Integrated Analysis of Cecal Microbiome and Metabolome Revealed Different Inflammatory Responses to Salmonella Enterica Serovar Enteritidis Between Reciprocal Crosses of Chicken

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Research

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Abstract

Background: *Salmonella enterica* serovar Enteritidis (*S. Enteritidis*) bacteria can colonize intestinal tract of chicken and transmit to humans, while the intestinal microbiota are resistant to their colonization. Our knowledge of the interplay between host, pathogen and microbiota is scarce, particularly in context of different genetic background of the host, such as the reciprocal cross.

Results: Comprehensive analysis of microbiome and metabolome showed that 23.4% of genera and 11.6% of metabolites in the Cross, and 17.2% of genera and 25.1% of metabolites in the Reverse-cross were altered ($P < 0.05$) in response to *Salmonella* infection. In comparison of the reciprocal crosses, all the co-differential genera and 73.1% of the co-differential metabolites were opposite in alteration trend. Phenylpropanoids and lipids were increased significantly in the Cross and in the Reverse-cross, respectively.

Conclusions: A more extensive inflammatory response to *S. Enteritidis* might be triggered in the Reverse-cross than in the Cross, and the regulatory modes were different. The Reverse-cross upregulated pro- and anti-inflammatory factors simultaneously, while the Cross was aided with the phenylpropanoids produced by intestinal bacteria.

Background

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 [1]. Unlike the host-specific serotypes, *S. Enteritidis* has a broad range of hosts and usually leads to a period of colonization of lower gastrointestinal tract, accompanied with activation of inflammatory responses and little or no signs of disease [2]. Due to this silent propagation, it is almost impossible to isolate contaminated animals and results in widespread at horizontal and vertical levels in poultry [3] and hence in food contamination. Increased resistance to *Salmonella* carrier state is therefore needed [4].

Salmonella bacteria have evolved an intimate relationship with their hosts that extends to their cellular and molecular levels [3]. As well known, the outcome of infection is the combined effect of the microbial gene set and the host genetic background. The situation is even more complex in the context of gut flora. Chicken's caeca are a pair of closed lumens and suitable for microbial colonization including *S. Enteritidis* [5]. Importantly, newly hatched chickens inoculated with cecal microbiota of 3-week-old or older chickens could be protected against the subsequent *S. Enteritidis* challenge [6], indicating a colonization resistance to *S. Enteritidis*. This resistance may not be produced by one or seldom bacteria [7], but may be the result of the joint action of microbiota. High throughput techniques, e.g. 16S rRNA gene sequencing and liquid chromatography-tandem mass spectrometry, can add to our capacity. Application of the new technologies, with careful experimental design anchored in the basic biology of *Salmonella* infection, will accelerate the discovery pipeline to define the mechanisms of immunity [1].

Reciprocal cross is a hybridization method by reversing the strains from which the dam and the sire are taken [8]. This method has been applied in the field of animal breeding very early and some stable traits were noted in the offspring of reciprocal crosses [9, 10]. Reciprocal effects are supposed to be due to the genetic effects of parents (i.e. maternal and paternal effects), cytoplasmic effects, and parent-of-origin effects [11]. Current research showed that the genetic resistance to *Salmonella* is not sex linked [12]. We thus pay more attention to maternal or cytoplasmic effects.

As the putative resistance genes are very probably segregating in outbred lines [13], two Chinese local chickens, Yao and Jining Bairi, which were geographically isolated over 1,400 km inline, were chosen to generate the reciprocal crosses [the Cross (Yao $\times$ Jining Bairi) and the Reverse-cross (Yao $\times$ Jining Bairi)]. Chicks were orally inoculated with 0.3 mL *S. Enteritidis* (1.0×10^8 CFU/mL) or PBS at 2-day old and sacrificed at 3 days post-inoculation (dpi), when the immune response of chicken transformed from acute resistance period to stable tolerance period [3]. Based on the integrated analyses of cecal microbiome and metabolome, we anticipated to understand the interplay between host, pathogen and microbiota, and find the host genetic background or reciprocal effects on *Salmonella* resistance.

Methods

Animal experiment

The Cross (Yao $\times$ Jining Bairi) and the Reverse-cross (Yao $\times$ Jining Bairi) were provided respectively by Shandong Bairi Chicken Breeding Co., Ltd (Jining, China), and therefore the experiment was carried out in two batches. Besides, all the materials and methods were the same. The *S. Enteritidis* strain (CVCC3377) was purchased from China Veterinary Culture Collection Center (Beijing, China) and stored at -80°C . After rejuvenation with nutrient broth (Hopebio, Qingdao, China), the bacterial solution was centrifuged at 4,000 g for 5 min and suspended again with sterilized phosphate buffered saline (PBS) to OD value 1, which was fine-tuned to 1.0×10^8 CFU/mL by the followed plating enumeration.

The operation of inoculation was previously described in detail [46]. In brief, one hundred of *Salmonella*-negative hybrid chicks were randomly divided into two groups, which were placed in different isolators of different houses with the same condition (32-35°C of temperature, 50-60% of humidity and 24h lighting scheme). Clean water and antibiotic-free feed were provided and all the chicks could *ad libitum* drink and eat. At 2-day old, one group was orally inoculated with 0.3 mL *S. Enteritidis* as Treatment, and the other group was mock inoculated with the same volume of PBS as Control. At 3 dpi, 12 chicks each group were euthanized. The cecal contents were taken out and transferred to the tube according to the aseptic procedure, then were quick-frozen in liquid nitrogen and stored at -80°C .

All the operation steps were repeated in experiment of another cross. The four groups were named as the Cross Control (CC), the Cross Treatment (CT), the Reverse-cross Control (RC) and the Reverse-cross Treatment (RT).

DNA extraction and 16S rRNA gene sequencing

Microbial DNA was extracted from the samples of cecal contents using the OMEGA DNA Kit (Omega Bio-Tek, Norcross, USA) according to manufacturer's protocols. The final DNA concentration and purification were determined by NanoDrop 2000 (Thermo Scientific, Wilmington, USA), and DNA quality was checked by 1% agarose gel electrophoresis. The V3-V4 region of the bacteria 16S rRNA gene was amplified by PCR using the following primer pair: 338F, ACTCCTACGGGAGGCAGCAG, and 806R, GGACTACHVGGGTWTCTAAT. PCR reactions were performed in 20 μ L mixture containing 4 μ L 5 $\times$ FastPfu Buffer, 2 μ L 2.5 mM dNTPs, 0.8 μ L 5 μ M each primer, 0.4 μ L FastPfu Polymerase and 10 ng template DNA under the following cycling conditions: 3 min at 95 $^{\circ}$ C followed by 27 cycles of 30 s at 95 $^{\circ}$ C, 30 s at 55 $^{\circ}$ C and 45 s at 72 $^{\circ}$ C, and a final 10 min at 72 $^{\circ}$ C. The pooled products were paired-end sequenced (2 $\times$ 300) on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) according to standard protocols.

Raw fastq files were demultiplexed, quality-filtered by Trimmomatic and merged by FLASH with the following criteria: (i) The reads were truncated at any site receiving an average quality score <20 over a 50 bp sliding window. (ii) Primers were exactly matched allowing 2 nucleotide mismatching, and reads containing ambiguous bases were removed. (iii) Sequences whose overlap longer than 10 bp were merged according to their overlap sequence. Operational taxonomic units (OTUs) were clustered with 99% similarity cut-off using UPARSE and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier algorithm against the Silva (SSU138) 16S rRNA database using confidence threshold of 70%. All samples were rarefied to 30,221 reads.

Liquid Chromatography-tandem Mass Spectrometry (LC-MS) analysis

To 50 mg of each sample, 400 μ L 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 $^{\circ}$ C for 6 min followed by ultrasound at 40 kHz for 30 min at 5 $^{\circ}$ C. The samples were placed at -20 $^{\circ}$ C for 30 min to precipitate proteins. After centrifugation at 13,000 g at 4 $^{\circ}$ C for 15 min, the supernatant was carefully transferred to a sample vial for LC-MS analysis. The quality control (QC) samples were prepared by mixing aliquots and were analyzed 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 $\times$ 2.1 mm i.d., 1.8 μ m; 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 μ L and the flow rate was set to 0.4 mL/min. The column temperature was maintained at 40 $^{\circ}$ C. During the period of analysis, all these samples were stored at 4 $^{\circ}$ C. The mass spectrometric data was collected using a Thermo UHPLC-Q Exactive Mass Spectrometer equipped with an electrospray ionization (ESI)

source operating in either positive or negative ion mode. The optimal conditions were set as followed: heater temperature, 400 °C; Capillary temperature, 320 °C; sheath gas flow rate, 40 arb; Aux gas flow rate, 10 arb; ion-spray voltage floating (ISVF), -2,800 V in negative mode and 3,500 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 (DDA) mode. The detection was carried out over a mass range of 70-1,050 m/z.

After UHPLC-MS/MS analyses, 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 (RT), 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 (RSD) of QC>30% were discarded. Following normalization procedures and imputation, statistical analysis was performed on log 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/>) and Metlin database (<https://metlin.scripps.edu/>). Concretely, the mass tolerance between the measured m/z values and the exact mass of the components of interest was ± 10 ppm. For metabolites having MS/MS confirmation, only the ones with MS/MS fragments score above 30 were considered as confidently identified.

Statistical analysis

Wilcoxon rank-sum test (Mann-Whitney U test) was adopted in comparison of two groups of alpha diversity (chao1, simpson and shannon index), genus and metabolites. Kruskal-Wallis (KW) sum-rank test was adopted in LEfSe analysis. Student's t-test was used to compare the difference of metabolites or predicted functions between two groups. The significant level in figures was marked as * ($0.01 < P \leq 0.05$), ** ($0.001 < P \leq 0.01$) and *** ($P \leq 0.001$). Most of the analyses were performed on the online platform of Majorbio Cloud Platform (www.majorbio.com).

Results

Alteration of cecal microbiome after S. Enteritidis infection

16S rRNA gene sequencing was performed on 41 samples of cecal contents (n = 12, 11, 9, 9 of CC, CT, RC and RT, respectively) and generated 2,439,579 high-quality sequence reads. The sequences were clustered into 942 operational taxonomic units (OTUs) (Additional file 1: Supplementary Table S1) with the threshold of 99% sequence identity. All of OTUs were taxonomically grouped into 10 phyla, 15 classes, 41 orders, 68 families, 125 genera and 201 species. Of phyla, Firmicutes had the most OTUs (794, 84.3%),

the next three were Proteobacteria (88, 9.3%), Bacteroidota (28, 3.0%) and Actinobacteria (12, 1.3%). The sole *Salmonella* strain was OTU607 of 942 OTUs and was only detected in CT and RT.

The rarefaction curve (Fig. 1) and Principal Co-ordinates Analysis (PCoA) (Fig. 2) showed that the amount of sequencing data was reasonable and the samples were gathered in groups. In comparison of CC and RC, there were 73 common species between CC and RC (Fig. 3; Additional file 2: Supplementary Table S2), which contributed to 97.5 (± 2.2 SD, $n = 12$) % and 97.3 (± 3.2 SD, $n = 9$) % of total abundance in CC and RC, respectively. The community diversity in Simpson and Shannon index had no significant difference (Figs. 4 and 5), though the community richness in Chao1 index of CC was higher than that of RC ($P=0.01$) (Fig. 6). Additionally, some dominant genus showed significant differences between CC and RC ($P < 0.05$), such as *Flavonifractor*, *Erysipelatoclostridium*, *Streptococcus*, *Klebsiella*, *Lachnospiraceae_NK4A136_group*, *Blautia* and *Lachnoclostridium* (Fig. 7). To prevent the batch effect, next analyses were performed within each cross.

LEfSe analysis can discover multi-dimensional biomarkers and omics features. As shown, 25 genera (23.4%) and 15 genera (17.2%) were altered ($P < 0.05$) compared to control in the Cross and in the Reverse-cross, respectively (Fig. 8A and B). Obvious differences could be seen between the reciprocal crosses. On family level, there were three co-differential families ($P < 0.05$), Erysipelotrichaceae, Lactobacillaceae and Eggerthellaceae. Only Erysipelotrichaceae altered in the same trend, and the other two altered in the opposite trend. On genus level, there were four co-differential genera ($P < 0.05$) *Tyzzarella*, *Negativibacillus*, *Lactobacillus* and *Salmonella*. Only *Salmonella* was consistent in trend, and the other three were contrary. Considering that *Salmonella* was the bacteria inoculated, that was to say, the alteration of cecal microbiome after *S. Enteritidis* infection was completely different. For the next correlation analysis, we merged the two parts of differential genera and obtained 36 genera (4 genera were overlapped) (Table 1). Among them, 11 genera (30.6%) were consistent in trend and 25 genera (69.4%) were contrary though some genera were insignificant in another cross.

Alteration of cecal metabolome after *S. Enteritidis* infection

Liquid chromatography-tandem mass spectrometry (LC-MS) was performed on 24 samples of cecal contents ($n = 4, 7, 6, 7$ of CC, CT, RC and RT, respectively, overlapped with the microbiome samples) and generated 22,358 mass spectrum peaks (Additional file 3: Supplementary Table S3). Therein, 1,468 peaks were annotated and used for analysis. Principal Component Analysis (PCA) (Fig. 9) and Venn analysis (Fig. 10) showed that the samples were gathered in groups and 1,306 metabolites were common in CC and RC, which accounted for 91.2% and 97.6% of their respective total number. The similarity within the Cross (1422/1450, 98.1%) or the Reverse-cross (1324/1381, 95.9%) was also high. According to the standards of Variable Importance in the Projection (VIP) > 1 and $P < 0.05$, 168 metabolites (11.6%) (Additional file 4: Supplementary Table S4) and 346 metabolites (25.1%) (Additional file 5: Supplementary Table S5) were screened in the Cross and in the Reverse-cross, respectively. There were 52 co-differential metabolites between the reciprocal crosses (Additional file 6: Supplementary Table S6). Of these, 31 and 21 metabolites were up- and down-regulated compared to control in the Cross, respectively;

it was 29 and 23 in the Reverse-cross. From another perspective, 14 metabolites (26.9%) altered in the same trend and 38 metabolites (73.1%) altered in the opposite trend.

Correlation between metabolites and microbes during *S. Enteritidis* infection

For further relationship between metabolites and microbes, the matrix was established based on the screened 52 metabolites and 36 bacterial genera (Fig. 11). The metabolites were divided into 4 classes (Class I-IV) according to the unsupervised clustering result and were labeled the up- or down-regulation with different colors. Notably, all the 11 co-upregulated metabolites (red) had positive correlation with *Salmonella* ($P < 0.05$), while all the 3 co-downregulated metabolites (blue) had negative correlation with it ($P < 0.01$). Another remarkable feature of Fig. 11 was that the correlation of Class I was almost completely contrary to Class II. For example, most metabolites of Class I were correlated positively with *Lactobacillus* and *Negativibacillus* ($P < 0.05$), while it was contrary in terms of Class II.

Phenotypic difference of phenylpropanoids between the reciprocal crosses after *S. Enteritidis* infection

Among the 52 co-differential metabolites, there were 11 phenylpropanoid metabolites (labeled A). Ten were upregulated and only 1 was downregulated compared to control in the Cross. On the contrary, it was 1 and 10 in the Reverse-cross, respectively. They were consistent when the search range was expanded to all the differential metabolites in each cross (not displayed). In a word, the phenylpropanoid metabolites were more accumulated in the Cross than the Reverse-cross.

For further information of a community's functional capabilities, we herein utilized PICRUSt approach to predict the functional composition of cecal microbiome (Additional file 7: Supplementary Table S7), and found it was not quite in line with the fact. For instance, there were three predicted functions related to phenylpropanoids biosynthesis, including the flavonoid biosynthesis, the flavone and flavonol biosynthesis and the phenylpropanoid biosynthesis (Fig. 12). They were enhanced by 2.7 folds ($P = 1.2E-03$), 4.2 folds ($P = 4.9E-04$) and 1.4 folds ($P = 0.009$) in the Cross compared to control, respectively, which was consisted with the actual result of the Cross. However, it was contradictory in the Reverse-cross. Most of the phenylpropanoid metabolites were decreased significantly in fact, while PICRUSt showed that the three pathways of phenylpropanoids biosynthesis were matched or enhanced ($P = 3.0E-05$) compared to control.

Phenotypic difference of lipids between the reciprocal crosses after *S. Enteritidis* infection

Furthermore, we noticed there were 23 lipid metabolites in the 52 co-differential metabolites. Ten were upregulated and 13 were downregulated compared to control in the Cross. It was 15 and 8 in the Reverse-cross, respectively. They were consistent when the search range was expanded to all the differential metabolites in each cross (not displayed). Thus the lipid metabolites were more accumulated in the Reverse-cross than the Cross. It was worth noting that there were two classes of bioactive lipids in them. The first class was lysophosphatidylcholines (lysoPCs), also named as 1-acyl-sn-glycero-3-phosphocholines, including lysoPC(17:0), lyso-PAF C-16 and lysoPC(0:0/18:0). All of them were increased

($P < 0.05$) in the Reverse-cross, but only 1 was increased ($P < 0.05$) and 2 were decreased ($P < 0.05$) in the Cross (Fig. 13). The second class was eicosanoids, including unoprostone, Δ^{17} -U-46619, 15(S)-HETrE and O-arachidonoyl ethanolamine (O-AEA). Three of them were increased ($P < 0.05$) and 1 was decreased ($P < 0.05$) in the Reverse-cross, but on the contrary, 1 was increased ($P < 0.05$) and 3 were decreased ($P < 0.05$) in the Cross (Fig. 14).

Discussion

As soon as entering the intestinal tract, *Salmonella* bacteria need to overcome the colonization resistance of microbiota, adhere and invade epithelial cells. The cellular events surrounding invasion and uptake of the bacteria by epithelial cells happen within minutes of contact with the mucosa [1]. *Salmonella* can cause inflammatory response via the notorious Type 3 Secretion System (T3SS), disturb microbiota and metabolism, and take opportunity for niches and nutrients [14–16]. In humans, *Salmonella* causes pathological inflammation; whereas, the same bacteria can be carried by poultry with virtually no ill effects on the host [17, 18]. Chicken and *S. Enteritidis* appear to have achieved a compromise that minimizes both the normal host response and the normal bacterial virulence in the long period of coevolution, which is considered as a kind of host defense strategy - tolerance [3].

After a comprehensive analysis to cecal microbiome, we found there were 23.4% and 17.2% of genera altered in response to *S. Enteritidis* infection in the Cross and in the Reverse-cross, respectively. In terms of cecal metabolome, 11.6% and 25.1% of metabolites were altered in the Cross and in the Reverse-cross, respectively. As chickens did not show any symptoms of illness, we speculated that this degree of alteration (20.3% of genera and 18.4% of metabolites in average) was within the tolerance of the hosts and would not likely affect their health.

Furthermore, we found none of the co-differential genera and only 26.9% of the co-differential metabolites showed same trend in alteration, while the larger proportion of co-differential genera and metabolites were opposite. On the premise of similar community diversity (Figs. 4 and 5), common species with over 97% abundance and common metabolites with over 91% numbers (Fig. 10) between two controls of reciprocal crosses, such a wide divergence after *S. Enteritidis* infection seemed unreasonable. We thought that the greater possibility might be attributed to the different immune responses of the host, despite the batch effect due to separate experiments. It should be pointed out that even if the experiments were carried out at the same time, the farm condition of the two parent populations should be identical first, because the maternal or environmental microbiota can be transferred to offspring via oviduct during egg formation [19, 20] or via contaminants during hatching [21]. In order to eliminate the batch effect as much as possible, we only selected the co-differential metabolites between the reciprocal crosses, which were speculated to represent some common or important metabolic reactions during *S. Enteritidis* infection. This approach was not suitable for microbiome analysis for only four co-differential genera between two crosses. We chose the merged 36 genera to correlate with the 52 co-differential metabolites.

The correlation analysis, function prediction of microbiome and function analysis of metabolites were performed to explore the nature of biology. In Fig. 11, we found all the co-upregulated metabolites (red) were correlated with *Salmonella* positively and all the co-downregulated metabolites (blue) were correlated with it negatively, which seemed to be consistent with the growth of *Salmonella* or even a strategy for its infection. For instance, the increase of 5-Formiminotetrahydrofolic acid, a kind of tetrahydrofolic acids which are essential for bacteria growth [22], might be beneficial for or accompanied with *Salmonella* multiplication. The decrease of 10-Oxo-11-octadecen-13-olide, a kind of macrolides which are generally considered toxic and antibiotic [23], should facilitate *Salmonella* colonization.

Additionally, most metabolites of Class Ⅱ were correlated positively with *Lactobacillus* and *Negativibacillus*, while it was contrary in terms of Class Ⅲ. *Lactobacillus* species are regarded as a kind of probiotics, which can antagonize the gastroenteric pathogens and immunomodulate human cells to reduce inflammations [24]. *Negativibacillus* is a kind of commensal bacteria, which was first isolated from human left colon with weak pathogenicity of anaerobic, catalase and oxidase negative, non-motile and not haemolytic properties [25]. We herein used *Lactobacillus* and *Negativibacillus* as biomarkers of caeca. According to biomarkers' phenotype, the cecal condition of the Cross was better than the Reverse-cross.

We found that the phenylpropanoid metabolites were more accumulated in the Cross than the Reverse-cross. Phenylpropanoids are a group of plant secondary metabolites derived from phenylalanine and have a wide variety of functions both as structural and signaling molecules (KEGG), including certain main classes of phenolic acids, flavonoids, stilbenes and lignans, most of which are generally involved in defense against ultraviolet radiation or aggression by pathogens in plant [26]. The beneficial functions in animal also have been identified, such as anti-oxidative, anti-inflammatory and anti-microbial of cinnamic acid [27], flavonoids [28], isoflavones [29–31] and stilbenes [32]. Most phenylpropanoids are poorly absorbed in the small intestine and pass into the hindgut [26], where they can be extensively metabolized by microbiota and release their bioactivity [33–35]. In Fig. 11, the probiotic effect could be reflected on the significant positive correlation between biomarkers and six phenylpropanoid metabolites (labeled Ⅱ in Class Ⅱ), all of which were increased in the Cross while decreased in the Reverse-cross. This fact further supported our opinion that the cecal condition of the Cross was better than the Reverse-cross because the normal microbiota were functioning.

Phenylpropanoid metabolites were increased in the Cross after *S. Enteritidis* infection, which was consistent with the PICRUST prediction. However, it was contradictory in the Reverse-cross. PICRUST showed that the pathways of phenylpropanoids biosynthesis were matched or enhanced compared to control (Fig. 12), but the fact was significant decrease of most phenylpropanoid metabolites. Maybe there existed some factors hindered the biosynthesis of phenylpropanoids in the Reverse-cross, which would be concerned in the end.

We also noticed the lipid metabolites were more accumulated in the Reverse-cross than the Cross. Importantly, there were two classes of bioactive lipids, lysoPCs and eicosanoids, which might be crucial in

inflammatory process. LysoPCs play as a kind of strong pro-inflammatory mediators, and may be responsible for various cellular processes such as regulation of monocyte adhesion molecule expression, chemoattractant properties, and monocyte proinflammatory cytokine secretion [36]. Furthermore, lysoPCs enhance arachidonic acid (AA) release from membrane phospholipid via phospholipase A₂ (PLA₂) in a time- and concentration-dependent manner [36–38]. AA is a substrate for the biosynthesis of several groups of lipid mediators collectively termed eicosanoids including prostaglandins (PGs), which were catalyzed by cyclooxygenases-1 (COX-1)/COX-2 [39]. PGs appear in the onset of inflammation and function as pro-inflammatory factors [40]. As a rate-limiting step [37], the enhancement of AA release from membrane phospholipids by lysoPCs (Fig. 13) could accelerate the synthesis of eicosanoids. Metabolite unoprostone, an analog of PGF_{2α} [41], and Δ^{17} -U-46619, an analog of thromboxane A₃ (TxA₃) [42], both belong to PGs and generally exert pro-inflammatory effect. At the same time, 15S-HETrE antagonizes the production of prostaglandins by suppressing COX-2 over-expression/PGE₂ biosynthesis [43]. The simultaneous increase of Δ^{17} -U-46619 and 15(S)-HETrE in the Reverse-cross (Fig. 14) might be a self-protection mechanism, because the excessive production of eicosanoids can lead to pathological injury [44].

The most fascinated compound O-AEA, also known as virodhamine, is a constitutional isomer of the well-characterized anti-inflammatory anandamide [*N*-arachidonyl ethanolamide (AEA)]. AEA belongs to a class of endocannabinoids (eCBs) which act on the same receptors (CB1 and CB2) as tetrahydrocannabinols. To date, it has been determined that O-AEA acts as a full agonist at the CB2 receptor and a partial agonist at the CB1 receptor. Interestingly, the CB1 receptor is primarily located in the brain and central nervous system, while the CB2 receptor is predominantly located in peripheral tissues and macrophages playing key role in inflammation [45]. O-AEA might function as an anti-inflammatory factor during *S. Enteritidis* infection in the Reverse-cross.

Overall, it seemed that there was a higher level of inflammation in the Reverse-cross than the Cross because more lysoPCs and eicosanoids were increased in the former. The inflammations of two crosses might be regulated in different modes, one of which was through 15S-HETrE and O-AEA in the Reverse-cross, and the other was through phenylpropanoids in the Cross. Violent inflammation can cause intestinal damage. Two functional compounds, choline and *N*[(3a,5b,7b)-7-hydroxy - 24-oxo-3-(sulfooxy)cholan-24-yl]-Glycine, which are involved in bile acid cycle and should be absorbed in the intestine, were found increased in the Reverse-cross but not in the Cross. Their abnormal rise indicated the reduction of absorption and enrichment of nutrients, which could lead to the decrease of pH and enzyme activity. This might be the reason why phenylpropanoid metabolites decreased but PICRUST prediction showed not in the Reverse-cross.

Conclusions

S. Enteritidis can colonize chicken caeca with weak pathogenicity, even for 2-day-old chicks in our experiment. After comprehensive analyses of cecal microbiome and metabolome, we found *Salmonella*

could take advantage of the alteration of partial metabolites to colonize and multiply, which might be caused by reprogramming the metabolic reaction of host or microbiota. On average, 20.3% of genera and 18.4% of metabolites in chicken caeca were disturbed by *S. Enteritidis*. This degree of alteration was within the tolerance of the hosts and would not likely affect their health. Between the reciprocal crosses, all the co-differential genera and 73.1% of the co-differential metabolites showed opposite trend in alteration. Such a wide divergence after infection was analyzed to be more attributed to the different immune responses. In-depth analysis manifested that a more extensive inflammation might be triggered in the Reverse-cross than the Cross, and the regulatory modes were different. It seemed that the former upregulated pro- and anti-inflammatory factors simultaneously, while the latter was aided with the phenylpropanoids produced by intestinal bacteria. Our research provided a valuable exploration and framework in understanding the interplay between host, pathogen and microbiota, and the effect of reciprocal crosses/host genetic background on *Salmonella* resistance.

Declarations

Availability of data and materials

The obtained 16S rRNA gene sequences were submitted to the China National Center for Bioinformation under the accession number CRA004605.

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Authors' contributions

L-YL and X-YL planned and designed the research and experiments. GH, X-XM, H-LL, L-WL and Y-ND undertook the field work and processing of samples. GH, X-XM, Y-NZ and Y-NP performed the experiments and analyzed the data. GH, L-YL, and X-YL wrote the paper. All authors read and approved the final manuscript.

Ethics approval

All experiments were approved by the Shandong Agricultural University Animal Care and Use Committee (Approval Number: # SDAUA-2018-058) and performed in accordance with China animal welfare laws.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Rarefaction curves of 41 samples

Figure 2

PCoA on OTU level of 41 samples

Figure 3

Venn on specie level of CC, CT, RC and RT

Figure 4

Simpson index on OTU level of CC, CT, RC and RT

Figure 5

Shannon index on OTU level

Figure 6

Chao1 index on OTU level

Figure 8

LEfSe cladograms of the Cross (A) and the Reverse-cross (B)

Figure 9

PCA of 24 samples

Venn plot

Figure 10

Venn of 1,468 annotated metabolites in CC, CT, RC and RT

Figure 12

PICRUST prediction

Figure 13

LysoPCs phenotype

Figure 14

Eicosanoids phenotype

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