimum metabolite values were imputed for specific samples in which the metabolite levels fell below the lower limit of quantitation and each metabolic features were normalized by sum. Metabolic features which the relative standard deviation of QC > 30% were discarded. Following normalization procedures and imputation, statistical analysis was performed on log2-transformed data to identify significant differences in metabolite levels between comparable groups. Mass spectra of these metabolic features were identified by using the accurate mass, MS/MS fragments spectra and isotope ratio difference with searching in reliable biochemical databases as Human metabolome database (HMDB) (http://www.hmdb.ca/), KEGG compound database (https://www.kegg.jp/kegg/compound/) and Metlin database (https://metlin.scripps.edu/).

Statistical analysis

The analyses of DESeq2, PICRUSt, Wilcoxon rank-sum test and Student's *t* test were run in OEBiotech Cloud (https://cloud.oebiotech.cn), Majorbio Cloud (www.majorbio.com) and Microsoft EXCEL 2016. The significant level in figures was marked as * ($0.01 \le p < 0.05$), ** ($0.001 \le p < 0.01$) and *** ($p < 0.001$). The bar charts were generated by GraphPad Prism 8.0 software with mean ± SEM.

Availability of data

The raw data of transcriptome sequencing, microbiome sequencing and metabolome determination were deposited in National Genomics Data Center (https://ngdc.cncb.ac.cn/) under BioProject accession number CRA005292, CRA004605 and OMIX718 respectively.

RESULTS

The phenotypic discrepancy in metabolome between the reciprocal crosses

As described in our previous study (Hu, Liu, Miao, Zhao, Peng, et al., 2022), the result of 16S rRNA gene

sequencing showed that *S.* Enteritidis was detected in a mean abundance of 1.09% (from 0.01% to 4.27%) in *Salmonella* groups (CT and RT) but not in control groups (CC and RC), suggesting *S.* Enteritidis can colonize in the cecal contents of chickens. We did not measure the colony-forming units of other organs. Several related studies had shown that oral inoculation of *S.* Enteritidis can infect chicken's internal organs such as liver and spleen (Matulova et al., 2013; Schokker et al., 2012).

In studies of transcriptome (n = 12), microbiome (n = 40) and metabolome (n = 24), we obtained 15,205 genes, 943 OTUs and 1348 known metabolites respectively. Based on the transcriptomic data and the quantitative verification, we had confirmed that the inflammatory response was triggered at mRNA level upon challenge, because all the 30 differentially expressed genes enriched in inflammatory response term (GO enrichment) were up-regulated in *Salmonella* group (Hu, Liu, Miao, Zhao, & Li, 2022). In light of the sharp up-regulation of regulatory genes *ACOD1*, *TNIP3* and *IL-10*, we deduced that the inflammatory response was transforming to anti-inflammatory tolerance at 3 dpi (Hu, Liu, Miao, Zhao, & Li, 2022).

Subsequently, we performed Principal Component Analysis (PCA) based on three omics datasets. As expected, the *Salmonella* groups were separated from the control groups in transcriptome (Figure 1A) by the first principal component (PC1), which was speculated to the factor of *Salmonella* challenge and contributed 41.09% to the total difference. At the same time, we found a batch effect between the two experiments of reciprocal crosses especially in microbiome (Figure 1B) and metabolome (Figure 1C), which was reflected by their PC1s of 36.87% and 37.20% respectively. Chicken genotype has been reported to have a limited effect on resistance to *S.* Enteritidis infection between two inbred lines (Mon et al., 2015). In theory, we proposed that the reciprocal crosses had similar genetic resistance to pathogen, which was less dependent on environmental and microbial factors.

Ignoring the batch effect, we found that *S.* Enteritidis challenge had a substantial impact on the Cross as well as on the Reverse-cross in respect of transcriptome and microbiome (Figure 1A,B). Intriguingly, in respect of metabolome, the impact of challenge on the Cross was slight though it was still effective on the Reverse-cross (Figure 1C). This situation was supported by the number of differential metabolites. With the threshold of False Discovery Rate (FDR) < 0.05 and Variable Importance in the Projection (VIP) > 1, there were 60 and 251 differential metabolites within the Cross (Table S1) and within the Reverse-cross (Table S2) respectively. Considering the approached material compositions of cecal contents between CC and RC (Figure 2), we pretty much excluded the external factors between reciprocal crosses. In the similar genetic and environmental background, the phenotypic discrepancy of metabolome between reciprocal crosses should be mainly attributed to the cecal microbiota.

The abnormal up-regulation of phenylpropanoids in the Cross

We further analysed the 60 and 251 differential metabolites in the Cross and in the Reverse-cross respectively. Lipids and phenylpropanoids were two superclasses with the most compounds according to HMDB taxology (Figure 3). In terms of lipids, there were 13 differential metabolites in the Cross (11 up-regulated and two down-regulated) and 76 in the Reverse-cross (57 up-regulated and 19 down-regulated) compared to control. The data in frequency (13/60 vs. 76/251) or tendency (11/2 vs. 57/19) was approximate between the reciprocal crosses. However, in terms of phenylpropanoids, there were 12 differential metabolites in the Cross (11 up-regulated and 1 down-regulated) and 36 in the Reverse-cross (12 up-regulated and 24 down-regulated) compared to control. The tendency of up-regulation in the Cross (11/1) was abnormally higher than that (12/24) in the Reverse-cross. It should be noted that, there were only three same metabolites between the differential phenylpropanoids of reciprocal crosses, but their changes upon challenge were opposite at all. It showed that the alterations of phenylpropanoids between reciprocal crosses were different completely, and multiple metabolites of phenylpropanoids in the Cross were notably increased compared to those in the Reverse-cross.

The consistent up-regulation of phenylpropanoid biosynthesis pathway in the Cross

Phenylpropanoids are a group of plant secondary metabolites (KEGG: map00940). Due to the xenobiotic nature and the approximate number of this kind in cecal contents between CC and RC (103 vs. 102 with 98 common, Figure 2), we speculated that the discrepancy in terms of phenylpropanoids between reciprocal crosses was caused by cecal microbiota. Based on the data of 16S rRNA gene sequencing, we utilized PICRUSt tool to predict functional pathways of cecal microbiome (Table S3). Consistently, the phenylpropanoid biosynthesis pathway (KEGG: map00940) was increased significantly compared to control in the Cross (p = 0.010) but not in the Reverse-cross (Figure 4). It was suggested that certain microbes in the Cross might contribute to this functional pathway.

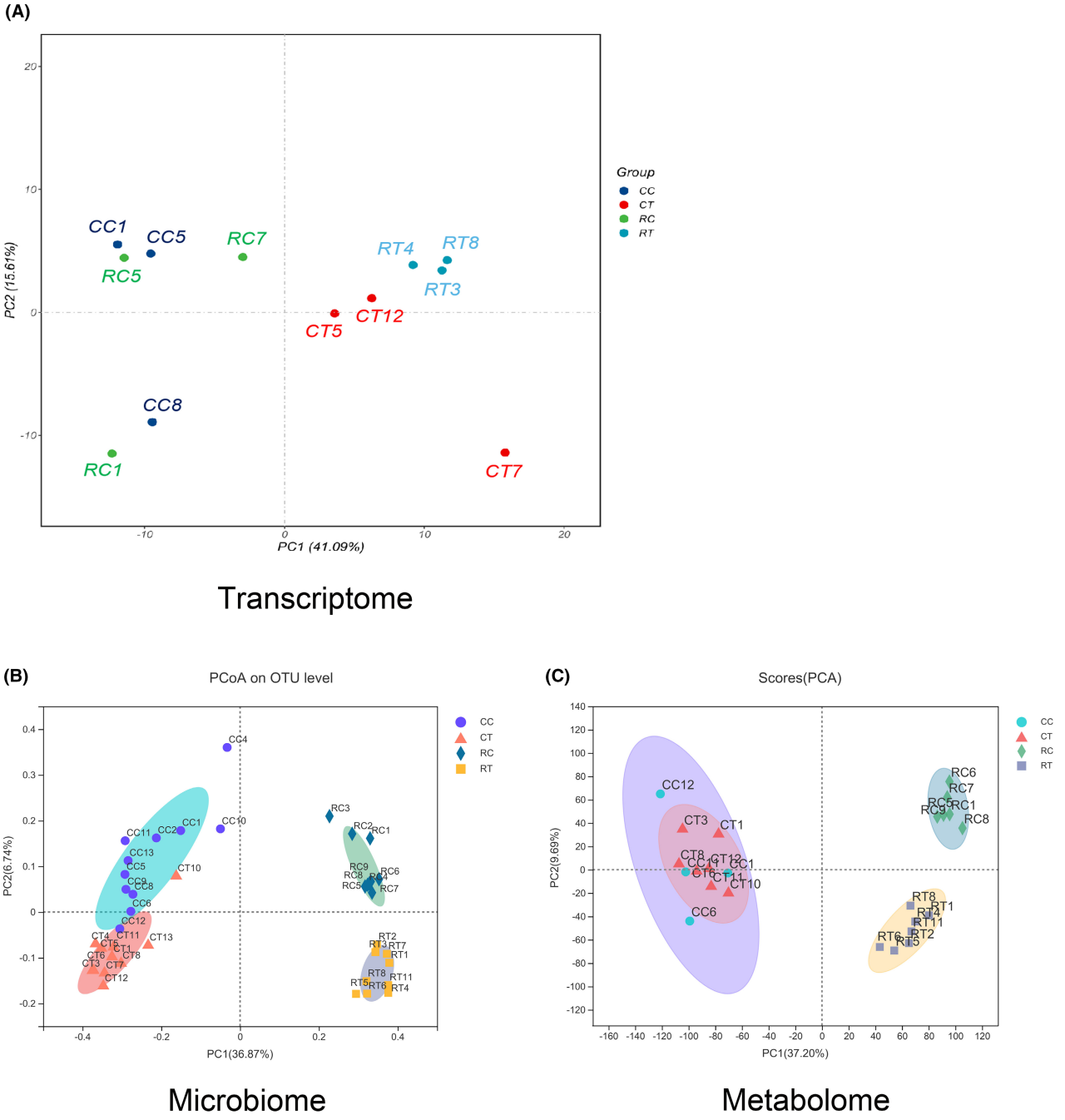
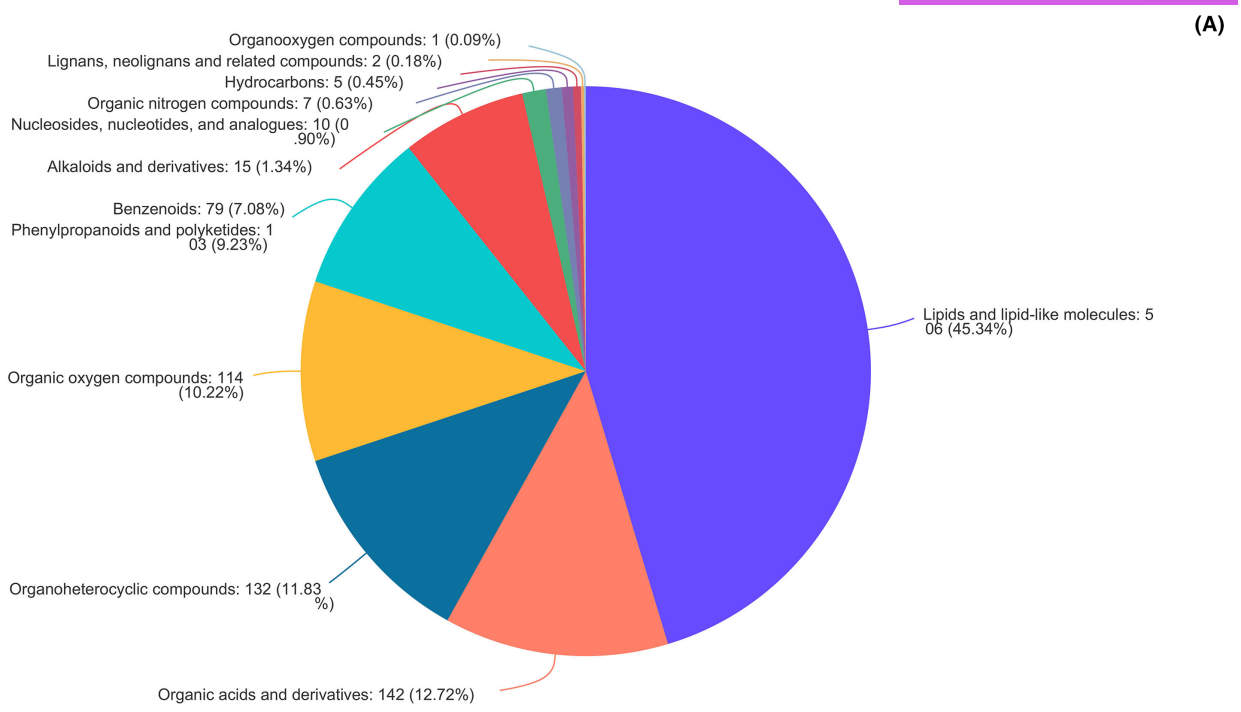


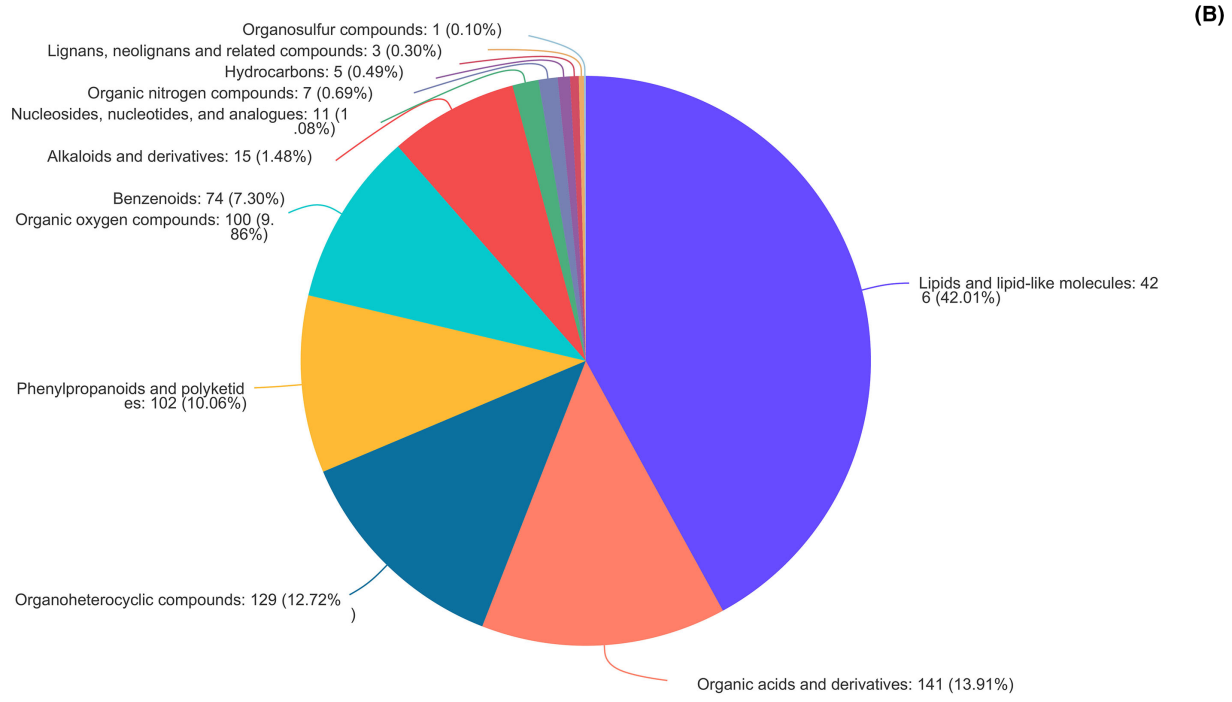
FIGURE 1 Principal component analysis (PCA) based on the data of transcriptome (A), microbiome (B) and metabolome (C).

The close correlation between phenylpropanoids and microorganisms

We constructed the spearman correlation analysis between the 12 differential phenylpropanoid metabolites and the 50 most abundant OTUs in the Cross (Figure 5). As shown, the uppermost metabolite in the figure was heterogeneous from the others because it was the only one decreased upon challenge in the Cross. Within the other metabolites, 12 OTUs (excluding the *Salmonella* OTU607) had significant and positive correlations (marked with asterisk and red background) with at least one metabolite, indicating a role in phenylpropanoids generation. Most of these OTUs belong to Clostridia class except one in Bacilli class. Due to the low resolution of 16S rRNA gene sequencing, our information of these OTUs only reaches the genus level at most (Figure 6), including one OTU in *Erysipelatoclostridium* (OTU485) of Erysipelatoclostridiaceae, one OTU in norank genus (OTU710) of [Eubacterium] coprostanoligenes group, seven OTUs in *Blautia* (OTU263), [Ruminococcus] torques group (OTU627) and unclassified genera (OTU886, OTU328, OTU761, OTU368, OTU439) of Lachnospiraceae, one OTU in



Material composition in cecal contents of the Cross



Material composition in cecal contents of the Reverse-cross

FIGURE 2 Material composition in the cecal contents of control group of the Cross (A) and control group of the Reverse-cross (B).

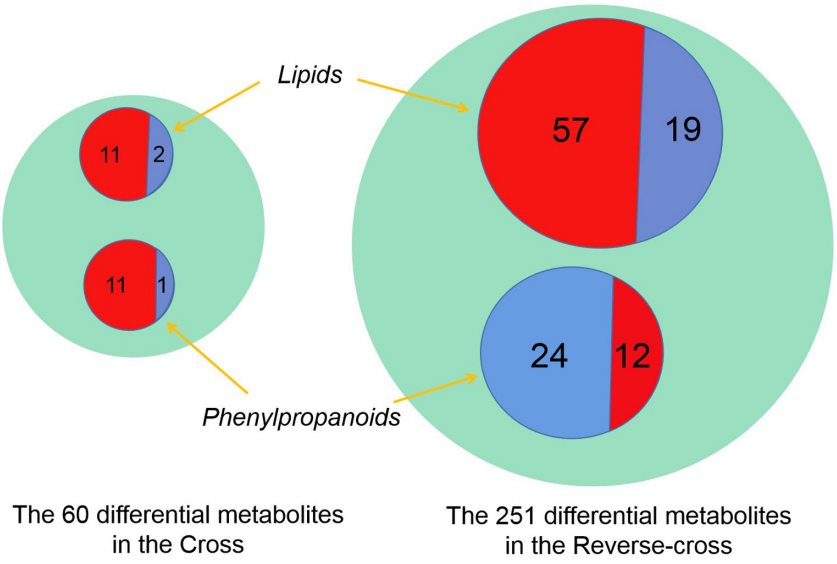


FIGURE 3 The alteration of lipids and phenylpropanoids in differential metabolites of the reciprocal crosses. The red area and the blue area represent the up-regulated metabolites and the down-regulated metabolites respectively.

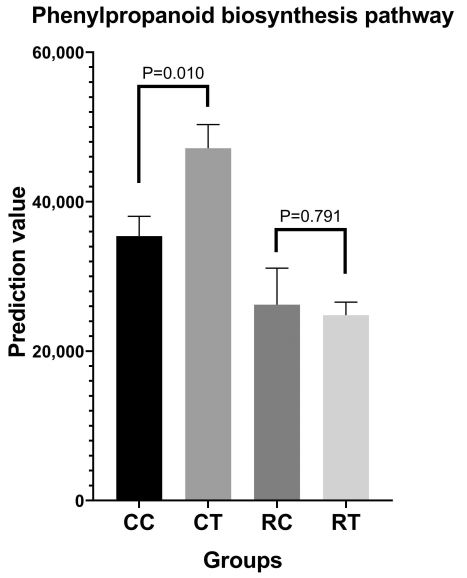


FIGURE 4 The phenylpropanoid biosynthesis pathway predicted by PICRUSt. n = 11, 11, 9, 9 in CC, CT, RC, RT, respectively.

Colidextribacter (OTU449) of Oscillospiraceae and two OTUs in *Negativibacillus* (OTU269) and unclassified genus (OTU391) of Ruminococcaceae.

Subsequently, we compared the abundance of the 12 OTUs between reciprocal crosses (Figure 7, Table S4). In the Cross, all the 12 OTUs were abundant and displayed a up-regulation tendency upon challenge. The increases of five OTUs (OTU710, OTU328, OTU368, OTU449, OTU269) were considerably significant ($p < 0.10$). In the Reverse-cross, however, seven of the 12 OTUs (OTU263, OTU269, OTU368, OTU627, OTU439, OTU391, OTU485) were sporadic or even none; three OTUs (OTU761, OTU710, OTU449) showed a down-regulation tendency upon challenge; only two OTUs (OTU886, OTU328) were abundant and increased significantly ($p < 0.001$). The increase of 12 OTUs might enhance the phenylpropanoid biosynthesis pathway and phenylpropanoids production in the Cross. Probably due to the absence and the decline of some species, the pathway and the related metabolites were not increased in the Reverse-cross.

DISCUSSION

The reciprocal crosses used in our experiment were generated from two China local chicken breeds, Guangxi Yao and Jining Bairi, which are geographically isolated over 1400 km. For a long period of geographical and reproductive isolation, local chicken can be regarded as a natural inbred line with relative homozygous genotype. Reciprocal cross is a hybridization method by reversing the parent strains (Vaiserman et al., 2013). The two hybrids of reciprocal crosses actually share the same genetic source. It was reported that chicken genotype has a limited effect on resistance to *S.* Enteritidis infection between two inbred lines (Mon et al., 2015). Compared to the two regional chickens, their reciprocal crosses will be less influenced by genetic background and help us to find out the common response across hybrids, which had been reflected in our new studies (Hu, Liu, Miao, Zhao, & Li, 2022; Hu, Liu, Miao, Zhao, Peng, et al., 2022).

According to the multi-omics analysis and the number of differential metabolites, we deduced that *Salmonella* challenge had a substantial impact on the Reverse-cross rather than on the Cross. Furthermore, 11 metabolites of phenylpropanoids were found to be increased significantly upon challenge in the Cross, but this rise did not happen in the Reverse-cross. Phenylpropanoids are a group of plant secondary metabolites derived from phenylalanine and featured for their

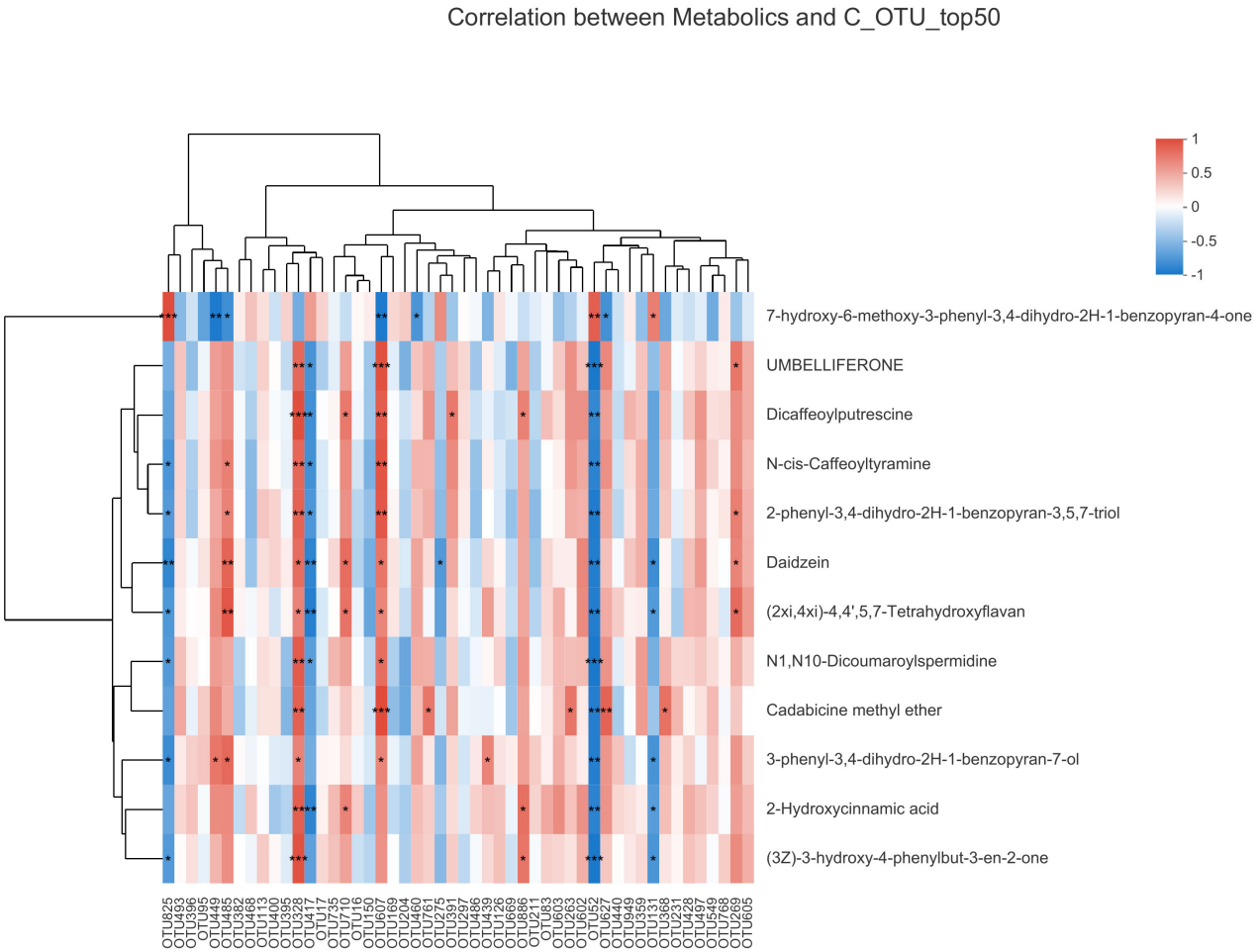
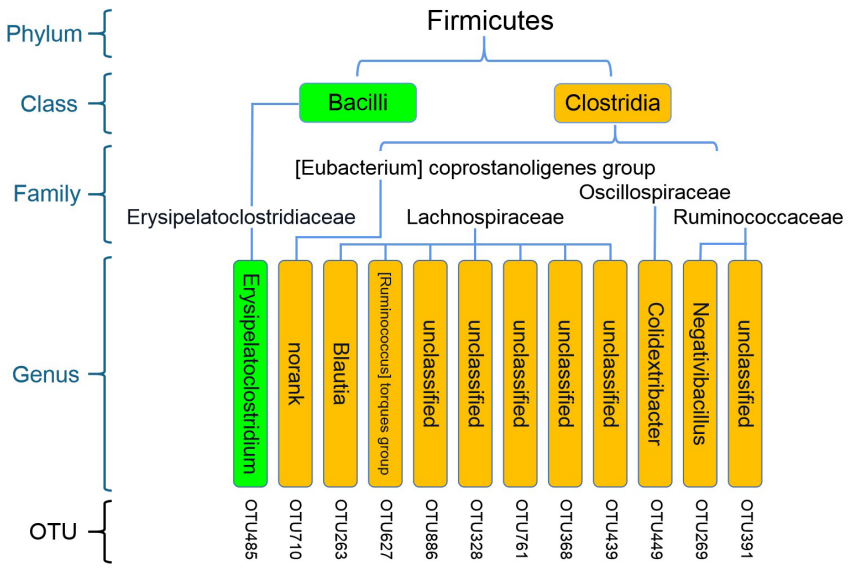


FIGURE 5 The spearman correlation analysis between the 12 differential phenylpropanoid metabolites and the 50 most abundant operational taxonomic units in the Cross.

FIGURE 6 The taxonomy of 12 operational taxonomic units.



phenylpropane (C6-C3) backbone, including cinnamic acid, coumaric acid, caffeic acid, ferulic acid, sinapic acid and flavonoids (KEGG: map00940). They can be metabolized by plants and certain microorganisms. Our 11 phenylpropanoids of interest belong to cinnamic acids (5), coumaric acids (1) and flavonoids (5). It was reviewed that phenylpropanoids and their derivatives possess a broad spectrum of antimicrobial

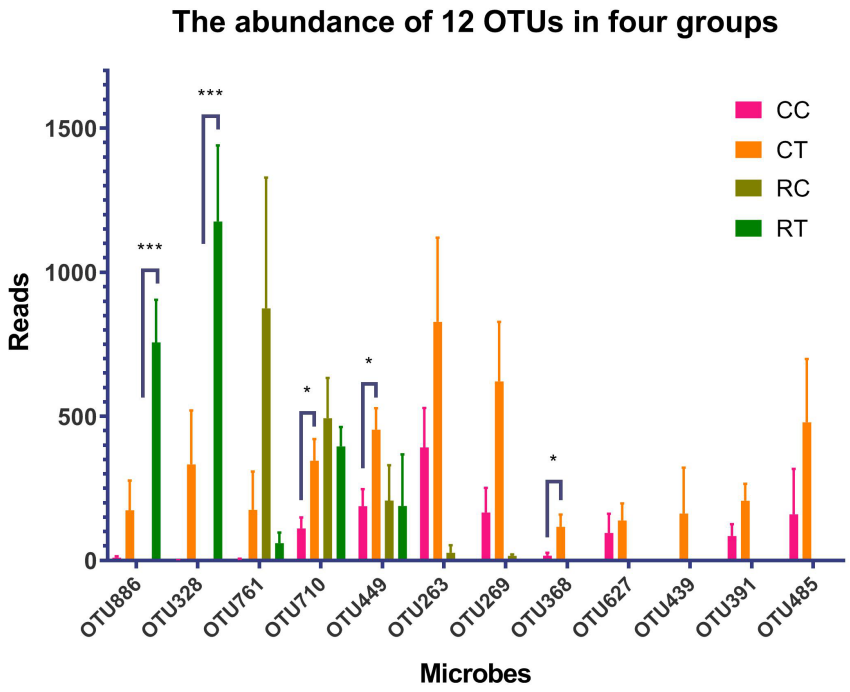


FIGURE 7 The abundance of 12 operational taxonomic units in four groups. All the samples were rarefied to 30,221 reads.

activity against human pathogens including *Salmonella* genus and its relatives in Enterobacteriaceae family, *Escherichia* and *Shigella* (Neelam et al., 2020). The antioxidant capacity of phenylpropanoids has been evaluated by numerous in vitro assays probably through free radical scavenging, metal chelation and protein binding (Amorati et al., 2006; Silva et al., 2000). Besides the aforementioned functions, phenylpropanoids and their derivatives have been proposed the anti-inflammatory potential. 4-hydroxycinnamic acid treatment suppressed pulmonary inflammation induced by cigarette smoke and LPS exposure in laboratory mice, with the decline of inflammatory cells accumulation, cytokines production and mitogen-activated protein kinases (MAPK) signalling (Park et al., 2017).

As a main component of the 11 phenylpropanoids, flavonoids have been reported to protect plants against damage from biotic and abiotic stresses via phytoalexin or antioxidant roles, and exert a variety of beneficial effects in humans (Liu et al., 2021). Flavonoids are classified into several subgroups such as flavans, flavanones, flavones and isoflavones. Daidzein is one of the most studied isoflavones and exists in our 11 compounds. Until recently, more attention has shifted to its product equol, which can be transformed by specific microflora in the gut and enhance the actions of soy isoflavones owing to its greater affinity for oestrogen receptors, unique antiandrogenic properties and superior antioxidant activity (HMDB: 0003312). Importantly, studies in humans showed that only approximately one-third to one-half of the population is able to metabolize daidzein to equol. The ability of equol production is presumably decided by the intestinal microflora (Jackman et al., 2007; Yuan et al., 2007). Based on the above literature, we speculated that these 11 phenylpropanoids might participate in maintaining or restoring the steady state after *Salmonella* challenge through antibacterial, antioxidant and anti-inflammatory effects, resulting in smaller fluctuation and fewer differential metabolites in metabolome of the Cross compared to the Reverse-cross.

Furthermore, we found that 12 OTUs had significant and positive correlation with the 11 phenylpropanoids. Notably, 11 of the 12 OTUs belong to Clostridia class, which has been reported of strong ability to catalyse complex organic substances (Tracy et al., 2012). To the best of our knowledge, the concrete bacterial strains metabolizing phenylpropanoids are not known. A few research mentioned species of *Clostridium*, *Eubacterium* and *Ruminococcus* were involved in flavonoids (a group of phenylpropanoids) metabolism (Hur et al., 2000; Křížová et al., 2019; Schoefer et al., 2002; Tamura et al., 2007). In the study of Schoefer et al. (2002), daidzein was in part degraded to 6′-hydroxy-O-desmethylangolensin by *Eubacterium ramulus*, the step which is crucial for further degradation. Consistently, the OTU710, an unclassified species of *Eubacterium* in our 12 screened OTUs, had a significant and positive correlation to daidzein (Figure 5). A recent article (Sun et al., 2022) found that the alteration of *Colidextribacter* and several unclassified genera from Lachnospiraceae and Ruminococcaceae were related to the enrichment of metabolites in phenylpropanoid biosynthesis pathway, which was close to our results. It was known that the commensal bacteria can actively coordinate the immune tolerogenic response of host (Fava & Danese, 2011). As a response to *Salmonella* invasion

and the highly possible inflammation in our study, some species mainly from Clostridia class thrived and presumably in turn played a role in colonization resistance to external pathogen by metabolizing phenylpropanoids.

The up-regulation of phenylpropanoids in the Cross could be attributed to the increase of some microorganisms and their functional enzymes, which was reflected in the increase of phenylpropanoid biosynthesis pathway (Figure 4). But why were the most of differential phenylpropanoids decreased notably at the same time that the biosynthesis pathway was stable relatively in the Reverse-cross?. We analysed it might be caused by two reasons. First, some key bacteria or enzymes were missing, resulting in a bottleneck effect. Second, the action conditions (e.g. pH) of enzymes were not in the optimum range, which led to an actual decline in productivity without significant change in the predicted value of functional pathway. The latter reason can be watched a clue by the metabolite catalogue of bile acids. In the Reverse-cross, there were three differential compounds of this catalogue, N-[(3a,5b,7b)-7-hydroxy-24-oxo-3-(sulfooxy)cholan-24-yl]-Glycine, Polyporusterone F and Glycocholic Acid, all of which were up-regulated upon challenge (Table S2); In the Cross, there was one differential compound of this catalogue, which was down-regulated upon challenge (Table S1). The bile acids and their derivatives are involved in bile acid cycle and should be absorbed in the hindgut (KEGG: map04976). Their significant increase in the Reverse-cross but not in the Cross indicated that the global absorption function of the Reverse-cross was reduced, probably leading to nutrient enrichment, pH alteration and the decrease of enzyme activity.

At last, although we did not measure the inflammatory indicators nor compare their differences between reciprocal crosses, several lines of clues showed that the fluctuation of intestinal environment in the Reverse-cross was greater than that in the Cross, including the omics profiles, the number of differential metabolites, the material reabsorption and an inflammatory biomarker of eicosanoids. Eicosanoids are a kind of inflammatory biomarker (de Cássia da Silveira E Sá et al., 2014). We found one metabolite (Iloprost) of eicosanoids significantly increased in the Reverse-cross but not in the Cross. These data further supported our deduction that, in the context of *S.* Enteritidis invasion, the intestinal environment of the Cross was more stable than that of the Reverse-cross due to the phenylpropanoids produced by special microbiota.

CONCLUSIONS

We identified 12 species of symbiotic bacteria which might contribute to the stability of chicken intestinal environment by producing probiotic phenylpropanoids. In fact, our results intensified an immunology consensus, that is, intestinal microbiota is involved in the process of host's anti-infection response. This symbiotic relationship with the host and the resistance to *Salmonella* can maintain the intestinal homeostasis. Although there was no obvious difference in clinical symptoms between the reciprocal crosses, the potential mechanism we found may be crucial in the context of some chronic diseases or facing more virulent pathogens in animals or humans.

AUTHOR CONTRIBUTIONS

Geng Hu: Data curation (lead); formal analysis (lead); investigation (lead); methodology (lead); software (lead); validation (lead); visualization (lead); writing – original draft (lead); writing – review and editing (lead). **Liying Liu:** Conceptualization (equal); funding acquisition (equal); writing – original draft (equal); writing – review and editing (equal). **Xiuxiu Miao:** Investigation (equal); writing – original draft (equal); writing – review and editing (equal). **Yanan Zhao:** Investigation (equal); writing – original draft (equal); writing – review and editing (equal). **Yanan Peng:** Validation (equal); writing – original draft (equal); writing – review and editing (equal). **Xianyao Li:** Conceptualization (lead); funding acquisition (lead); methodology (lead); project administration (lead); resources (lead); supervision (lead); writing – original draft (lead); writing – review and editing (lead).

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China (31872343, 31601980), National Key Research and Development Program of China (2021YFD1300102), Modern Agricultural Technology Industry System of Shandong province (SDAIT-11-02) and Natural Science Foundation of Shandong Province (ZR2018MC026).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Hu, G., Liu, L., Miao, X., Zhao, Y., Peng, Y. & Li, X. (2023) Symbiotic bacteria stabilize the intestinal environment by producing phenylpropanoids. *Microbial Biotechnology*, 16, 88–98. Available from: https://doi.org/10.1111/1751-7915.14180