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VETERINARY MICROBIOLOGY - RESEARCH PAPER

Shifts in microbiota and gene expression of nutrient transporters, mucin and interleukins in the gut of fast-growing and slow-growing chickens infected by *Salmonella* Enteritidis

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Abstract

Slow-growing breeds are more resistant to *Salmonella* infection compared to fast-growing broilers. However, it is unclear whether that is associated with innate resistance or rather rely on differences in *Salmonella*-induced gut responses. We investigated the microbial composition and gene expression of nutrient transporters, mucin, and interleukin in the gut of a fast-growing (Cobb500) and a slow-growing naked neck (NN) chicken breeds challenged with *Salmonella* Enteritidis. Hatchlings were inoculated at two days of age using sterile broth (sham) or *Salmonella* Enteritidis (SE) and distributed according to a completely randomized design into four treatments: Cobb-sham; Cobb-SE; NN-sham; and NN-SE. Cecal SE counting and microbial composition by 16 S rRNA sequencing were determined at 24-, 96-, and 168-hours post-inoculation (hpi). Gene expression of amino acid (*Asct1*) and peptide transporters (*PepT1*), glucose transporters (*Sglt1*, *Glut2* and *Glut5*) and mucin (*Muc2*) in the jejunum and expression of interleukins (*IL1 beta*, *IL8*, *IL17* and *IL22*) in the cecum was assessed by qPCR at 24 and 168 hpi. NN birds were colonized by SE just as Cobb birds but showed innate upregulation of *Muc2*, *IL8* and *IL17* in comparison to Cobb. While nutrient transporter mRNA expression was impaired in SE-challenged Cobb birds, the opposite was observed in NN. There were no differences in microbial diversity at different sampling times for Cobb-SE, whereas the other groups had higher diversity and lower dominance at 24 hpi compared with 96 hpi and 168 hpi. NN birds apparently develop earlier gut microbial stability, have higher basal level of mucin gene expression as well as differential nutrient transporter and interleukin gene expression in the presence of SE which might mitigate the effects of SE infection compared to Cobb birds.

Keywords Broiler genotype · Gut microbiota · Gene expression · Interleukin · Intestinal transporter

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Introduction

Gut health is crucial to support animal performance because of greater ability to utilize and metabolize dietary nutrients. The uptake of protein and carbohydrate digestion products in the intestine relies on transporters present on enterocytes. Apical transporters involved in the absorption of proteolysis products include *Pept1* (peptide transporter, SLC15A1) and *Asct1* (alanine, serine, cysteine, threonine transporter, SLC1A4), and there are many transporters located in the basolateral membrane for specific amino acids [1–4]. Glucose and fructose transporters are *Sglt1* (sodium-dependent glucose transporter; SLC5A1) and *Glut5* (fructose transporter, SLC2A5) in the apical membrane, and *Glut2* (glucose transporter, SLC2A2) in the basolateral membrane [3, 5].

Besides competing for nutrients, enteric pathogens may damage the epithelium, negatively affecting digestion and absorption [6] and consequently, compromising performance. *Salmonella enterica* is one of the most important zoonotic agents associated with the consumption of poultry products worldwide [7], and Enteritidis (*S.* Enteritidis, SE) is one of the leading serovars involved in foodborne salmonellosis outbreaks in humans [8]. *Salmonella* infection in the birds is age dependent [7], probably related to the development of the immune system and lower leukocyte population in the gut lamina propria [9].

Birds selected for fast growth and high production may become more susceptible not only to respiratory and cardiovascular diseases [10, 11], but also to infectious diseases, whereas slow-growing birds have genetic elements associated with improved resistance against pathogens [12]. Susceptibility might be associated with differences in immunological mechanisms between fast- and slow-growing birds. While the activation of macrophages and T cells favoring the oxidative process is observed in meat-type chickens both in homeostasis and after sanitary challenges [13], slow-growing birds have a rapid pro-inflammatory response, with greater heterophil numbers, as observed in Fayoumis birds, a rustic African breed showing resistance against *S.* Enteritidis infection [14]. Fast-growing birds inoculated with *S.* Enteritidis showed higher bacterial counts in the liver and more evident weight loss compared with slow-growing birds [15]. In addition to genetic background, different response patterns are affected by age, the organ or intestinal segment, native intestinal microbiota, diet, and type of pathogen [16, 17]. Therefore, comparative physiological, immunological and microbiological investigations considering slow- and fast-growing birds could shed light on important drivers affecting gut health in modern poultry production.

Previous studies in our laboratory have evidenced a less compromised performance of naked neck birds challenged with SE than Cobb birds, even though SE colonization was similar in both breeds (non-published results). It is hypothesized that these results are not associated with innate resistance of naked neck birds against SE but rather rely on putative differences in *Salmonella*-induced gut responses between slow and fast-growing birds. Therefore, this study assessed the *Salmonella* counts, microbial composition and expression of interleukin genes in the cecum of Cobb and naked neck (NN) two-day-old chicks inoculated with SE or nutrient broth (sham), as well as mucin and nutrient transporter gene expression in the jejunum.

Materials and methods

Animals and management practices

Cobb500 and naked neck fertile eggs ($n = 120$/each) were incubated at 37.7°C and relative humidity of 60%. Eggs were automatically turned every two hours and candling was performed at 11 days to discard dead embryos and infertile eggs. After hatch, individual hatchling weight was recorded, and the negative *Salmonella* status was confirmed using cloacal swabs from 20 birds per incubator. Hatchlings ($n = 50$/breed) with similar average weight were then distributed in a completely randomized design according to 2×2 factorial with two breeds (Cobb and naked neck, NN) and two inoculation conditions (*Salmonella* Enteritidis culture - SE; or sterile nutrient broth - sham). In this study, each bird was considered the experimental unit, since animals from a treatment were submitted to the same environment and conditions. Therefore, there were four treatments: Cobb-sham; Cobb-SE; NN-sham; and NN-SE.

Birds were individually identified with leg bands and kept in boxes with a minimum area of at least 0.05 m^2 per bird (1.25 m x 1 m). Boxes were covered with nylon to avoid *Salmonella* cross-contamination between boxes by flies and other vectors. Water and food were provided *ad libitum*, and environment and management conditions were similar for both breeds. Room temperature and relative humidity were monitored with thermo-hygrometers (Oregon Scientific, Portland, USA). Corn and soybean meal-based diets were formulated with 22.20% CP, 2,950 kcal/kg ME, 1.31% digestible lysine, 0.94% digestible methionine + cystine and 0.852% digestible threonine in the initial phase (1 to 10 d) according to Rostagno et al. [18].

All management practices, as well as slaughter and sampling procedures were approved by the Ethical Committee for the Use of Animals from Universidade Federal da Paraiba (protocol 186/15) in compliance with the National Council for Animal Experimentation Control – CONCEA (Federal Law nº 11.794/08) as established in Art. 225 of the Brazilian National Constitution on the guidance for the use of animals for scientific purposes.

Inoculation and bacterial counts

Inoculation was performed according to Moreira Filho et al. [19]. All birds in each box were inoculated at 2 days of age (d) into the crop using either 0.5 mL sterile nutrient broth or 0.5mL of nalidixic acid-resistant *Salmonella* (1.6×10^9 CFU/mL). Cecal contents were sampled individually from five birds per treatment for bacterial counts when birds were 3d (24 hpi), 6d (96 hpi) and 9d of age (168 hpi). Samples were weighed and serially diluted with PBS pH 7.4 and twenty-microliter aliquots from each dilution were streaked onto brilliant green agar with nalidixic acid (100 µg/mL), followed by incubation at 37ºC for 24 h. Colonies were counted and values were expressed as

colony forming units per gram of cecal content (CFU/g). The 72 h-interval between samplings was chosen based on the epithelial turnover rate in chickens [20].

Microbial composition analyses

Total DNA was extracted from cecal contents sampled individually from three birds at 3d (24 hpi), 6d (96 hpi) and 9d of age (168 hpi) using a commercial kit (PowerSoil DNA Isolation, Qiagen, Germany) following the provided protocol. The microbial 16S rRNA gene (V3-V4 region) was amplified using the primers 341F and 785R (5'TCG TCGGCAGCGTCAGATGTGTATAAGAGACAGCCT ACGGGNGGCWGCAG-3' and 5'GTCTCGTGGGCTCGG AGATGTGTATAAGAGACAGGACTACHVGGGTATCTA ATCC-3'). Polymerase chain reaction (PCR) conditions were 95ºC for 3 min, followed by 25 cycles at 95ºC for 30 s, 55ºC for 30 s, and 72ºC for 30 s and a final extension to 72ºC for 5 min. Library preparation was performed *as per* the standard Illumina 16 S rRNA gene protocol. Amplification products were evaluated in 1.5% agarose gel, purified using AMPure beads (Beckman Coulter, USA) and quantified by fluorometry (Qubit2.0, Life Invitrogen, USA). Quality was assessed using a capillary electrophoresis system (Fragment Analyzer, Agilent, USA) before sequencing in the Illumina MiSeq with Illumina V2 kit (2 × 250 cycles).

Demultiplexed paired-end reads in fastq format were processed using QIIME2 [21]. Sequences were joined, selected by size (> 240 bp), quality filtered (minimum Phred score > 20) and dereplicated using VSEARCH. Chimeras were removed using UCHIME. *De novo* clusterization with 99% of similarity was performed to obtain the amplicon sequence variants (ASVs). The sample with lowest number of sequences was used to standardize the number of sequences per sample. Taxonomic classification was attributed using the Naïve Bayes method with SILVA database (https://www.arb-silva.de/) with 99% for region V3-V4. Alpha and beta diversity were assessed with phyloseq and DESEq2 packages in R [22]. Multiple groups were analyzed with STAMP (https://beikolab.cs.dal.ca/software/STAMP), using the Farthest Neighbor and ANOVA with significance level of 5%.

Gene expression analyses

Jejunal and cecal mucosa was individually sampled at 24 and 168 hpi from five birds per treatment, snap-frozen and kept at -80ºC. Total RNA was isolated from individual jejunum samples using the RNeasy Mini kit (Qiagen, Germany). Concentration and purity were determined at 260/280 and 260/230 using a microvolume spectrophotometer (Colibri, Titertek-Berthold, Germany). Reverse transcription was performed with AffinityScript QPCR cDNA Synthesis Kit (Agilent, USA) and relative gene expression was determined by real time PCR using Brilliant III Ultra-Fast SYBR QPCR Master Mix (Agilent) according to provided guidelines. Cycling was carried out in a Stratagene Mx3005P thermocycler (Agilent). Primer sequences targeting sodium-dependent glucose transporter (*Sglt1*), glucose transporter (*Glut2*), fructose transporter (*Glut5*), peptide transporter (*Pept1*), alanine, serine, cysteine, threonine transporter (*Asct1*), interleukin 1 beta (*IL1-beta*); interleukin 8 (*IL8*); interleukin 17 (*IL17*); interleukin 22 (*IL22*) and reference genes (glyceraldehyde-3-phosphte dehydrogenase/*Gapdh*, and hydroxymethylbilane synthase/*Hmbs*) are shown in Online Resource 1.

Statistical analyses

Bacterial counts (CFU/g) were transformed in Log10 to be analyzed in a completely randomized design, comparing the groups of SE-inoculated birds of the two genotypes ($n = 5$ per breed per sampling point). Relative gene expression was calculated using the method $2^{-\Delta\Delta Ct}$ [23]; Ct values of each sample were standardized for the reference gene *Gapdh*. Gene expression data was analyzed in each post-inoculation sampling, considering four treatments in a 2 × 2 factorial (Cobb or NN; SE or sham), and each bird as repetition ($n = 5$ per tissue per treatment). A significant interaction indicates that changes in gene expression caused by SE-inoculation are different between breeds and/or indicate that, within a single breed, SE-inoculation will affect gene expression. Thus, when there was interaction, the four treatments were compared by Tukey's test at 5% of probability. When there was no interaction, means were compared either between breeds (independent of inoculation) or between inoculation treatments (independent of breed), using Tukey's test at 5% probability.

Results

Salmonella cecal counts

Salmonella counts were not different between breeds at 24 and 96 hpi ($p > 0.05$). At 168 hpi, counts were lower in NN birds ($p < 0.05$; Fig. 1). All sham-inoculated birds showed negative results for SE in all sampling periods.

Gene expression

The statistically significant results of gene expression are shown in Table 1 and complete gene expression data is shown in Online Resource 2. Data with significant interaction ($p < 0.05$) were further analyzed to show differences in gene expression caused by SE-inoculation within a single breed and/or differences between breeds within each inoculation group (Table 2).

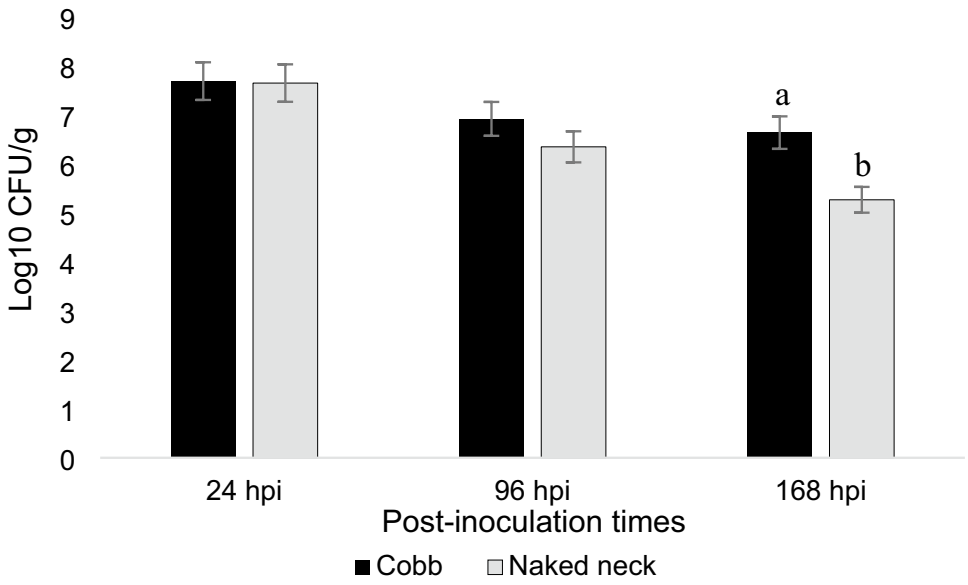

Fig. 1 Cecal bacterial count (CFU/g) of Cobb and naked neck birds inoculated with *Salmonella* Enteritidis. Within each evaluation period, differences between breeds are indicated by different letters

Table 1 Gene expression at 24 and 168 h post-inoculation (24 hpi and 168 hpi) in Cobb and naked neck birds inoculated with nutrient broth (sham) or *Salmonella* Enteritidis at 2 days of age

Breed	*Muc2* (24 hpi)	*IL1 beta* (24 hpi)	*Glut2* (168 hpi)	*Glut5* (168 hpi)	*Pept1* (168 hpi)	*IL8* (168 hpi)	*IL17* (168 hpi)
Cobb	1.081	1.013	1.487^{b}	0.845	0.451^{b}	1.284	0.990
Naked neck	1.245	1.339	2.586^{a}	1.047	1.263^{a}	1.622	1.351
Inoculation							
Sham	0.819^{b}	0.836^{b}	1.922	1.155^{a}	1.082	1.167^{b}	1.018
Salmonella	1.507^{a}	1.517^{a}	2.152	0.737^{b}	0.632	1.739^{a}	1.322
SEM	0.189	0.229	0.253	0.143	0.178	0.202	0.181
Factor							
Breed (B)	0.5439	0.323	0.0041^{Z}	0.3248	0.0028	0.2431	0.1673
Inoculation (I)	0.0141^{Z}	0.042	0.5250	0.0455	0.0827	0.0523^{Z}	0.2431
B * I	0.0014	0.921	0.0002	0.9051	0.5911	0.0108	0.0742

Cytokines were analyzed in the cecum and mucin and transporters in the jejunum. Complete table is presented as Online Resource 2

$^{a, b}$Different letters within the column indicate differences between the means by Tukey's test

^{Z}These differences are analyzed and discussed within each main factor due to significant interaction (refer to text and Table 2)

Table 2 Gene expression of *Muc2* (jejunum, 24 hpi), *Glut2* (jejunum, 168 hpi), *IL8* and *IL17* (cecum, 168 hpi) in Cobb and naked neck birds inoculated with nutrient broth (sham) or *Salmonella* Enteritidis at 2 days of age

		Muc2 (24 hpi)		*Glut2* (168 hpi)	
	Breed	Sham	*Salmonella*	Sham	*Salmonella*
Jejunum	Cobb	0.277^{bB}	1.886^{aA}	2.109^{aA}	0.866^{bB}
	Naked neck	1.362^{aA}	1.128^{aA}	1.735^{bB}	3.438^{aA}
	SEM	0.267		0.358	
		IL8 (168 hpi)		*IL17* (168 hpi)	
	Breed	Sham	*Salmonella*	Sham	*Salmonella*
Cecum	Cobb	0.614^{bB}	1.953^{aA}	0.602^{bB}	1.377^{aA}
	Naked neck	1.719^{aA}	1.525^{aA}	1.434^{aA}	1.267^{aA}
	SEM	0.285		0.252	

Cytokines were analyzed in the cecum and mucin and transporters in the jejunum

$^{a, b}$For each gene, small letters indicate difference between breeds within each inoculation group (in the column)

$^{A, B}$For each gene, capital letters indicate difference between inoculation within each breed (in the row)

There was no interaction between factors on the expression of cytokines in the cecum and transporters in the jejunum at 24 hpi (Table 1), therefore, inoculation and breeds are discussed separately. *IL1 beta* ($p = 0.042$) expression was upregulated when the birds were inoculated with *Salmonella*, but there was no effect of genotype ($p = 0.323$). These effects were not seen at 168 hpi. Neither breed nor inoculation affected *IL8*, *IL17* and *IL22* at 24 hpi, or *IL1 beta* and *IL22* at 168 hpi.

There was interaction between breed and inoculation for *Muc2* ($p = 0.0014$) at 24 hpi and for *Glut2* ($p = 0.0002$), *IL8* ($p = 0.011$) and *IL17* ($p = 0.074$) at 168 hpi (Table 2). The analysis within each factor evidenced that *Muc2* expression in Cobb-sham birds was lower than NN-sham birds (0.277 vs. 1.362) and was upregulated when Cobb birds were inoculated with SE (1.886). In naked neck birds, *Muc2* expression was similar between sham- and SE-inoculated animals (1.362 vs. 1.128). Similarly, at 168 hpi, *IL8* and *IL22* were upregulated in Cobb-SE compared with Cobb-sham birds (3X higher and 2X higher, respectively), whereas expression was not changed between sham- and NN-SE birds at 168 hpi. *IL8* and *IL22* expression in Cobb-sham was also lower when compared with NN-sham birds (3X and 2X lower, respectively) (Table 2). *Glut2* expression was higher in Cobb than in naked neck when birds were sham-inoculated (2.109 vs. 1.735). *Muc2* expression significantly decreased in Cobb-SE (0.866, 2X lower) and significantly increased in NN-SE birds (3.438, 2X higher) when compared to the sham-inoculated birds within the same breed (Table 2).

Microbial composition analyses

Ten different phyla and 192 genera were identified considering all groups. These data are presented in Online Resources 3 and 4.

Alpha diversity analyses evidenced that Cobb-sham, NN-sham and NN-SE had lower ($p < 0.05$) observed ASVs and Shannon indexes and higher ($p < 0.05$) dominance index at 24 hpi when compared to 96 hpi and 168 hpi. There were no differences in ASVs, Shannon or dominance indexes at different sampling times for Cobb-SE (Table 3).

Differential abundance analyses were carried out for genera with significant statistical difference ($p < 0.05$) between sampling times within each treatment (Figs. 2 and 3, Online Resources 5–8). In Cobb-sham chicks, abundance of *Dickeya* and unclassified Enterobacterales abundance was higher at 24 hpi when compared with 96 and 168 hpi. The abundance of *Erysipelatoclostridium*, *Flavonifractor*, *Lactobacillus*, *Sellimonas*, *Streptococcus* and *Tepidibacter* abundance was higher at 96 hpi. Finally, at 168 hpi, higher abundance was seen for [Ruminococcus]_torques_group, *Anaerostignum*, *Anaerostipes*, *Anaerotruncus*, *Butyricicoccus*, *Candidatus*_Arthromitus, *Lachnoclostridium*, Lachnospiraceae_unclassified, *Negativibacillus*, *Oscillibacter*, Oscillospiraceae_unclassified, Oscillospiraceae_uncultured, Ruminococcaceae_Incertae_Sedis and Ruminococcaceae_unclassified.

Cobb-SE presented higher abundances of the genera *Clostridium*_sensu_stricto_1, Enterobacterales_unclassified, *Epulopiscium* and *Escherichia-Shigella* at 24 hpi. [*Clostridium*]_methylpentosum_group, *Clostridioides* and *Sellimonas* abundances were higher at 96 hpi. Clostridia_vadinBB60_group, Ruminococcaceae_Incertae_Sedis and *Tyzzerella* abundances were higher at 168 hpi.

At 24 hpi, NN-sham birds had higher abundances of the genera *Acinetobacter*, *Dickeya* and Enterobacterales_unclassified, while at 96 hpi, higher abundances of Lachnospiraceae_CHKCI001, *Lactobacillus*, *Sellimonas* and *Tepidibacter* were observed. Genera [Ruminococcus]_gauvreauii_group, *Anaerostignum*, *Colidextribacter*, Lachnospiraceae_UCG-010, *Negativibacillus*, *Oscillibacter* and Ruminococcaceae_Incertae_Sedis showed higher abundances at 168 hpi.

Only Enterobacterales_unclassified had higher abundance at 24 hpi compared with 96 and 168 hpi in NN-SE chicks. Abundance at 96 hpi was higher for *Anaerostipes*, *Clostridioides*, *Lactobacillus* and Oscillospirales_unclassified. At 168 hpi, abundance was higher for *Anaerostignum*, *Bifidobacterium*,

Table 3 Alpha diversity indexes observed at 24, 96, and 168 h post-inoculation (24 hpi, 96 hpi and 168 hpi) in Cobb and naked neck (NN) birds inoculated with nutrient broth (sham) or *Salmonella* Enteritidis (SE) at 2 days of age

		Cobb-sham			Cobb-SE	
	24 hpi	96 hpi	168 hpi	24 hpi	96 hpi	168 hpi
Observed ASVs	53.6^{b}	105.0^{a}	136.0^{a}	98.4^{a}	139.4^{a}	146.2^{a}
Shannon	2.9^{c}	3.9^{b}	4.3^{a}	3.7^{a}	4.2^{a}	4.2^{a}
Dominance	0.08^{a}	0.03^{b}	0.02^{b}	0.05^{a}	0.03^{a}	0.03^{a}
		NN-sham			NN-SE	
	24 hpi	96 hpi	168 hpi	24 hpi	96 hpi	168 hpi
Observed ASVs	54.6^{b}	122.2^{a}	147.4^{a}	80.6^{a}	163.4^{ab}	174.6^{b}
Shannon	2.8^{b}	3.8^{a}	4.0^{a}	3.3^{b}	4.2^{a}	4.4^{a}
Dominance	0.10^{a}	0.05^{b}	0.04^{b}	0.07^{a}	0.03^{b}	0.03^{b}

Complete description of microbial genera and phyla is presented in Online Resource 3

$^{a, b}$For each treatment group, small letters indicate difference between post-inoculation times in the row

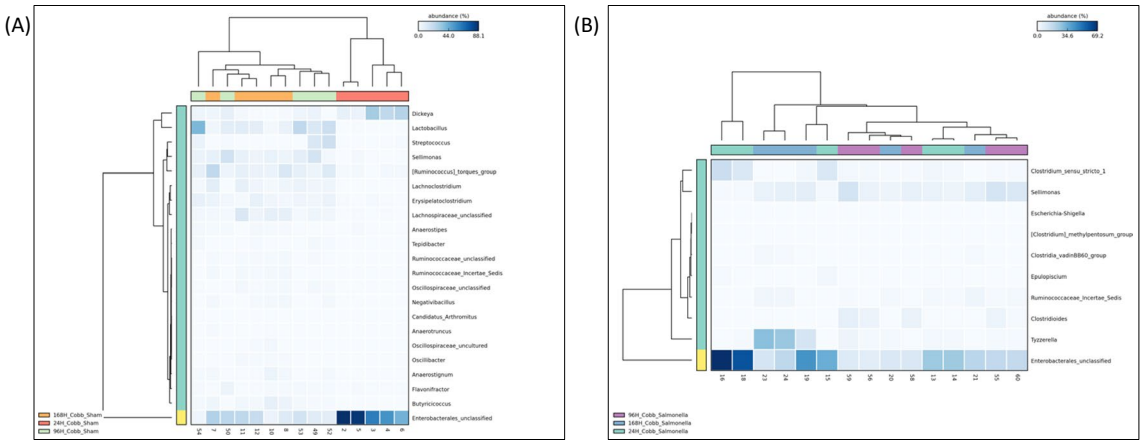

Fig. 2 Differential abundance in Cobb chicks at 24, 96 and 168 hpi. **A** Sham-inoculated. **B** *Salmonella*-inoculated

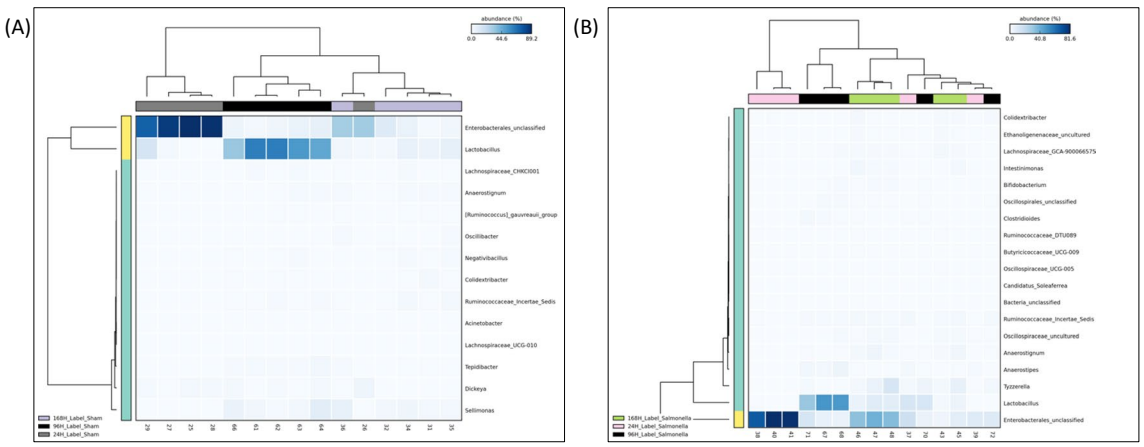

Fig. 3 Differential abundance in naked neck chicks at 24, 96 and 168 hpi. **A** Sham-inoculated. **B** *Salmonella*-inoculated

Butyricicoccaceae_UCG-009, Candidatus_Soleaferrea, *Colidextribacter*, Ethanoligenenaceae_uncultured, *Intestinimonas*, Lachnospiraceae_GCA-900,066,575, Oscillospiraceae_UCG-005, Oscillospiraceae_uncultured, Ruminococcaceae_DTU089, Ruminococcaceae_Incertae_Sedis and *Tyzzerella*.

Discussion

Salmonella counts in the cecum and gene expression

Differences in gastrointestinal ontogeny between fast- and slow-growing birds have been reported earlier and correlated to lower colonization and faster clearance of *Salmonella* in the latter [16]. Mucin is one of the major components of the mucosal barrier and has a fundamental role in the prevention of pathogen invasion [24]; it is a simple and effective measure to prevent *Salmonella* adhesion and provide clearance by peristalsis [7]. The higher expression of *Muc*2 in the jejunum of NN-sham birds indicate an innate ability for clearance of bacteria and may be responsible for the lower counts in SE-inoculated birds at 168 hpi. This assumption is corroborated by the fact that, in Cobb breed, increased expression of *Muc*2 was observed only in SE-challenged birds. The lower SE counts (Fig. 1) are in line with the *Muc*2 expression data and indicate that the more rustic slow-growing breed might use interrelated mechanisms that improve the response to the pathogen such as mucin production.

Further evidence of the importance of mucin for gut protection is that mucin production is started as early as 20–21 days of incubation with an increased density of goblet cells [25]. A steady number of goblet cells was reported in the duodenum of Cobb birds at hatch, whereas the density of goblet cells increased until 4 days of age, suggesting that the mucus layer must be well-formed earlier to provide a protective barrier in the newly hatched chick against oral infection by pathogens [26].

Forder et al. [27] have reported lower *Muc*2 expression in Cobb birds challenged with *Eimeria* spp. and *Clostridium perfringens*, suggesting an impairment of the mucosal activity with time due to the deterioration of the intestinal mucosa, resulting in less replenishment of the mucus layer and greater susceptibility to other bacterial infections. The expression of *Muc2* was more prominent in the naked neck birds in the present study and was not changed in this breed in the presence of SE; on the other hand, *Muc2* expression increased approximately five times in Cobb-SE birds. These apparently contradictory responses might be explained by different inoculation ages, intestinal segments or by different effects of the bacterial agents on the mucus layer. *C. perfringens* and *Eimeira* spp. cause a more severe enteritis and greater desquamation of epithelial and goblet cells, decreasing *Muc*2 expression and consequently increasing the probability of new infections [27, 28].

Prominent morphophysiological changes occur in the end of incubation and first days after hatch, enabling birds to properly digest and absorb exogenous food. Studies on the gene expression of intestinal transporters in slow-growing birds compared to fast-growing breeds are scarce. On the other hand, the expression of intestinal transporters is vastly reported in Cobb birds considering factors such as diet supplementation, feed restriction and age [29–31]; however, no changes were reported in *Glut*2 expression in these previous studies. In the results presented herein, the downregulation in *Glut*2 expression in Cobb-SE compared to Cobb-sham birds might reflect greater mucosal damage, whereas upregulation was seen in naked neck birds. According to Gilbert et al. [5], the upregulation of transporter genes is probably related to a greater ability of absorption of its substrate. Thus, glucose and peptide absorption might be improved in naked neck chicks in the presence of SE as part of the response against the pathogen. Downregulation of *Asct1* and *Eaat3* mRNA levels has been reported in *Clostridium perfringens*-challenged birds, suggesting reduced uptake of amino acids and glutamate, the latter an energy source, which might respond for changes in morphology and growth performance [32]. The authors also reported downregulation of *Glut*2 in *Eimeria*-challenged birds, similar to our findings.

Diet utilization depends not only on these complex physiological changes, but also on the establishment of a commensal microbiota that is essential for gastrointestinal homeostasis. In this sense, the gut immune system must be able to differentiate between putatively deleterious antigens and inoffensive antigens, such as those from commensal bacteria and dietary proteins, and gut health depends on the balance between response and tolerance [33, 34]. The inflammatory response is minimized to reduce negative effects on the host health [35], and disease tolerance in the gut evolves concomitantly with microbiota development, thus, proinflammatory cytokine levels are seen as a specific defense mechanism of the host.

The cecum of newly hatched birds shows greater abundance of heterophils in the lamina propria, followed by macrophages and T lymphocytes. Macrophage and heterophils play a key role on the response to *Salmonella* and are directly related to pro-inflammatory cytokine expression (*IL1 beta* and *IL8*) [7]. These pro-inflammatory mediators activate the recruitment of leukocytes in the developing gut [36], a process that lasts one to two weeks during the development of the immune system. Inoculation was performed at 2d-old chicks in our study and *IL1 beta* expression was higher in SE-inoculated birds at 24 hpi, although there were no differences between breeds (Table 1). IL1 beta production is related to the rapid inflammatory response as an attempt to fight the bacterial invasion [37]. On the other hand, *IL8* expression increased in Cobb-SE chicks at 168 hpi, whereas levels were higher in NN birds, including sham-inoculated birds. These findings show that the higher *IL8* expression levels in NN is not necessarily associated with SE infection. It can be inferred that basal activation is more prominent in naked neck birds or that macrophage response is activated in Cobb birds only in the presence of SE. Indeed, Rychlik et al. [7] also reported higher expression of *IL1* and *IL8* in slow-growing birds.

IL17 and *IL22* are frequently expressed belatedly, but in specific conditions. Although in the present study only *IL17* expression was affected by SE-infection in Cobb breed (Table 2), Chranova et al. [38] reported increased expression of both cytokines 24 h after *Salmonella*-infection. Van Hemert et al. [16] also reported greater expression of interleukins that are produced by Th17 cells after *Salmonella* inoculation, including *IL17* and *IL22*. *IL17* stimulates heterophils against microbial invasion and promotes increased number of regulatory T cells in the cecum of *Salmonella*-infected birds, which is associated with greater expression of anti-inflammatory cytokines and lower expression of pro-inflammatory cytokines.

IL17 receptors were seen in dendritic cells, macrophages and T lymphocytes, indicating its potential to regulate the immune response [39]. On the other hand, IL22 receptor was observed only in non-immune cells, stimulating these cells to produce antimicrobial peptides, and also stimulating the growth and regeneration during infection. Normal microbiota, therefore, stimulates the pro-inflammatory response that, due to the absence of positive regulation of IL22, is not deleterious to tissues [40].

Once *Salmonella* adheres to gut mucosa cells, a response is mounted to restrict *Salmonella* dissemination to other tissues [7]. Heterophil numbers do not change significantly,

but macrophage infiltration is fast followed by a decrease in macrophage numbers at 6 days post-inoculation (dpi), whereas T-lymphocytes will decrease only at 10dpi in chicks from a commercial fast-growing breed [10]. The upregulation of cytokines in sham-inoculated birds at 168 hpi observed in our study indicate that leukocyte recruitment might be affected by commensal microbiota establishment, or it may be different from Cobb birds.

Microbial composition analyses

Colonization of the gastrointestinal tract in hatchlings occurs rapidly and the commensal microbiota complexity and diversity evolves, until stabilization [41]. The commensal microbiota is important for the gut morphofunctional development and is crucial for protection against pathogens, competing for colonization sites. It also affects the development of Peyer patches and immunoglubulin A production [42].

According to our results, *Salmonella* inoculation did not induce dramatic shifts in microbial composition of birds, corroborating Videnska et al. [43]. Nevertheless, we observed a significant reduction in microbial dominance and increased diversify at 96 and 168 hpi in NN-birds (Table 3), indicating a faster recovery of the microbial stability, which is also supported by the faster clearance of *Salmonella* Enteritidis in the gut of NN-birds at 196 hpi compared with the Cobb breed (Fig. 1). Interestingly, the SE challenge caused greater shifts in the expression of intestinal transporters and cytokines in the gut of Cobb birds compared with NN-birds (Tables 1 and 2). Therefore, these findings indicate that NN chicks had a better response against *Salmonella* infection.

Diversity indexes in sham-inoculated chicks showed a common pattern independent of the breed, i.e., Shannon index was lower at 24 hpi and increased at 96 and 168 hpi in both NN-sham and Cobb-sham. On the contrary, dominance index was higher at 24 hpi and decreased at 96 and 168 hpi. Interestingly, *Salmonella* inoculation did not change this pattern in naked neck birds, whereas in Cobb birds the alpha diversity was similar between 24 hpi, 96 hpi and 168 hpi. These results suggest that *Salmonella* inoculation affected the development, succession, and balance/stability of the microbiota of Cobb birds, but not in NN birds. This can be also supported by the marked increased abundance of *Tyzzerella* in SE-inoculated Cobb birds at 168 hpi compared with 24 and 96 hpi (Online Resources 8). Increased *Tyzzerella* abundance has been reported in heat-stressed broilers showing damaged intestinal villus-crypt structures, and microbial gut disbiosis [44].

The similar pattern of microbiota development shared by NN-SE and sham-inoculated birds is further supported by the genera that are more abundant in each time post-inoculation. These three groups of birds showed dominance of *Dickeya* and unclassified Enterobacterales at 24 hpi, followed by an increase in *Lactobacillus* abundance at 96 hpi and greater diversity at 168 hpi. On the other hand, Cobb-SE birds showed no dominance of taxonomic groups at 24 hpi. Furthermore, this is the only group showing a greater abundance of *Clostridium, Escherichia* and *Epulopiscium* at 24 hpi when compared with 96 e 168 hpi. Differently from the other groups, Cobb-SE did not show differential abundance of *Lactobacillus* at 96 hpi, and only three genera were highly abundant at 168 hpi. The lower microbial diversity in Cobb-SE birds at 168 hpi can favor *Salmonella* persistence in the gut, as *Salmonella* abundance in chickens appears to be decreased with higher diversity of the microbial population [45]. Equilibrium during microbial succession is important for the development and immune response of the host. The change in the succession patterns and in the cecal microbiota establishment in Cobb birds associated with *Salmonella*-challenge may be related to a lower ability to respond to bacterial infections, probably due to a later maturation of the immune system, which is corroborated by cytokine expression patterns.

In summary, naked neck chicks do not seem to be more resistant to *Salmonella* Enteritidis infection, i.e., they are colonized just as Cobb birds, as evidenced by similar *Salmonella* counts in the cecum of both breeds until 96 hpi. On the other hand, innate upregulation of *Muc*2 (mucin production), and of the cytokines *IL8* and *IL17* in comparison to Cobb birds might play an important role on pathogen clearance at 168 hpi. Furthermore, this can be also affected by significant differences in the expression of nutrient transporters between the two breeds. While glucose uptake could be impaired in Cobb-SE birds, the opposite is observed in NN-SE birds. Nonetheless, peptide uptake may also be improved in this breed due to the higher *Pept1* gene expression. Lastly, the greater microbiota stability observed in naked neck compared with Cobb chicks possibly contribute to a better response against *Salmonella* infection.

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Author contributions ALBMF, PENG, MFSA and CJBO conceived and designed research. MRBS, MFSA, NMVS, MLPL, GFCS conducted experiment, laboratorial and bioinformatic analyses. CJBO and OCFN contributed with reagents. MRBS, PENG, ALBMF, OCFN, MFSA, NMVS, GS, MLPL and CJBO analyzed data. MRBS, PENG, CJBO and GFCS wrote the manuscript. All authors read and approved the manuscript.

Data availability The datasets generated during and/or analyzed during the current study are not publicly available but can be made available from the corresponding author on reasonable request.

Declarations

Ethics approval All management practices, as well as slaughter and sampling procedures were approved ty the Ethical Committee for the use of Animals from Universidade Federal da Paraiba (protocol 186/15) in compliance with the National Council for Animal Experimentation Control – CONCEA [Conselho Nacional de Controle de Experimentação Animal - CONCEA] (Federal Law nº 11.794/08, Lei Arouca) as established in art. 225 of the Brazilian National Constitution on the guidance for the use of animals for scientific purposes.

Competing interests The authors state that there is no conflict of interest.

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