FUNCTIONAL MECHANISMS AT THE AVIAN GUT MICROBIOME-INTESTINAL IMMUNITY INTERFACE AND ITS REGULATION OF AVIAN PHYSIOLOGICAL RESPONSES

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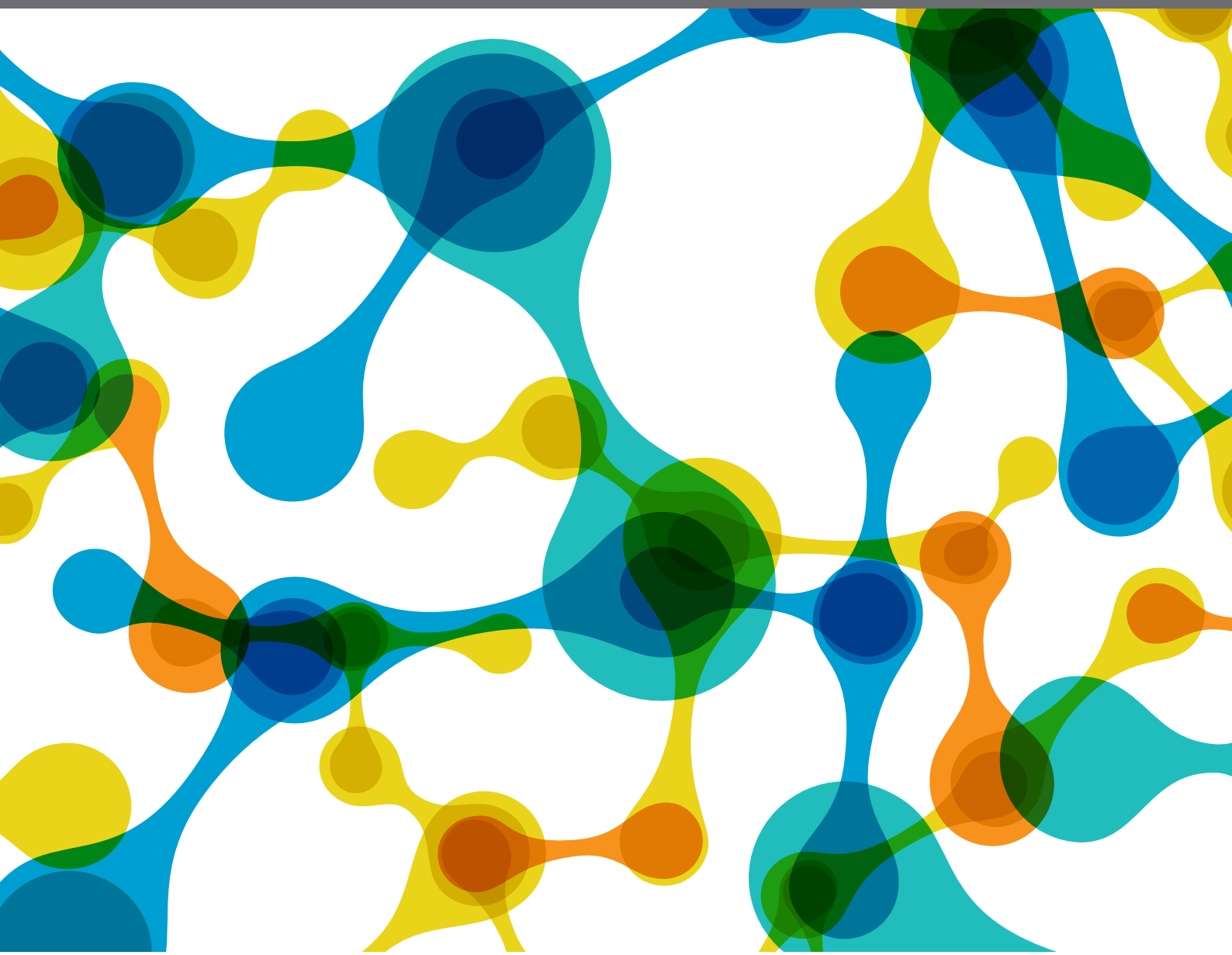

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FUNCTIONAL MECHANISMS AT THE AVIAN GUT MICROBIOME-INTESTINAL IMMUNITY INTERFACE AND ITS REGULATION OF AVIAN PHYSIOLOGICAL RESPONSES

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Editorial: Functional mechanisms at the avian gut microbiome-intestinal immunity interface and its regulation of avian physiological responses

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gut health, microbiota, mucosal immunity, poultry, physiology

Editorial on the Research Topic
Functional mechanisms at the avian gut microbiome-intestinal immunity interface and its regulation of avian physiological responses

Our understanding of the interface between the gut microbiome and avian host immunity is almost exclusively based on descriptive, associative studies which have not established causality. Clearly, the need to elucidate the causal relationships and the molecular mechanisms by which the gut microbiome influences the avian host immune system, both locally and systemically, is fundamental for the translational success of intestinal microbiota-based diagnostics, therapeutics, and adjunct therapies for avian immune development and function that impacts the poultry industry worldwide. The intestinal microbiome:innate immune interactome is a signaling hub that integrates environmental inputs of poultry, especially diet, with genetic and immune signals to translate the signals into host physiological responses and the regulation of microbial ecology. Based on mammalian studies, this network of interactions characterizes the interdependence between the innate immune system and the microbiota with the two systems affecting one another to orchestrate local intestinal and whole-organism physiology. As the basic tools for characterizing microbiomes are now widely accessible, the future of poultry (chickens, turkeys ducks, geese) microbiome research is to broaden the vision and approach to enhance understanding the functional mechanisms at the avian microbiome:immunity interface and its regulation of avian physiological responses. Thus, microbiome

studies in poultry are at a challenging transition from descriptive studies of association towards mechanistic studies. Essential for this transition is a diversity of thinking (chemical and systems biology, metabolism, microbiology, physiology and immunology) and the development of novel approaches (assays and models).

Heat stress and gut health

Heat stress (HS) is an ongoing concern to commercially produced poultry worldwide particularly now with the onset of global warming and climate change. Heat stress affects meat and egg quality, predominately through the impairment of gut function (feed intake, nutrient transport, dysbiosis, inflammation) that ultimately can compromise the sustainability of the poultry industry. Cao et al. reviewed the effects of heat stress on the gut-brain axis of poultry describing the effects of high ambient temperatures on not only on gut physiology, but also the dramatic effect on the microbiota and microbiota-derived metabolites and neurotransmitters on host metabolism, behavior, and health. Emami et al. presented new data proving that the adverse effects of heat stress on intestinal integrity, physiological performance, and carcass quality was independent of the reduced feed intake normally associated with heat stress. Further work done by the Dridi group (Abdelli et al.) demonstrated that heat stress altered nutrient transporters in the intestine of different modern broiler breeds and their ancestral jungle fowl. Interestingly, the phenotype differences in growth between lines (slow, moderate, rapid) appear to play a role in the susceptibility of the lines to the adverse effects of heat stress. Lastly, two submissions by the Jha group in Hawaii (Liu et al.; Liu et al.) reported the profound negative effects of heat stress on the intestinal physiology, barrier function, immunity, the microbiome and metabolome of the slow-growing yellow-feather broiler line indigenous to China. Many of the negative effects appear to be dependent upon the increased production of pro-inflammatory cytokine-induced microbiota dysbiosis.

Gut microbiota and intestinal immune development

Rodrigues et al. demonstrated that activation of the immune response of chicks at- or immediately post-hatch increased the proficiency of the neonatal avian immune system to sense and react to pathogens. Especially enlightening was that neonatal immune function was dependent on specific microbiota (lactic acid bacteria) exposure on the day-of-hatch when compared to exposure at 10-day post-hatch.

Gut health and developmentof a natural subclinical ne model

Current models of NE involve pre-exposure to disease risk factors, in combination with exogenous *C. perfringens* inoculation. He and colleagues described the development of a new model based on the natural uptake of *C. perfringens* presented in the housing environment by the chicken. This group incorporated multiple NE-associated predisposing factors to promote the natural development of Clostridium *perfringens* without inoculation and successfully reproduced subclinical NE infections.

Alternatives to antibiotics on gut health under field conditions

The development of antibiotic-free poultry production requires the identification and characterization of defined feed additives that increase the functional components of gut health (nutrient digestion and absorption, metabolism and energy generation, a stable microbiome, mucus layer development, barrier function, and mucosal immune responses) under field conditions. Further, ability to target alternatives to antibiotic growth promoters (ATA) to specific gut compartments is of upmost importance, especially under field conditions. Bortoluzzi et al. reported that encapsulating organic acids and essential oils assured their locating to the cecum which resulted in the decease in intestinal inflammation and the maintenance of the microbiota composition similar to that observed with AGPs only; thus, demonstrating the potential of encapsulating ATAs for improved intestinal health and integrity under field conditions.

Host-microbiota interactions and hologenomics

To meet sustainability issues associated with changing environmental conditions worldwide that can affect poultry health and welfare, Tous et al. provided insights into a novel technology called hologenomics. Hologenomics provides a multi-omics platform that moves from the traditional trial and error approach to a knowledge-based strategy in which

understanding the biological processes that underlie the administration of feeds, feed additives or pharmaceuticals and the observed interindividual variation is prioritized. This platform, albeit with large data sets, allows provides the interpretational understanding of how host genomic features, microbiota development dynamics and host-microbiota interactions shape animal welfare and performance.

Author contributions

Both authors contributed equally to the writing of the Editorial. MF-K was responsible for compiling each contribution into a single document.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Heat Stress and Feed Restriction Distinctly Affect Performance, Carcass and Meat Yield, Intestinal Integrity, and Inflammatory (Chemo)Cytokines in Broiler Chickens

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This study was conducted to distinguish the effects of heat stress (HS) and feed intake (FI) on broiler chicken's physiological responses. Day-old male Cobb 500 broilers (n = 672) were allocated to three treatments: (1) control (CTL): birds raised under normal temperature (23°C) from day 29 to 42; (2) cyclic heat stress (CHS): birds exposed to high temperatures (8 h/day at 35°C; from 9:30 am to 5:30 pm) from day 29 to 42; (3) pair-fed (PF): birds raised under thermoneutral condition but fed the same amount of feed as CHS from day 29 to 42. On day 42, 15 birds/pen were processed, to measure carcass and meat yields. To measure blood parameters and gut integrity (using fluorescein isothiocyanate-dextran), on day 42, CHS birds were sampled before (Pre-CHS) and 2 h after (Post-CHS) the temperature increased. Furthermore, after sampling CTL birds, they were exposed to 2h heat and sampled (acute heat stress, AHS). Data were analyzed using one-way ANOVA (JMP Pro15) and significance between treatments identified by LSD ($P < 0.05$). BW and relative carcass yield were significantly higher in CTL compared to CHS and PF. Compared to CHS, PF had significantly higher BW and lower relative carcass yield. Breast yield was significantly higher for CTL and PF, while leg quarters and wings yield were significantly lower compared to CHS. Gut barrier integrity was significantly altered in Post-CHS and AHS compared to CTL. mRNA abundances of tumor necrosis factor-α, C-C motif chemokine ligand-20, heat shock protein (*HSP*)-*27*, and *HSP70* were significantly higher in Post-CHS and AHS compared to CTL. AHS had significantly higher mRNA abundances of CARD domain containing (*NLRC*)-*3* and *NLRC5* inflammasomes, and lower superoxide dismutase (*SOD*)-*1* and *SOD2* abundance compared with CTL. PF had significantly higher liver weight (% BW) compared to all other groups; while abdominal fat was significantly higher in Pre-CHS compared to CTL, PF, and AHS. Together, these data indicate that the negative effects of HS are partially due to reduced FI. However, the negative effect of HS on gut integrity, average daily gain, feed conversion ratio, and meat yield are direct and independent of the reduced FI during the HS. Thus, warrant investigating the underlying mechanisms in future research.

Keywords: heat stress, broiler chicken, performance, body parts yield, immune response, gut integrity

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INTRODUCTION

Global warming is threatening all kinds of life on earth, especially avian species, which are highly susceptible to heat stress (HS) due to the lack of sweat glands and higher core body temperatures compared to mammals (42°C compared to 37°C) (Warren et al., 2018; Emami et al., 2020b). Demand for poultry meat is on the rise and poultry production is expected to increase more than double by 2050 (O'keefe, 2014). However, several factors, including HS, negatively impact the efforts of the poultry industry to meet the high demands by eliciting physiological, behavioral, and production changes in poultry (Emami et al., 2020b; Wasti et al., 2020).

Therefore, evaluating the effects of HS on bird's physiology, immune response and gut integrity is of substantial importance, and a thorough understanding of these responses is necessary to better design targeted treatments or interventions. The negative effects of acute and cyclic heat stress (AHS and CHS) on performance parameters of broiler chickens is well defined by our research group in previous experiments. During the AHS (2 h) and CHS (3 weeks, 12 h/day at 35°C), feed intake (FI) was significantly reduced in broiler chickens compared to their counterparts maintained under thermoneutral conditions (Flees et al., 2017; Greene et al., 2021a). This, in turn, resulted in a significant reduction in body weight in the CHS birds compared to their thermoneutral counterparts (Greene et al., 2021a). Comparison of four genetically distinct chicken lines with different feed efficiency showed that AHS (2 h) decreased FI in modern broilers (1995 random bred and modern random bred lines) but not in jungle fowl and Athens Canadian random bred chickens (Tabler et al., 2020). Several other studies have shown the negative impacts of HS on broiler chickens' performance (Del Vesco et al., 2017; Awad et al., 2020).

Heat stress can negatively affect carcass traits as well. Indeed, CHS increased fat deposition, and decreased the proportion of breast muscle, while increasing the proportion of thigh muscle in broiler chickens (Lu et al., 2007; Zhang et al., 2012).

Besides negative effects on performance and carcass traits, the immunosuppressing effects of HS in poultry is well indicated (Lara and Rostagno, 2013). HS causes multiple immune abnormalities in broiler chickens by impairing the developmental process and functional maturation of T- and B-cells in both primary and secondary lymphoid tissues (Hirakawa et al., 2020). Pro-inflammatory cytokines, including interleukin (*IL*)-*1*, *IL2*, *IL6*, *IL18*, and tumor necrosis factor (*TNF*)-α, are involved in the inflammatory response under HS, and excessive pro-inflammatory response may result in tissue damage (Helwig and Leon, 2011; Goel et al., 2021; Greene et al., 2021b). Plasma titers of anti-bovine serum albumin (BSA) immunoglobulin (Ig)Y, IgM, and IgA were lower in broiler chickens exposed to constant HS (35°C) from day 22 to 36 than those of thermoneutral chickens immunized with BSA (Hirakawa et al., 2020). Recently, we have shown that gene expression of circulating inflammatory factors are dysregulated during CHS (Greene et al., 2021b). CHS (12 h/day at 35°C) upregulated the expression of superoxide dismutase (*SOD*)-*1*, *TNF*-α, and C-C motif chemokine ligand (*CCL*)-*4* and *CCL20*; but, downregulated glutathione peroxidase (*GPX*)-*3*, *IL18*, and nucleotide-binding, leucine-rich repeat and pyrin domain containing (*NLRP*)-*3* inflammasome. Heat shock proteins (*HSP*) and nucleotide-binding oligomerization domain, leucine-rich repeat, and CARD domain containing (*NLRC*)-*3* and nucleotide-binding, leucine-rich repeat containing X1 (*NLRX1*) inflammasomes mRNA were unaffected by HS (Greene et al., 2021b).

As mentioned, most of the negative effects of HS on broiler chickens has been attributed to lower FI in heat-stressed birds with no efforts to distinguish the direct and indirect (through reduction in FI) effects of HS on bird's physiological response. Interestingly, there are indications that the physiological effects of HS are not limited to reduced FI in heat-stressed birds. Heat exposure at 34°C for 15 days significantly increased *IL4* and *IL12* mRNA abundance and decreased interferon (*IFNG*), with no effect on mRNA abundance of *IL6*, *IL10*, *IL13*, and *IL18*. mRNA abundance of *IL4* in the feed-restricted group was higher than that in the control group. Further, *IFNG* abundance increased and *IL12* abundance was not affected by the reduction of FI, suggesting that the FI reduction induced by HS does not modulate splenic cytokine expression in broiler chickens. These data suggest that HS induces spleen involution and affects the expression of splenic cytokines such as *IL12* and *IFNG* in broiler chickens independently of the FI reduction (Ohtsu et al., 2015).

Exposure to HS increased carcass and abdominal fat percentages, and reduced breast, liver and heart percentages which was completely different from pair-fed (PF; feed restricted) chickens that had the lowest fat percentage, and breast percentage similar to birds raised at thermoneutral condition (Zeferino et al., 2016). Recently, researchers indicated that changes in the intestinal morphology and permeability in heat-stressed chickens (24–72 h at 33°C) were due to the HS conditions and not due to the reduced FI (Nanto-Hara et al., 2020). However, the mentioned studies were limited due to addressing a single aspect (such as performance, carcass yield, immune response, or gut integrity) with no measurement of other parameters. In addition, no study has evaluated the effect of CHS and AHS in comparison with feed-restricted birds.

Thus, we conducted this experiment to have a holistic understanding about the physiological changes in broiler chickens during a CHS and distinguish the direct effects of HS from the indirect effects (which are related to the reduction in FI) on broiler chickens' performance, mortality, and carcass and meat yield. In addition, we evaluated the effect of CHS, AHS, and feed restriction on organs weight, gut integrity and circulating (chemo)cytokines.

MATERIALS AND METHODS

Birds, Diets, and Management

All animal care and procedures were approved by the Institutional Animal Care and Use Committee at the University of Arkansas. On d of hatch, 672 Cobb 500 male broilers were neck tagged, individually weighed, and

housed in environmentally controlled chambers in the Poultry Environmental Research Laboratory at the University of Arkansas. There were twelve environmental chambers, each equipped with separate controllers to enable temperature adjustments. Each chamber consisted of two equally sized pens (1.2 × 2.4 m) and all pens were covered with 7 cm pine shavings. Four chambers (eight pens) were allocated to CHS group. Rest of the chambers (eight chambers) were allocated to control (CTL) and PF groups (one pen for each group/chamber) as these groups were raised under the same environmental conditions. Birds were randomly allocated to one of 24 pens with 28 birds/pen. Each pen was equipped with a bucket-type feeder and drinker, and feed and water intake were measured on daily basis from day 0 to 42. Lighting schedule was 24 h light for the first 3 days, reduced to 23 h light:1 h dark day 4 to 7, and reduced further to 18 h light:6 h dark thereafter. Birds were raised in environmentally controlled chambers and temperature and humidity in each pen were recorded every day. Temperature was maintained as follows: 32°C for the first 3 days, then gradually reduced approximately 3°C each week until it reached 23°C on day 21. All birds were fed the same corn-soybean meal basal diet in the form of crumble during the starter (day 0–14), or pellet during the grower (day 15–28) and finisher (day 29–42) period (**Table 1**). Birds were assigned to one of the three treatments, each with eight replicate pens as follows:

1) Control (CTL): birds raised under thermoneutral condition (23°C) from day 29 to 42.
2) CHS: birds exposed to cyclic high ambient temperature (35°C) for 8 h/day (9:30 am to 5:30 pm) from day 29 to 42.
3) Pair-fed (PF): birds raised under thermoneutral condition (23°C) from day 29 to 42, but PF to CHS group, receiving each day 1.05 times the average FI recorded in the HS group in the previous day.

Two days before the CHS, two chickens per pen were randomly selected and a Thermochron temperature logger (iButton, Embedded Data Systems, KY, United States) was placed in the crop *via* oral gavage for continuous monitoring of core body temperature. The environmental temperature and humidity were also continuously recorded in each chamber, inside and outside the barn.

TABLE 1 | Composition of basal diets (as fed basis, %).

Ingredients (%)	Period (Days)		
	Starter (0–14) Crumble	Grower (15–28) Pellet	Finisher (29–42) Pellet
Corn (7.81% CP)	60.53	60.99	66.52
Soybean meal (48% CP)	32.95	32.55	27.22
Poultry fat (9000 kcal/kg)	1.80	2.38	2.46
Dicalcium phosphate (18.5% P, 22% Ca)	2.08	1.85	1.67
Limestone (37% calcium)	1.10	1.00	0.91
Sodium chloride	0.38	0.40	0.44
DL-methionine (990 g/kg)[1]	0.38	0.30	0.27
L-lysine hydrochloride (788 g L-lysine/kg)[2]	0.37	0.22	0.20
L-threonine (985 g/kg)[3]	0.16	0.08	0.08
Choline chloride (60%)	0.10	0.08	0.08
Vitamin/trace mineral premix[4]	0.15	0.15	0.15
Calculated analysis (% unless specified)			
ME (kCal/kg)	2994	3038	3108
Crude protein	21.71	21.30	19.18
Total phosphorus	0.77	0.71	0.66
Available phosphorus	0.45	0.42	0.38
Calcium	0.90	0.84	0.75
Chlorine	0.33	0.32	0.34
Sodium	0.16	0.17	0.19
Potassium	0.84	0.83	0.74
Methionine	0.67	0.59	0.54
Methionine + cysteine	0.98	0.89	0.82
Lysine	1.32	1.18	1.04
Threonine	0.86	0.78	0.70
Linoleic acid	1.46	1.47	1.57
Dietary cation-anion balance	192	196	176

[1] *Rhodimet NP9, ADISSEO, GA, United States.*
[2] *L-Lysine HCl, AJINOMOTO HEARTLAND, INC., Eddyville, IA, United States.*
[3] *FENCHEM Ingredient Technology, Nanjing, China.*
[4] *Vitamins supplied per kg diet: retinol 3.33 mg, cholecalciferol 0.1 mg, α-tocopherol acetate 23.4 mg, vitamin K3 1.2 mg, vitamin B1 1.6 mg, vitamin B2 9.5 mg, niacin 40 mg, pantothenic acid 9.5 mg, vitamin B6 2 mg, folic acid 1 mg, vitamin B12 0.016 mg, biotin 0.05 mg, and choline 556 mg. Minerals supplied per kg diet: Mn 144 mg, Fe 72 mg, Zn 144 mg, Cu 16.2 mg, I 2.1 mg, and Se 0.22 mg.*

Performance

On day 0, 28, and 42 birds were weighed individually, while feed and water intake were measured on daily basis. Finally, adjusted average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were calculated for day 0–28, day 29–42, and overall experimental period (day 0–42).

Mortality

Starting at placement, birds were monitored twice daily. For each dead bird, date, neck tag number, body weight, and cause of death were recorded. This procedure continued throughout the study (up to day 42) to record mortality/treatment for each period thus allowing for adjusting performance parameters for daily mortality.

Carcass and Meat Yield

On day 42, 15 birds/pen (eight pens/treatment) were randomly selected, and wing tagged. Birds were processed using a commercial inline system at the University of Arkansas Pilot Processing Plant (Fayetteville, AR, United States). Birds were electrically stunned (11 V, 11 mA for 11 s), exsanguinated, scaled at 53.8°C for 2 min, and defeathered using a commercial, inline equipment (Foodcraft Model 3; Baker international, MI, United States). Carcasses were manually eviscerated and rinsed

before prechilling at 12°C for 15 min. Then, carcasses were chilled for 90 min at 1°C in immersion chilling tanks with manual agitation at 15 min regular intervals. Slaughter weight, and prechill carcasses were recorded, and following a 2 h chill at 4°C, the weight of breasts, tenders, leg quarters, and wings were recorded.

Gut Integrity, Organ Weights, and Blood Gene Expression Profile

To measure organ weights, gut integrity, and expression of (chemo)cytokines, inflammasomes, *HSP*, and antioxidants in the blood, on day 42 we further divided the birds into five treatments as follow:

1) Control (CTL): birds raised under thermoneutral condition (23°C) from day 29 to 42.
2) AHS: birds raised under thermoneutral condition (23°C) from day 29 to 42, but exposed to 2 h AHS (35°C) before sampling on day 42.
3) Pre-CHS: birds exposed to high ambient temperature (35°C) for 8 h/day (9:30 am–5:30 pm) from day 29 to 42, and sampled before starting the CHS.
4) Post-CHS: birds exposed to high ambient temperature (35°C) for 8 h/day (9:30 am–5:30 pm) from day 29 to 42, and sampled 2 h after starting the CHS.
5) Pair-fed (PF): birds raised under thermoneutral condition (23°C) from day 29 to 42, but PF to CHS group. These birds received each day 1.05 times the average FI recorded in the CHS group in the previous day.

Gut Integrity

On day 42, gut integrity was evaluated with the use of fluorescein isothiocyanate-dextran (FITC-d). Two birds/pen from each of the five treatments were selected, and after 1 h fasting, birds were weighed individually, and 8.32 mg/kg FITC-d (4 kDa, Sigma-Aldrich, St. Louis, MO, United States) that were diluted in water were orally gavaged to each bird. Blood was collected 1 h post gavage, and serum collected by centrifugation at 2,000 rpm for 10 min. Sera were diluted 1:5 with PBS, and FITC-d concentration was measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm (Synergy HT, multimode microplate reader, BioTek Instruments, Inc., Winooski, VT, United States). Pooled sera of three birds from the thermoneutral group that did not receive FITC-d were used for normalization.

Organs Weight

On day 42, three birds were selected from each replicate of the five treatments, and liver and abdominal fat weight were recorded, and results were reported as a % of live body weight.

RNA Isolation and Quantitative Real-Time PCR

On day 42, 250 μl of whole blood was collected in 750 μl of TRIzol LS (Life Technologies, Carlsbad, CA, United States) from one bird/pen from each of the five treatments, and total RNA was extracted according to the manufacturer's instruction. cDNA synthesized using qScript cDNA Synthesis Supermix (Quanta Biosciences, Gaithersburg, MD, United States). Finally, target genes (*HSP27*, *HSP60*, *HSP70*, *HSP90*, *GPX1*, *GPX3*, *SOD1*, *SOD2*, *IL6*, *IL10*, *IL18*, *TNF-α*, *CCL4*, *CCL5*, *CCL20*, *NLRC3*, *NLRC5*, *NLRP3*, and *NLRX1*) were amplified using SYBR green master mix (Life Technologies, Carlsbad, CA, United States) and 7500 Real-Time PCR system (Applied Biosystems). Oligonucleotide primer sequences specific for chicken are listed in **Table 2**. Each reaction was performed in duplicate. Product specificity was confirmed by analysis of the melting curves generated by the 7500 software (version 2.0.3). mRNA abundance was analyzed using tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) as an endogenous control. Average mRNA abundance relative to *YWHAZ* for each sample was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008) with the CTL group as a calibrator.

Statistical Analyses

Statistical analysis was performed using the one-way ANOVA procedure of JMP software (2015) and significance between treatments ($P < 0.05$) determined by the LSD test. Pen was considered as experimental unit ($n = 8$). The statistical model for data analysis is outlined below:

$$Yij = \mu + Ai + eij \quad (1)$$

where Yij, measured value for each observation (data); μ, grand mean; Ai, treatment effect; and eij, experimental error.

RESULTS

Core Body Temperature

Core body temperature, inside chamber as well as environmental (inside and outside barn) temperature and relative humidity are shown in **Figures 1**, **2**. On average, core body temperature was approximately 1°C higher in CHS group compared to CTL and PF groups (**Figure 2**).

Mortality

Mortality data is presented in **Table 3**. Mortality was low in all the treatments during the starter, grower, finisher, and overall experimental period and there was no difference between treatments.

Performance

Table 4 indicates performance parameters during various phases of the experiment. Before the start of CHS (day 0–28) performance including BW, ADFI, ADG, and FCR was similar for all the treatments. However, for the finisher phase (day 29–42), birds in the CTL group had significantly higher ADFI, and ADG compared to CHS and PF groups. In addition, PF birds had higher ADG compared to the CHS group. FCR was significantly better for both CTL and PF groups compared to CHS, and there was no difference in FCR for CTL and

TABLE 2 | Oligonucleotide primers for real-time qPCR.

Gene	Accession #	Primer sequence (5′ → 3′)	Orientation	Product size (bp)
CCL4	NM_204720.1	CCTGCTGCACCACTTACATAACA	Forward	63
		TGCTGTAGTGCCTCTGGATGA	Reverse	
CCL5	NM_001045832.1	TTTCTACACCAGCAGCAAATGC	Forward	59
		GCCCCTTCCTGGTGATGAA	Reverse	
CCL20	NM_204438.2	TGCTGCTTGGAGTGAAAATGC	Forward	62
		CAGCAGAGAAGCCAAAATCAAA	Reverse	
GPX1	NM_001277853.2	TCCCCTGCAACCAATTCG	Forward	57
		AGCGCAGGATCTCCTCGTT	Reverse	
GPX3	NM_001163232.2	GGGCGCTGACCATCGAT	Forward	59
		CATCTTCCCCGCGTACTTTC	Reverse	
HSP27	XM_001231557	TTGAAGGCTGGCTCCTGATC	Forward	58
		AAGCCATGCTCATCCATCCT	Reverse	
HSP60	NM_001012916	CGCAGACATGCTCCGTTTG	Forward	55
		TCTGGACACCGGCCTGAT	Reverse	
HSP70	JO2579	GGGAGAGGGTTGGGCTAGAG	Forward	55
		TTGCCTCCTGCCCAATCA	Reverse	
HSP90	X07265.1	TGACCTTGTCAACAATCTTGGTACTAT	Forward	68
		CCTGCAGTGCTTCCATGAAA	Reverse	
IL6	NM_204628.1	GCTTCGACGAGGAGAAATGC	Forward	63
		GGTAGGTCTGAAAGGCGAACAG	Reverse	
IL10	NM_001004414.2	CGCTGTCACCGCTTCTTCA	Forward	63
		CGTCTCCTTGATCTGCTTGATG	Reverse	
IL18	NM_204608.1	TGCAGCTCCAAGGCTTTTAAG	Forward	63
		CTCAAAGGCCAAGAACATTCCT	Reverse	
NLRC3	XM_015294675.2	CTCCAACGCCTCACAAACCT	Forward	93
		GCCTTTGGTCATTTCCATCTG	Reverse	
NLRC5	NM_001318435.1	CTCGAAGTAGCCCAGCACATT	Forward	80
		CATGTCCAGAGGTGTCAGTCTGA	Reverse	
NLRP3	NM_001348947.1	GTTGGGCAGTTTCACAGGAATAG	Forward	63
		GCCGCCTGGTCATACAGTGT	Reverse	
NLRX1	XM_004948038.3	GGCTGAAACGTGGCACAAA	Forward	59
		GAGTCCAAGCCCAGAAGACAAG	Reverse	
SOD1	NM_205064.1	TGGCTTCCATGTGCATGAAT	Forward	58
		ACGACCTGCGCTGGTACAC	Reverse	
SOD2	NM_204211.1	GCTGGAGCCCCACATCAGT	Forward	61
		GGTGGCGTGGTGTTTGCT	Reverse	
TNF-α	NM_204267.1	CGTTTGGGAGTGGGCTTTAA	Forward	61
		GCTGATGGCAGAGGCAGAA	Reverse	
YWHAZ	NM_001031343.1	ACGCCGTAGGTCATCTTGGA	Forward	58
		ACGGCCTTCCGTCTTTTGT	Reverse	

PF groups. Differences in the finisher phase led to significant difference in performance among the treatments during the overall experimental period. Birds in the CTL group had the highest ADFI, and ADG which was significantly higher than CHS and PF groups. Likewise, PF birds had significantly higher ADG compared to CHS, despite the same ADFI. Birds in the CTL and PF group had similar FCR which was significantly better that the CHS group.

Carcass and Meat Yield

Carcass and meat yield is shown in **Table 5**. Hot carcass weight (% live body weight), was significantly higher in the CTL and CHS compared to PF group. Breast yield (% hot carcass weight) was significantly higher in CTL compared to CHS and PF. In addition, PF group had significantly higher breast yield compared to CHS group. On the contrary, leg quarters and wings (% hot carcass weight) were significantly higher in CHS compared to CTL and PF groups. Furthermore, tender yield (% hot carcass weight) was higher in CHS compared to CTL but not PF group.

Organs Weight

Table 6 indicates liver and abdominal fat (including fat surrounding gizzard, proventriculus, bursa of fabricius, cloaca,

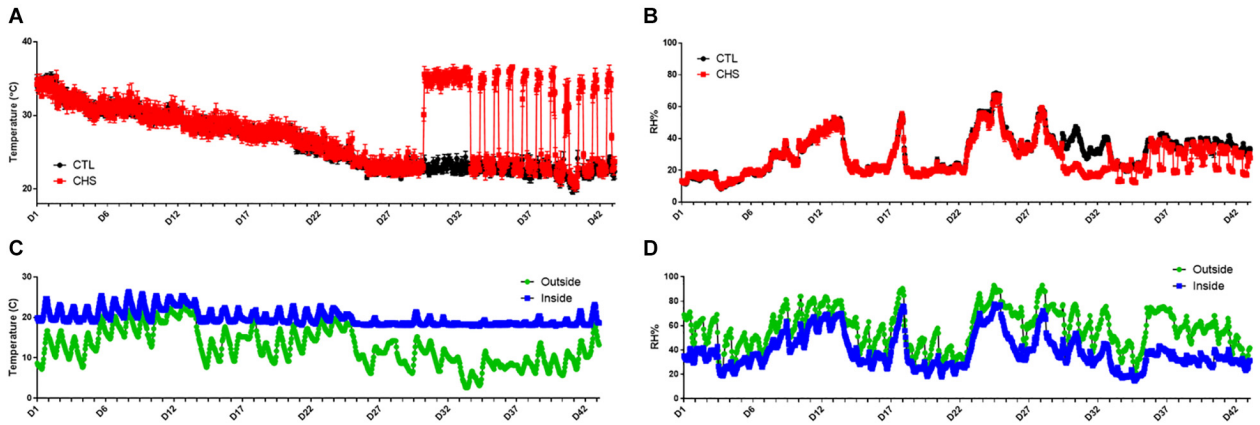

FIGURE 1 | Temperature and relative humidity (RH) fluctuation in the environmental chambers **(A,B)**, and inside and outside the barn **(C,D)** during the cyclic heat stress (CHS) experiment. Birds raised under recommended conditions from day 0 to 28. From day 29 to 42, birds either raised at thermoneutral temperature (CTL, 23°C) or exposed to CHS (35°C). Data are presented as mean ± SEM (n = 1/chamber or 8/group).

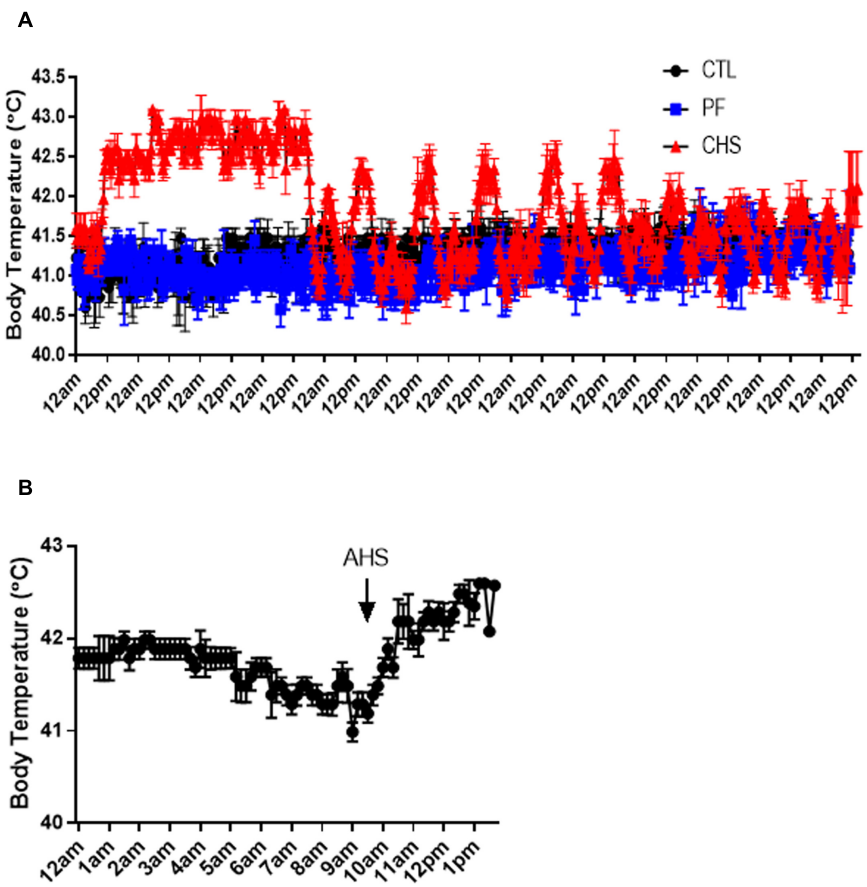

FIGURE 2 | Core body temperature fluctuation during heat stress (HS). **(A)** Treatments include: (1) control (CTL): birds raised under normal temperature (23°C) from day 29 to 42 and had free access to the diet; (2) cyclic heat stress (CHS): birds exposed to high temperatures (8 h/day at 35°C; from 9:30 am to 5:30 pm) from day 29 to 42 and had free access to the diet; (3) pair-fed (PF): birds raised under the same condition as CTL group, but fed the same amount of feed as CHS group from day 29 to 42. **(B)** Core body temperature fluctuation during an acute heat stress (AHS). Birds in this group raised under thermoneutral condition (23°C) from day 29 to 42 but exposed to 2 h AHS (35°C) before sampling on day 42. Data are presented as mean ± SEM (n = 8; two birds/pen).

TABLE 3 | Effect of rearing temperature and feed restriction on mortality (%) of Cobb 500 male broiler chickens.

Treatments[1]	Time period (day)		
	0–28	29–42	0–42
CTL	0.89	1.34	2.23
CHS	3.12	0.93	4.01
PF	1.78	0.46	2.23
SEM[2]	0.96	0.58	1.12
P-value[3]	0.282	0.573	0.444

[1]Treatments include: (1) control (CTL): birds raised under normal temperature (23°C) from day 29 to 42 and had free access to the diet; (2) cyclic heat stress (CHS): birds exposed to high temperatures (8 h/day at 35°C; from 9:30 am–5:30 pm) from day 29 to 42 and had free access to the diet; (3) pair-fed (PF): birds raised under the same condition as CTL group, but fed the same amount of feed as CHS group from day 29 to 42. All birds fed the same corn-soybean meal basal diet.
[2]SEM: pooled standard error of means.
[3]P-value: based on one-way ANOVA.

TABLE 4 | Effect of rearing temperature and feed restriction on Cobb 500 male broiler chicken's performance.[1]

	Treatments[2]			Statistics	
Item[3]	CTL	CHS	PF	SEM[4]	*P*-value[5]
Body weight (g)					
Day 0	38.37	38.28	38.59	0.19	0.523
Day 28	1839.54	1812.25	1828.44	17.16	0.537
Day 42	3701.42	3215.29	3381.62	28.88	<0.001
Day 0–28					
ADFI, g	87.57	87.47	87.66	0.71	0.982
ADG, g	64.32	63.35	63.92	0.61	0.537
FCR	1.36	1.38	1.37	0.01	0.112
Day 29–42					
ADFI, g	216.09[a]	181.95[b]	183.67[b]	2.31	<0.001
ADG, g	132.99[a]	100.68[c]	110.03[b]	1.86	<0.001
FCR	1.62[b]	1.81[a]	1.66[b]	0.02	<0.001
Day 0–42					
ADFI, g	128.32[a]	117.43[b]	118.10[b]	0.91	<0.001
ADG, g	87.21[a]	75.79[c]	79.29[b]	0.71	<0.001
FCR	1.47[b]	1.55[a]	1.48[b]	0.01	<0.001

[a–c]In each row, means with different letters are significantly different (P < 0.05).
[1]Data represent the mean value of eight replicate pens of 28 birds/pen (n = 8).
[2]Treatments include: (1) control (CTL): birds raised under normal temperature (23°C) from day 29 to 42 and had free access to the diet; (2) cyclic heat stress (CHS): birds exposed to high temperatures (8 h/day at 35°C; from 9:30 am to 5:30 pm) from day 29 to 42 and had free access to the diet; (3) pair-fed (PF): birds raised under the same condition as CTL group, but fed the same amount of feed as CHS group from day 29 to 42.
[3]ADFI, average daily feed intake; ADG, average daily gain; FCR, feed conversion ratio. [4]SEM: pooled standard error of means. [5]P-value: based on one-way ANOVA.

TABLE 5 | Effect of rearing temperature and feed restriction on carcass and meat yield of Cobb 500 male broiler chickens on day 42.[1]

Treatments[2]	Hot carcass (% body weight)	Meat yield (% hot carcass weight)			
		Breast	Tender	Leg quarters	Wings
CTL	71.99[a]	31.07[a]	5.05[b]	28.84[c]	10.42[b]
CHS	71.37[a]	29.18[c]	5.24[a]	30.44[a]	10.71[a]
PF	70.60[b]	29.99[b]	5.12[ab]	29.72[b]	10.37[b]
SEM[3]	0.25	0.17	0.05	0.13	0.05
P-value[4]	0.003	<0.001	0.036	<0.001	<0.001

[a–c]In each column, means with different letters are significantly different (P < 0.05).
[1]Data represent the mean value of eight replicate pens of 15 birds/pen (n = 8).
[2]Treatments include: (1) control (CTL): birds raised under normal temperature (23°C) from day 29 to 42 and had free access to the diet; (2) cyclic heat stress (CHS): birds exposed to high temperatures (8 h/day at 35°C; from 9:30 am to 5:30 pm) from day 29 to 42 and had free access to the diet; (3) pair-fed (PF): birds raised under the same condition as CTL group, but fed the same amount of feed as CHS group from day 29 to 42.
[3]SEM: pooled standard error of means.
[4]P-value: based on one-way ANOVA.

TABLE 6 | Effect of temperature and feed restriction on organ weight of Cobb 500 male broiler chickens on day 42.[1]

Treatments[2]	Organ weight (% body weight)	
	Liver	Abdominal fat[3]
CTL	1.75[b]	1.02[ab]
PF	1.94[a]	0.96[b]
Pre-CHS	1.75[b]	1.14[a]
Post-CHS	1.44[c]	1.04[ab]
AHS	1.53[c]	0.96[b]
SEM[4]	0.05	0.05
P-value[5]	<0.001	0.048

[a–c]In each column, means with different letters are significantly different (P < 0.05).
[1]Data represent the mean value of eight replicate pens of three birds/pen (n = 8).
[2]Treatments include: (1) control (CTL): birds raised under normal temperature (23°C) from day 29 to 42 and had free access to the diet; (2) acute heat stress (AHS): birds from CTL group after a 2 h heat exposure on day 42 (35°C); (3 and 4) cyclic heat stress (CHS): birds exposed to high temperatures (8 h/day at 35°C; from 9:30 am to 5:30 pm) from day 29 to 42 and sampled before the start (Pre-CHS) or 2 h after the start of CHS (Post-CHS) on day 42; (5) pair-fed (PF): birds raised under the same temperature as CTL group, but fed the same amount of feed as CHS group from day 29 to 42.
[3]Fat surrounding gizzard, proventriculus, bursa of fabricius, cloaca, and adjacent muscles.
[4]SEM: pooled standard error of means.
[5]P-value: based on one-way ANOVA.

and adjacent muscles) as a % of live body weight in different groups. Birds in the PF group had the highest liver weight compared to all other groups. On the contrary, Post-CHS and AHS birds had the lowest liver weight that was significantly different from Pre-CHS and CTL groups. Pre-CHS had the highest abdominal fat which was significantly different from PF and AHS groups.

Gut Integrity

Figure 3 shows the FITC-d concentration in the sera of different groups. There was a significant difference in the concentration of FITC-d in Post-CHS and AHS groups compared to the CTL. Furthermore, Post-CHS group had higher FITC-d concentration in the sera (lower gut integrity) compared to the PF group.

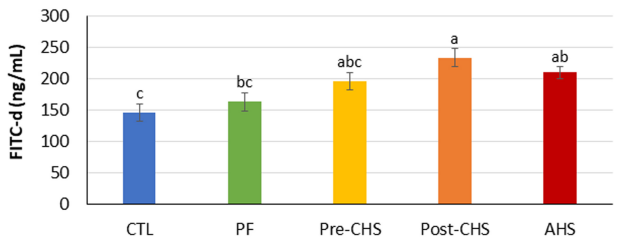

FIGURE 3 | FITC-d concentration in the serum of Cobb 500 male broiler chickens on day 42. Birds in all the groups were raised under the recommended conditions from day 0 to 28. On day 42, birds fasted for 2 h, then individually weighed and orally gavage with 8.32 mg FITC-d/kg body weight, and blood was collected 2 h post gavage. Treatments include: (1) control (CTL): birds raised under thermoneutral condition (23°C) from day 29 to 42; (2) AHS: birds raised under thermoneutral condition (23°C) from day 29 to 42, but exposed to 2 h AHS (35°C) before sampling on day 42; (3) Pre-CHS: birds exposed to high ambient temperature (35°C) for 8 h/day (9:30 am–5:30 pm) from day 29 to 42, and sampled before starting the CHS; (4) Post-CHS: birds exposed to high ambient temperature (35°C) for 8 h/day (9:30 am–5:30 pm) from day 29 to 42, and sampled 2 h after starting the CHS; (5) pair-fed (PF): birds raised under thermoneutral condition (23°C) from day 29 to 42, but pair-fed to CHS group (these birds received each day 1.05 times the average feed intake recorded in the CHS group in the previous day). Results are given as means (n = 8; two birds/pen) for each group. Error bars indicate standard errors. Columns with different letters are significantly different ($P < 0.05$).

Blood (Chemo)Cytokines, Inflammasomes, Heat Shock Proteins, and Antioxidants

mRNA abundances of HSPs, antioxidant enzymes, cytokines, chemokines, and inflammasomes are shown in **Figures 4–8**, respectively. *HSP27* abundance was significantly higher in Post-CHS and AHS compared CTL and PF. Post-CHS was the only group with significantly higher abundance of *HSP60* compared to CTL, PF and Pre-CHS; while, *HSP90* was significantly higher in the AHS group compared to CTL, PF and Pre-CHS. Both Post-CHS and AHS had significantly higher *HSP70* mRNA abundance in the circulation compared to other groups (**Figure 4**).

Control group had significantly lower *GPX1* abundance compared to PF and AHS, while *GPX3* was significantly lower in the CTL group compared to Pre-CHS and AHS. AHS significantly upregulated mRNA abundance for *GPX1* compared to other groups except PF. In addition, AHS group had the highest *GPX3* abundance that were significantly different from all other groups. On the contrary, mRNA abundance of *SOD1* was significantly lower in PF, Pre-CHS, and AHS compared to CTL. In addition, mRNA abundance of *SOD2* was significantly lower in Pre-CHS, and AHS compared to CTL group (**Figure 5**).

mRNA abundances of *IL10* and *IL18* in the circulation showed no difference among the treatments. *IL6* abundance was significantly lower in AHS compared to CTL and PF; while, *IL6* abundance in Pre-CHS and Post-CHS was significantly lower compared to PF. In addition, mRNA abundance of TNF-α was significantly higher in AHS group compared to all other groups. However, for the Post-CHS group, mRNA abundance of TNF-α was significantly higher only compared to the CTL (**Figure 6**).

Chemokine expression profile showed no differences in mRNA abundance of *CCL5*, and *CCL20* in the blood samples from different groups. However, birds in the AHS group had significantly higher abundance of *CCL4* compared to other groups (**Figure 7**).

Circulatory inflammasomes including *NLRP3* and *NLRX1* abundance was not affected by any of the treatment groups. However, mRNA abundance of *NLRC3* was significantly higher in the AHS group compared to CTL, PF, and Pre-CHS groups. In addition, mRNA abundance of *NLRC5* was significantly higher in AHS compared to other groups. Interestingly, *NLRC5* abundance was higher in PF group compared to the CTL (**Figure 8**).

DISCUSSION

Birds raised under thermoneutral condition had significantly higher ADG, and ADFI compared to CHS and PF groups. Previously, researchers including our group reported that both chronic and constant HS reduced ADG and ADFI, and increased FCR compared to birds raised under thermoneutral condition (Zeferino et al., 2016; Baxter et al., 2020; Tabler et al., 2020; Greene et al., 2021a). Similarly, heat exposure (6 h/day at 34°C from day 22 to 35) significantly reduced FI and body weight gain (day 22–35 and 1–35), while increased FCR (day 22–35 and 1–35) and serum levels of acute phase proteins (APPs) in both Cobb and Ross broiler chickens (Awad et al., 2020).

Despite similar ADFI, PF group had significantly higher ADG compared to CHS group in the current experiment, which is different from the previous study where growth performance was similar between PF and heat-stressed chickens (Zeferino et al., 2016). These inconsistencies might be due to differences in HS regimes (CHS in our study vs. constant HS) or type of housing (floor pens in our study vs. cages) among trials. Lower ADG in CHS compared to PF group in our experiment might be justified by the higher mRNA abundance of HSP's in the circulation, and disrupted gut integrity. This might be indicative of nutrient allocation toward maintaining gut integrity and homeostasis instead of growth in order to deal with the negative impacts of the CHS, and brings the theory of resource allocation into attention (Siegel and Honaker, 2009). The impact of HS on the inflammatory response in poultry may be a significant contributor to the reductions seen in growth and productivity (Lara and Rostagno, 2013). HS causes multiple immune abnormalities in broiler chickens by impairing the developmental process and functional maturation of T- and B-cells in both primary and secondary lymphoid tissues, and increase the expression of pro-inflammatory cytokines, presumably through an enhanced proliferation of lymphocytes and macrophages (Hirakawa et al., 2020; Goel et al., 2021). Broilers subjected to HS had lower levels of total circulating antibodies, as well as lower specific IgM and IgG levels, both during primary and secondary humoral responses (Bartlett and Smith, 2003). HS- induced decrease in the number of lymphocytes in the cortex and medulla areas of the bursa was

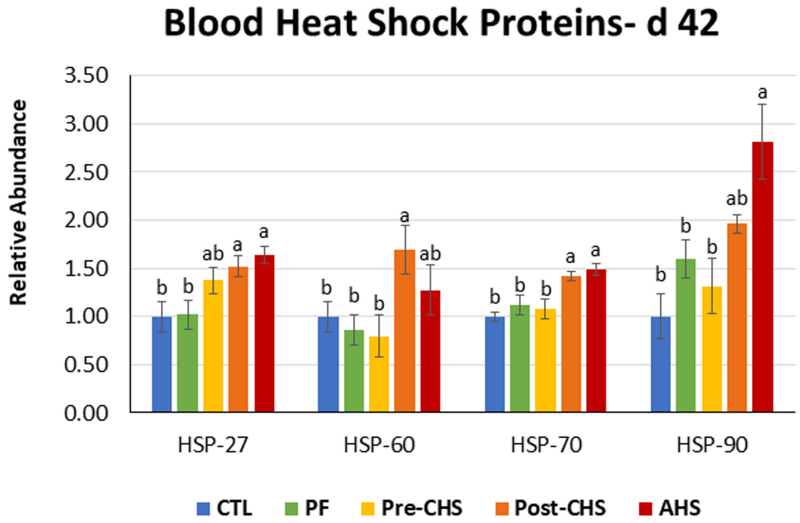

FIGURE 4 | Relative mRNA abundance of heat shock protein (*HSP*)-*27*, *HSP60*, *HSP70*, and *HSP90* in the blood of Cobb 500 male broiler chickens on day 42. Birds in all the groups were raised under the recommended conditions from day 0 to 28. Treatments include: (1) control (CTL): birds raised under thermoneutral condition (23°C) from day 29 to 42; (2) AHS: birds raised under thermoneutral condition (23°C) from day 29 to 42, but exposed to 2 h AHS (35°C) before sampling on day 42; (3) Pre-CHS: birds exposed to high ambient temperature (35°C) for 8 h/day (9:30 am–5:30 pm) from day 29 to 42, and sampled before starting the CHS; (4) Post-CHS: birds exposed to high ambient temperature (35°C) for 8 h/day (9:30 am–5:30 pm) from day 29 to 42, and sampled 2 h after starting the CHS; (5) pair-fed (PF): birds raised under thermoneutral condition (23°C) from day 29 to 42, but pair-fed to CHS group (these birds received each day 1.05 times the average feed intake recorded in the CHS group in the previous day). Values are represented as a *n*-fold difference relative to the calibrator (CTL). Results are given as means ($n = 8$) for each group. Error bars indicate standard errors. For each gene, bars with different letters are significantly different ($P < 0.05$).

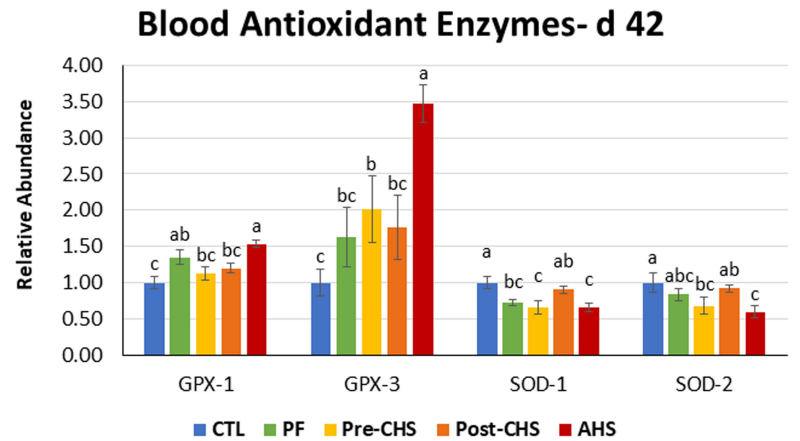

FIGURE 5 | Relative mRNA abundance of superoxide dismutase (*SOD*)-*1*, *SOD2*, glutathione peroxidase (*GPX*)-*1*, and *GPX2* in the blood of Cobb 500 male broiler chickens on day 42. Birds in all the groups were raised under the recommended conditions from day 0 to 28. Treatments include: (1) control (CTL): birds raised under thermoneutral condition (23°C) from day 29 to 42; (2) AHS: birds raised under thermoneutral condition (23°C) from day 29 to 42, but exposed to 2 h AHS (35°C) before sampling on day 42; (3) Pre-CHS: birds exposed to high ambient temperature (35°C) for 8 h/day (9:30 am–5:30 pm) from day 29 to 42, and sampled before starting the CHS; (4) Post-CHS: birds exposed to high ambient temperature (35°C) for 8 h/day (9:30 am–5:30 pm) from day 29 to 42, and sampled 2 h after starting the CHS; (5) pair-fed (PF): birds raised under thermoneutral condition (23°C) from day 29 to 42, but pair-fed to CHS group (these birds received each day 1.05 times the average feed intake recorded in the CHS group in the previous day). Values are represented as a *n*-fold difference relative to the calibrator (CTL). Results are given as means ($n = 8$) for each group. Error bars indicate standard errors. For each gene, bars with different letters are significantly different ($P < 0.05$).

reported (Aengwanich, 2008). Pro-inflammatory cytokine family, including interleukin *IL1*, *IL2*, *IL6*, *IL18*, and *TNF*-α, have been shown to play an active role in the inflammatory response under high ambient temperature (Helwig and Leon, 2011). HS (31°C from day 35 to 42) did not affect mRNA abundance of *IL1B*, *IL6*, *IL10*, *IL12*, *IL13*, *IFNG* in the spleen and cecal tonsils of broiler chickens; However, it increased mRNA abundance of *TGFB* in the cecal tonsils, with no effect on mRNA abundance of *TGFB* in spleen (Quinteiro-Filho et al., 2017). The mRNA abundance of *IL1B* significantly decreased in the spleen of Three-yellow chicken during AHS, whereas it was not affected in the Kirin chicken. The mRNA abundance of *IL6* and *IL10* were increased in

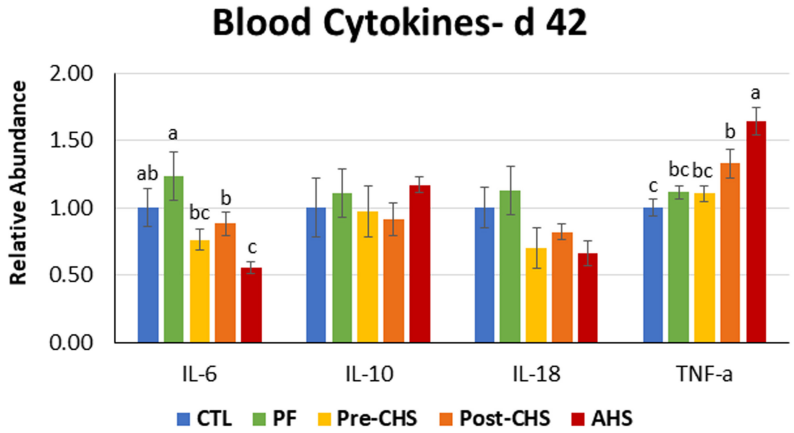

FIGURE 6 | Relative mRNA abundance of interleukin (*IL*)-*6*, *IL10*, *IL18*, and tumor necrosis factor-α in the blood of Cobb 500 male broiler chickens on day 42. Birds in all the groups were raised under the recommended conditions from day 0 to 28. Treatments include: (1) control (CTL): birds raised under thermoneutral condition (23°C) from day 29 to 42; (2) AHS: birds raised under thermoneutral condition (23°C) from day 29 to 42, but exposed to 2 h AHS (35°C) before sampling on day 42; (3) Pre-CHS: birds exposed to high ambient temperature (35°C) for 8 h/day (9:30 am–5:30 pm) from day 29 to 42, and sampled before starting the CHS; (4) Post-CHS: birds exposed to high ambient temperature (35°C) for 8 h/day (9:30 am–5:30 pm) from day 29 to 42, and sampled 2 h after starting the CHS; (5) pair-fed (PF): birds raised under thermoneutral condition (23°C) from day 29 to 42, but pair-fed to CHS group (these birds received each day 1.05 times the average feed intake recorded in the CHS group in the previous day). Values are represented as a *n*-fold difference relative to the calibrator (CTL). Results are given as means (n = 8) for each group. Error bars indicate standard errors. For each gene, bars with different letters are significantly different ($P < 0.05$).

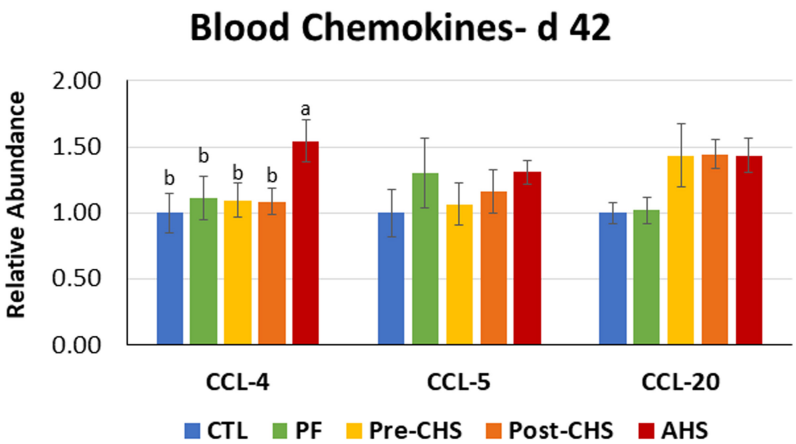

FIGURE 7 | Relative mRNA abundance of C-C motif chemokine ligand (*CCL*)-*4*, *CCL5*, and *CCL20* in the blood of Cobb 500 male broiler chickens on day 42. Birds in all the groups were raised under the recommended conditions from day 0 to 28. Treatments include: (1) control (CTL): birds raised under thermoneutral condition (23°C) from day 29 to 42; (2) AHS: birds raised under thermoneutral condition (23°C) from day 29 to 42, but exposed to 2 h AHS (35°C) before sampling on day 42; (3) Pre-CHS: birds exposed to high ambient temperature (35°C) for 8 h/day (9:30 am–5:30 pm) from day 29 to 42, and sampled before starting the CHS; (4) Post-CHS: birds exposed to high ambient temperature (35°C) for 8 h/day (9:30 am–5:30 pm) from day 29 to 42, and sampled 2 h after starting the CHS; (5) pair-fed (PF): birds raised under thermoneutral condition (23°C) from day 29 to 42, but pair-fed to CHS group (these birds received each day 1.05 times the average feed intake recorded in the CHS group in the previous day). Values are represented as a *n*-fold difference relative to the calibrator (CTL). Results are given as means (n = 8) for each group. Error bars indicate standard errors. For each gene, bars with different letters are significantly different ($P < 0.05$).

the spleen of Kirin chicken subjected to AHS, but their abundance was not affected in Three-yellow chicken due to the AHS (Adu-Asiamah et al., 2021). AHS (7 h at 40°C) significantly decreased *IFNB*, and *IL4* concentration in the plasma of broiler chickens (Saleh and Al-Zghoul, 2019).

Dysregulation of circulating inflammatory factors during CHS was previously reported by our group which is not in line with the results of the current study. Compared to birds raised under thermoneutral condition, CHS (12 h/day; 35°C) increased mRNA abundance of *SOD1*, *TNF-α*, *CCL4*, and *CCL20*, downregulated *GPX3*, *IL18*, and *NLRP3* inflammasome, with no effect on HSPs, *NLRC3* and *NLRX1* inflammasomes (Greene et al., 2021b). Discrepancies might be due to the duration of HS (8 vs. 12 h/day).

Regulated gut integrity is essential for controlling the transfer of pathogenic bacteria and immunogens and is considered a very essential and critical checkpoint for the regulation of immune responses (Emami et al., 2019, 2020a). Thus, altered gut integrity in the CHS group but not in the PF, confirm that the

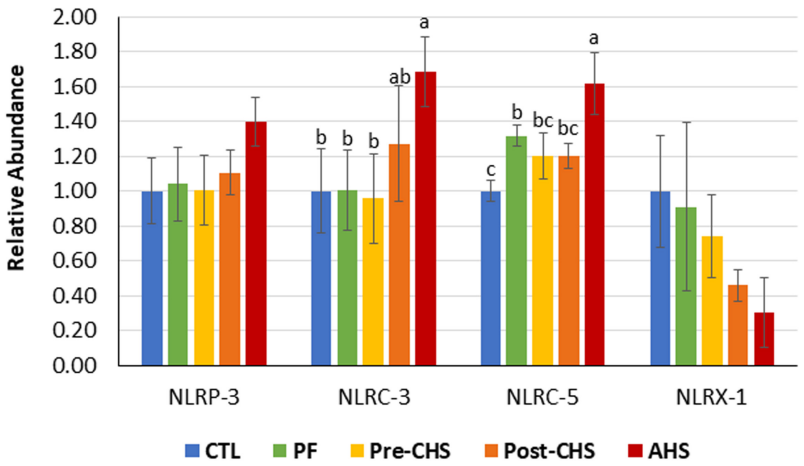

FIGURE 8 | Relative mRNA abundance of nucleotide-binding, leucine-rich repeat and pyrin domain containing (*NLRP*)-*3*, nucleotide-binding oligomerization domain, leucine-rich repeat, and CARD domain containing (*NLRC*)-*3*, *NLRC5*, and nucleotide-binding, leucine-rich repeat containing X1 (NLRX1) in the blood of Cobb 500 male broiler chickens on day 42. Birds in all the groups were raised under the recommended conditions from day 0 to 28. Treatments include: (1) control (CTL): birds raised under thermoneutral condition (23°C) from day 29 to 42; (2) AHS: birds raised under thermoneutral condition (23°C) from day 29 to 42, but exposed to 2 h AHS (35°C) before sampling on day 42; (3) Pre-CHS: birds exposed to high ambient temperature (35°C) for 8 h/day (9:30 am–5:30 pm) from day 29 to 42, and sampled before starting the CHS; (4) Post-CHS: birds exposed to high ambient temperature (35°C) for 8 h/day (9:30 am–5:30 pm) from day 29 to 42, and sampled 2 h after starting the CHS; (5) pair-fed (PF): birds raised under thermoneutral condition (23°C) from day 29 to 42, but pair-fed to CHS group (these birds received each day 1.05 times the average feed intake recorded in the CHS group in the previous day). Values are represented as a *n*-fold difference relative to the calibrator (CTL). Results are given as means ($n = 8$) for each group. Error bars indicate standard errors. For each gene, bars with different letters are significantly different ($P < 0.05$).

negative effects of CHS cannot be attributed only to the lower FI. Similarly, others showed that the changes observed in the intestinal permeability in chickens subjected to HS for 24–72 h are due to the HS conditions and not due to reduced FI (Nanto-Hara et al., 2020). In addition, results of current experiment showed that CHS and AHS both had a negative impact on the gut integrity, suggesting the rapid onset and continuation of leaky gut during the HS.

Birds in the CTL group had significantly higher hot carcass yield compared to PF but not CHS group. In contrast, HS reduced slaughter and carcass weights compared to birds raised under thermoneutral condition (Zeferino et al., 2016; Greene et al., 2021a). Despite the lowest ADG and final live body weight, birds in the CHS group had higher hot carcass yield compared to the PF group on day 42. Others reported increased carcass and abdominal fat percentages in broilers exposed to HS compared to their PF counterparts (Zeferino et al., 2016). Higher carcass yield in the CHS compared to the PF might be due to significantly lower liver weight (in %) compared to PF group. Previous studies confirm that birds exposed to HS had lower internal organ weights including thymus, bursa, spleen, liver, and heart (Bartlett and Smith, 2003; Zeferino et al., 2016). Furthermore, reduced liver weight was reported in laying hens subjected to CHS conditions (Felver-Gant et al., 2012). Besides lower organs weight, higher hot carcass yield in the CHS group might be due to higher leg quarters and wing% in the CHS group compared to PF.

Cyclic heat stress exposure decreased breast yield, which was significantly lower than that in CTL and PF groups. It has been reported that the effects of CHS is breed-dependent and HS negatively affects breast yield in fast growing broilers (arbor acres), but not slow growing chickens (Beijing You Chicken) (Lu et al., 2007). Similarly, CHS decreased the proportion of breast muscle, while increasing the proportion of thigh muscle and fat deposition in broilers (Zhang et al., 2012). Previously, we showed the negative effects of CHS on breast weight (Greene et al., 2021a). Exposure to HS reduced breast %; however, in contrast to the results of our experiment, PF chickens' breast % was similar to thermoneutral raised birds (Zeferino et al., 2016). Higher leg quarters and wings %, despite lower breast yield due to CHS might be due to the allocation of resources to the muscles that are involved in the movement (legs and wings), rather than breast which is mostly considered as a storage. On the top of that, blood which contains the nutrients might have been directed toward the limbs (extremities) as a mechanism for heat dissipation.

As mentioned, PF group had the highest liver % which was significantly higher compared to other groups, while abdominal fat was significantly higher in birds exposed to CHS. Lower abdominal fat % in the PF group compared to the birds raised under CHS was previously reported by other researchers (Zeferino et al., 2016). This brings an interesting hypothesis into consideration which is the distinct regulatory mechanisms of energy metabolism in the body of PF vs. CHS birds. With this regard, it seems that the energy metabolism in PF group leans toward glycogen metabolism (short-term); while, in the CHS birds the regulation of energy metabolism seems to be directed toward the fat metabolism (long-term). Further consideration, assessment and comparison

of the key regulators of lipogenesis, lipolysis, and glycogenesis in the liver and fat tissue of CHS compared to the PF group is warranted.

CONCLUSION

Chronic CHS exerts its negative effects on performance, and body composition of broiler chickens through direct mechanisms that are independent of FI depression. Additionally, CHS and AHS both exert negative effects on broiler chickens' gut integrity. However, at the mRNA level, only AHS dysregulate inflammatory responses in the circulation which suggests that the involved mechanisms occur rapidly during the HS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

All animal care and procedures were approved by the Institutional Animal Care and Use Committee at the University of Arkansas.

AUTHOR CONTRIBUTIONS

SD conceptualized and designed the experiment. NE, EG, and SD performed the experiment. NE wrote the first draft. SD edited the manuscript with a critical review by MK. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Proper Immune Response Depends on Early Exposure to Gut Microbiota in Broiler Chicks

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The successional changes in the early intestinal microbiota occur concomitantly with the development, expansion, and education of the mucosal immune system. Although great attention of researchers has been focused on understanding the linkage between microbiota and immune functions, many essential details of the symbiotic relationship between the intestinal pioneer microbiota and the avian immune system remain to be discovered. This study was conducted to understand the impact of different early life intestinal colonizers on innate and adaptive immune processes in chicks and further identify immune-associated proteins expressed in the intestinal tissue. To accomplish it, we performed an *in ovo* application of two apathogenic *Enterobacteriaceae* isolates and lactic acid bacteria (L) to determine their influences on the intestinal proteome profile of broilers at the day of hatch (DOH) and at 10 days old. The results indicated that there were predicted biological functions of L-treated chicks associated with the activation and balanced function of the innate and adaptive immune systems. At the same time, the *Enterobacteriaceae*-exposed birds presented dysregulated immunological mechanisms or downregulated processes related to immune development. Those findings suggested that a proper immune function was dependent on specific gut microbiota exposure, in which the prenatal probiotic application may have favored the fitting programming of immune functions in chicks.

Keywords: immune programming, probiotics, *Enterobacteriaceae*, lactic acid bacteria, intestinal microbiome, innate immune system, proteome, pathways

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INTRODUCTION

In recent years, attention has focused on understanding the linkage between microbiota and immune functions. Such an intimate relationship creates mechanisms for mutual benefits to both microbes and the host (Chow et al., 2010). At homeostasis, this mutualistic partnership enables the maintenance of microbial tolerance in the intestinal ecosystem, and, in turn, the proper microbiota colonization contributes to the development, maturation, and function of the immune system (Bar-Shira et al., 2003; Kelly et al., 2007; Brisbin et al., 2008; Chung et al., 2012).

Nevertheless, the cooperative arrangements between gut microbiota and host mucosal immunity are constantly threatened during the early life of chickens. Evidence has suggested

that undesirable microbial colonization during the prenatal period may negatively influence the function and expansion of the immune system in broilers (Bar-Shira et al., 2003; Rodrigues et al., 2020a). Previous work has also demonstrated that perturbation of the intestinal pioneer microbiota with antibiotic exposure affects early immune programming and has been shown to boost negative antibody response in hens after cessation of antibiotic treatment (Simon et al., 2016). Besides, our previous reports indicated that pre-hatch colonization by *Enterobacteriaceae* promoted immune dysregulation and chronic inflammation (Rodrigues et al., 2020a,b; Wilson et al., 2020). Another example of dysregulated immune response triggered by early disturbance of enteric microbiota in chicks was shown by Schokker et al. (2010). It was revealed that early colonization by *Salmonella enterica* serotype Enteritidis delayed the morphological processes of the jejunum, thereby interrupting the spatial-temporal development of the immune system in chicks. On account of this fact, intestinal dysbiosis during the early post-hatch phase in chicks may have short- and long-term consequences on immune responses. Against this background, many essential details of the symbiotic relationship between the intestinal pioneer microbiota and the avian immune system remain to be discovered, and an important outstanding question was whether a proper early immune response depends on host-specific gut microbiota.

Recently, the emergence of *in ovo* techniques made it possible to manipulate the intestinal bacteria colonization before chicks have even been hatched or exposed to farm environments (Pedroso et al., 2016; Roto et al., 2016; Teague et al., 2017). Our lab has previously used the *in ovo* technique as an experimental model to address how early intestinal colonization shapes the microbiome composition in hatching chicks (Wilson et al., 2020). The technique of delivering various nutrients, supplements, immunostimulants, vaccines, and drugs *via* the *in ovo* route is gaining wide attention among researchers for boosting the production performance and immunity and for safeguarding the health of poultry (Saeed et al., 2019; Alagawany et al., 2021; Hassan et al., 2021).

In order to better understand the impact of different early life intestinal colonizers on innate and adaptive immune processes in chicks and further identify immune-associated proteins expressed in the intestinal tissue, we performed an *in ovo* application of two apathogenic *Enterobacteriaceae* isolates and lactic acid bacteria to determine their influences on the intestinal proteome profile of broilers at the day of hatch (DOH) and 10 days of age. Proteomics pathway enrichment and Gene Ontology (GO) function annotation analyses were performed to reveal the effect of different pioneer intestinal colonizers on innate and adaptive immune processes in chicks.

MATERIALS AND METHODS

Study Design

The trial was performed on Ross 708 fertile eggs obtained from a local hatchery and incubated under standard conditions at the poultry research farm of the Ohio Agricultural Research and Development Center. All eggs were in the same incubator prior to inoculation. Once eggs were confirmed fertile, at embryonic day 18, inoculations containing one of the following: 0.9% sterile saline (S), $\sim 10^2$ cells of *Citrobacter* (CF), *Citrobacter* 2 (C2), or a lactic acid bacteria probiotic (L) were administered *via in ovo* injection into the amnion. After inoculation, the eggs were allocated by treatments into separate benchtop hatchers (Hova-Bator model 1602N, Savannah, GA, USA). Each inoculation treatment was separated into three hatchers, which contained up to 30 eggs. The chicks hatched between 48 and 72 h post-inoculation. The hatchability ranged from 86 to 100% among the *in ovo* treatments (Wilson et al., 2020). The bacterial inoculum was selected from our previous trial (Bielke et al., 2003), and the homology of strains was confirmed by next-generation sequencing. The L isolate was composed of a mixed culture of *Lactobacillus salivarius* and *Pediococcus* ssp. The CF strain was composed of *Citrobacter freundii*, and the C2 was identified as *Citrobacter* spp. Bacterial inoculum was prepared as described by Wilson et al. (2020). All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Ohio State University.

Sample Collection

Once all chicks were hatched, 10 chicks were randomly chosen from among the hatchers within each of the treatments ($n = 40$) and were immediately euthanized *via* cervical dislocation. The intestine was aseptically removed, from the duodenum to the cloaca. Tissues were placed in individual 2 ml tubes and flash-frozen in liquid nitrogen. Immediately post-hatch, the remaining 128 chicks were placed into treatment-separate brooder battery cages and had *ad libitum* access to a standard corn-soy diet and water (Nutrient Requirements of Nutrient Requirements of Poultry., 1994). At 10 days post-hatch, 12 chicks per treatment were randomly selected for ileal proteome analysis. The tissue of the region above the ileocecal junction, designated as lower ileum, was aseptically collected ($n = 45$). Since there were three mortalities in the CF, only nine birds were sampled for this treatment. Ileum tissue was also frozen and stored at −80°C until further use.

Once intestinal samples were thawed, a cumulative total of 0.1 g was individually placed into 5 ml of buffer (8 M urea/2 M thiourea, 2 mM DTT, 50 mM Tris, 5% SDS, pH 6.8). The extraction protocol is a modified version described previously by Iqbal et al. (2004) and Kong et al. (2016). In brief, samples were homogenized for 5 s (PRO250 Homogenizer; Pro Scientific, Oxford, CT, USA), and then, 500 μl of the solution was placed in 2 ml tubes containing 0.1 g stainless steel beads (SSB14B; Next Advance, Averill Park, NY, USA). Samples were homogenized for 3 min total in 30-s intervals (MiniBeadbeater-16, Model 607; BioSpec Products, Bartlesville, OK, USA) and centrifuged at 4°C at 14,000 *g* for 20 min. The supernatant was collected, aliquoted, and placed at −80°C until further use.

To ensure proper extraction, the concentration of total protein was quantified with the Bradford assay (Bradford reagent; VWR, Suwanee, GA, USA) and a standard bovine serum albumin curve (VWR, Suwanee, GA, USA) on a Synergy HTX multi-mode plate reader (BioTek Instruments, Winooski, VT, USA). Samples were mixed to create pooled samples from two birds

per treatment/time and were sent to the Ohio State University Proteomics Core lab for performing in solution digestion and mass spectrometry *via* established methods.

Proteomics Analyses

Samples were precipitated with 25% (w/v) trichloroacetic acid (TCA) and then resuspended in 50 mM ammonium bicarbonate. A total of 5 ml of DTT (5 μg/μl in 50 mM ammonium bicarbonate) was added, and the samples were incubated at 56°C for 15 min. After incubation, 5 μl of iodoacetamide (15 mg/ml in 50 mM ammonium bicarbonate) was added, and the samples were kept in the dark at room temperature for 30 min. Sequencing grade-modified trypsin (Promega, Madison, WI, USA) prepared in 50 mM ammonium bicarbonate was added to each sample with an estimation of 1:20/1:100 enzyme-substrate ratio set at 37°C overnight. The reaction was quenched the following day by adding acetic acid for acidification. Once samples were quenched, the peptide concentration was measured by Nanodrop (Thermo Scientific Nanodrop 2000; Thermo Scientific, Waltham, MA, USA).

Capillary-liquid chromatography-nanospray tandem mass spectrometry (Capillary-LC/MS/MS) of global protein identification was performed on a Thermo Fisher Fusion mass spectrometer (Thermo Scientific, Waltham, MA, USA). Samples were separated on a Thermo Nano C18 column (UltiMate™ 3000 HPLC system; Thermo Scientific, Waltham, MA, USA). The MS/MS data sequences were scanned, and the scan sequence was based on the preview mode data-dependent TopSpeed™ method with CID and ETD as fragmentation methods. The raw data were searched on Sequest *via* Proteome Discoverer (Proteome Discoverer™ software; Thermo Scientific, Waltham, MA, USA). The data were searched against the most recent Uniprot *Gallus gallus* database for the identification of proteins. Only proteins with <0.05 false discovery rate (FDR) were reported. Proteins with a Mascot score of 50 or higher with a minimum of two unique peptides from one protein having a -b or -y ion sequence tag of five residues or better were accepted. Any modifications or low score peptide/protein identifications were manually checked for validation.

BioInformatics and Statistical Analysis

Label-free quantitation was performed using the spectral count approach, in which the relative protein quantitation is measured by comparing the number of MS/MS spectra identified from the same protein in each of the multiple LC/MSMS datasets. Comparisons between *in ovo* bacterial treatments and S control group were performed in Scaffold (Scaffold 4.8.4; Proteome Software, Portland, OR, USA). The student's *t*-test ($P < 0.05$) was performed to identify significance across the fold-change values. Differentially expressed proteins (DEPs) were assigned at level $P \leq 0.1$.

From the significant proteins were performed GO annotation enrichment terms analysis using the STRING v10.0 database (https://string-db.org). Pathway enrichment was assessed using upregulated DEPs based on Reactome databases. Then to evaluate the role of overexpressed proteins in the immune system, we searched for immune pathways based on biological processes using Reactome pathways (https://reactome.org/). $P < 0.05$ was set as the threshold value, and FDR was corrected using the Benjamini-Hochberg method. Immune-related DEPs were identified and their predicted functional partners were searched (STRING v10.0 database). Then, functional clusters in the protein-protein interaction (PPI) networks were determined. Active interaction sources, including experiments, databases, co-occurrence, and co-expression, as well as species limited to "*Gallus gallus*," and an interaction score > 0.4 were applied to construct the PPI networks.

RESULTS

Identification of DEPs

A total of 78, 107, and 39 proteins were identified as DEPs in the ileum of L, CF, and C2 treatments, based on the set threshold ($P \leq 0.1$) at DOH. By 10 days of age, a total of 61, 44, and 63 proteins showed differential expression in L, CF, and C2, respectively, compared with the *in ovo* S control.

Functional Enrichment Analysis

To investigate whether different early colonizers in the gastrointestinal tract (GIT) could affect biological processes linked to the immune system, GO enrichment analyses were performed for overexpressed and underexpressed proteins in each dataset. The top 20 enriched GO terms, relative to the control, were exhibited for each *in ovo* treatment at two time points. Proteins associated with response to stimulus and immune annotation roles were displayed in **Figure 1**.

The upregulated DEPs in L revealed that the major biological processes at DOH were related to protein and cellular metabolic process, cellular organization, and response to a stimulus (Blue bars in **Figure 2A**). On the 10th day, cellular differentiation and assembly, regulation of stress response, and immune system process were enriched. In terms of downregulated DEPs in the L dataset, the most enriched processes were biological regulation, cell communication, and response to stress at DOH. While by 10 days of age, proteolysis, cellular metabolic process, and immune system process were overrepresented (Red bars in **Figure 2B**).

The upregulated DEGs in CF were involved in different GO terms, including cellular and metabolic processes, and protein folding at DOH (**Figure 3A**), whereas terms associated with protein stabilization and regulation of cellular were evident by 10 days of age (**Figure 3B**). Conversely, the downregulated DEPs in CF were related to transport, localization, and cellular organization component at DOH (**Figure 3A**). By 10 days of age, the GO terms were allied to the catabolic process and response to a stimulus (**Figure 3B**).

The most significant GO annotation based on the overexpressed proteins in C2 was associated with a cellular process, biological regulation, and organelle organization at DOH (**Figure 4A**), whereas it was associated with cellular metabolic process, primary metabolic process, and response to a stimulus at 10 days of age (**Figure 4B**). The underrepresented proteins enhanced only a few terms at DOH, including cellular process and transport. Biological functions enriched by 10 days

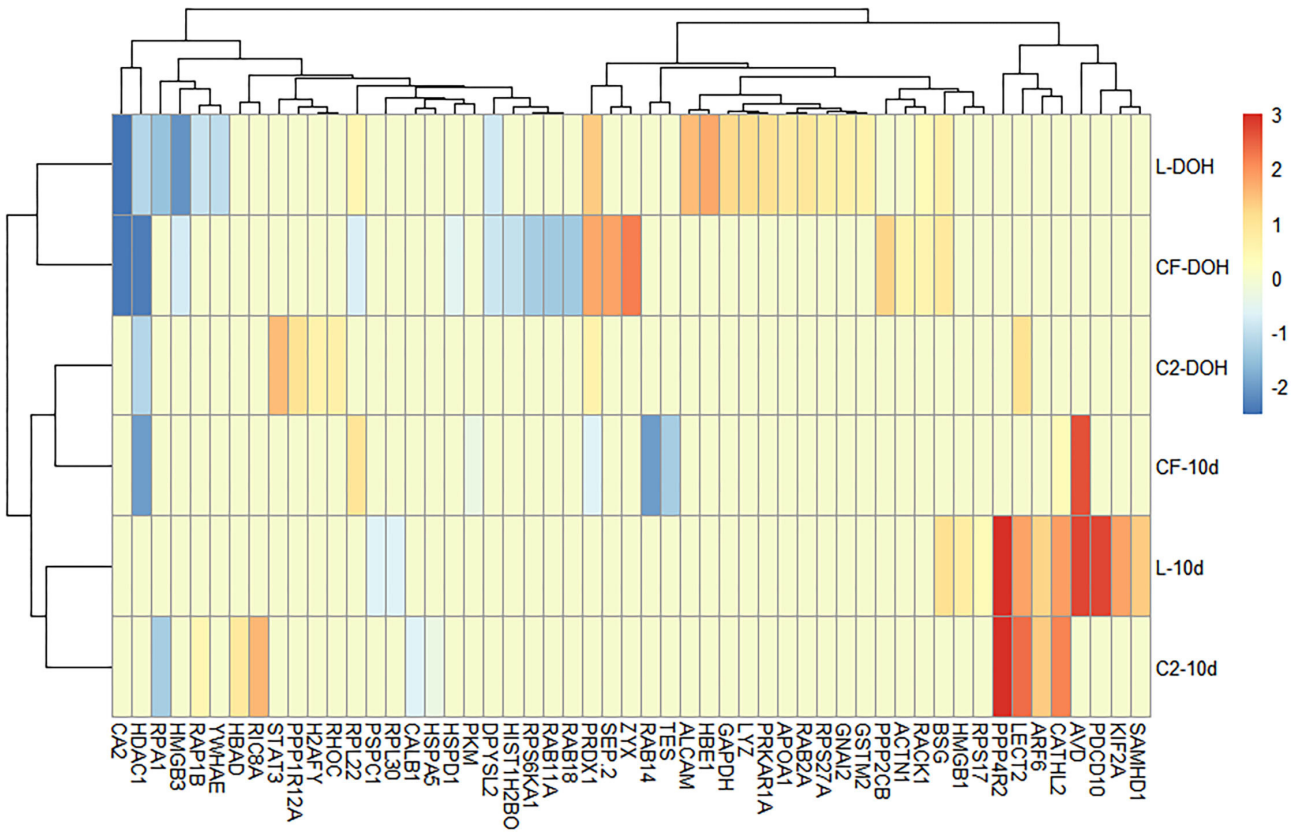

FIGURE 1 | Differentially expressed proteins (DEPs) related to immune processes based on Gene Ontology analysis. Heatmap plot represents the log2-fold changes of proteins expressed intestine samples of broilers treated with lactic acid bacteria (L), *Citrobacter freundii* (CF) or *Citrobacter* spp. (C2) in relation to control treatment at the day of the hatch (DOH) and 10 days of age (10d). Blue shades represent downregulation, while red shades indicate upregulation of the particular protein. Light yellow cells indicate no expression.

of age were related to protein metabolic, homeostatic, and cellular protein metabolic processes (**Figure 4B**).

Pathways With DEPs

The five most significant pathways in L at DOH included amyloid fiber formation, glutathione conjugation, cellular responses to stress, HSF1 activation, and cellular responses to external stimuli (**Table 1**). The enriched pathways by 10 days of age were muscle contraction, smooth muscle contraction, kinesins, axon guidance, and COPI-dependent Golgi-to-ER retrograde traffic.

The most relevant predicted pathways in CF were syndecan interactions, integrin cell surface interactions, assembly and cell surface presentation of NMDA receptors, cellular responses to external stimuli, and extracellular matrix organization at DOH (**Table 1**). By 10 days of age, pathways associated with P75NTR negatively regulate the cycle *via* SC1, and phase II conjugation of compounds, glutathione conjugation, acetylation, and FOXO-mediated transcription were enriched.

The inoculation of C2 affected pathways related to the regulation of Toll-like receptors (TLRs) by endogenous ligand, signaling by RAF1 mutants, paradoxical activation of RAF signaling by kinase inactive BRAF, signaling by moderate kinase activity BRAF mutants, and signaling downstream of RAS mutants at DOH; whereas cellular response to starvation, response of EIF2AK4 (GCN2) to amino acid deficiency, eukaryotic translation elongation, GRB2:SOS linkage to MAPK signaling for integrins, and L13a-mediated translational silencing of ceruloplasmin expression was activated at 10 days of age (**Table 1**).

The presence of several immune genes prompted us to investigate whether the early exposure to different bacterial isolates could affect the immune biological processes in the GIT of broilers. Then, we performed the broad immune response pathways in Reactome for up- and downregulated proteins. Analyses showed substantially more upregulated DEPs in L treatment that accomplishes functions on immune signaling than the other *in ovo* treatment by DOH (**Figure 5**). Accordingly, an enrichment of heterophil degranulation ($P = 0.0005$; FDR = 0.034) and antimicrobial peptides pathways ($P = 0.022$; FDR = 0.129) at DOH was observed (**Figure 5**). By 10 days of age, MHC class II antigen presentation was overexpressed in L treatment ($P = 0.003$; FDR = 0.076). Using downregulated DEPs, Reactome analyses showed that Rap1 signaling was enriched in L at DOH ($P = 0.004$; FDR = 0.053; **Figure 5**), while the pathways heterophil degranulation ($P = 0.018$; FDR= 0.223) and Butyrophilin (BTN) family interactions ($P = 0.037$; FDR= 0.223) were significant at 10 days of age.

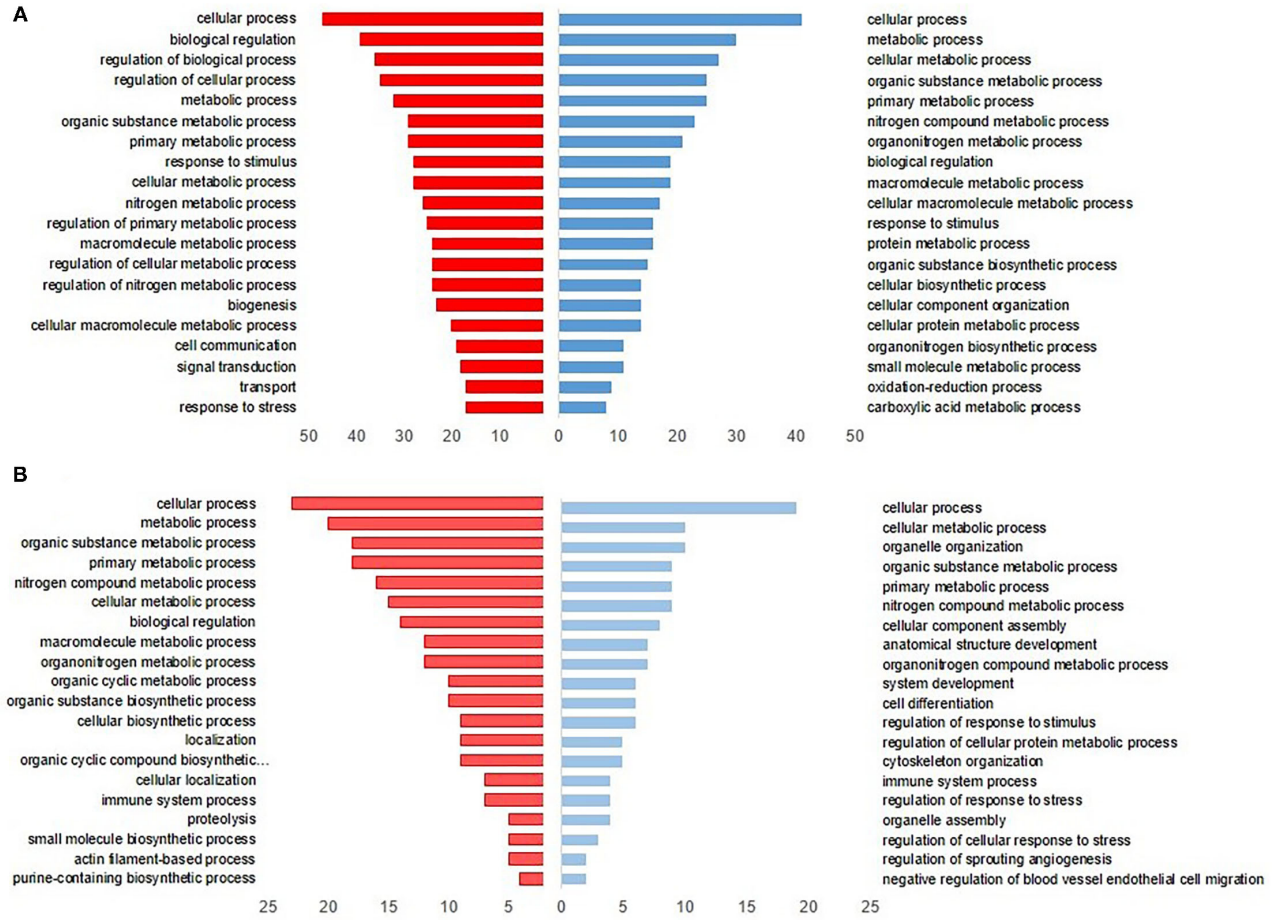

FIGURE 2 | Gene Ontology (GO) enrichment analysis of intestinal differentially expressed proteins from broilers exposed to lactic acid bacteria (L) *in ovo* **(A)** at the day of hatch and **(B)** by 10 days of age. The blue bars to the right indicate the top 20 GO-enriched terms by upregulated proteins. The red bars to the left represent the top 20 GO terms enriched by downregulated proteins, while the axis at the bottom is the number of proteins in each biological process.

While there was not any pathway enhanced with upregulated DEPs at DOH in CF, the pathway Fcgamma receptor (FCGR)-dependent phagocytosis ($P = 0.025$; FDR = 0.100) was overrepresented in downregulted DEPs. Regarding C2 treatment, within upregulated DEPs, there were enriched pathways related to heterophil degranulation ($P = 0.001$; FDR = 0.022) and TLRs cascades ($P = 0.001$; FDR = 0.023) at DOH. By 10 days of age, Rap1 pathway ($P = 0.003$; FDR = 0.019) was significant. Within the underexpressed DEPs profile, the FCGR-dependent phagocytosis was enriched at DOH ($P = 0.0002$; FDR = 0.007). By 10 days of age, antimicrobial peptides ($P = 0.031$; FDR = 0.130) and heterophil degranulation ($P = 0.022$; FDR= 0.130) were enhanced.

Identification of Immune-Related DEPs

Proteins related to immunity among the treatments were ranked based on a functional comparison involving the GO terms. The key immune-related DEPs included leukocyte cell-derived chemotaxin-2 (LECT2), avidin (AVD), high mobility group protein B1 (HMGB1), activated leukocyte cell adhesion molecule (ALCAM), cathelicidin-2 (CAMP), and lysozyme C (LYZ). The DEPs associated with immune function were obtained from the following categories: immune system process, immune response, response to stress, cellular response to stimulus, and related functions (**Supplementary Data**).

Then, a PPI network for each key protein was screened for a better understanding of their roles and underlying mechanisms involving immune processes. **Figure 6** shows the interactome networks for LECT2, AVD, HMGB1, ALCAM, CAMP, and LYZ, in which the nodes represent proteins, while the edges are physical, biochemical, or functional interactions between them.

DISCUSSION

The presence of distinct intestinal proteome profiles in chicks inoculated *in ovo* with *Enterobacteriaceae* isolates or L-probiotic poses the question of whether the expression of intestinal immune-related proteins is dependent on specific early microbiota exposure. Here, we conducted different proteomic approaches as pathway enrichment and GO function annotation

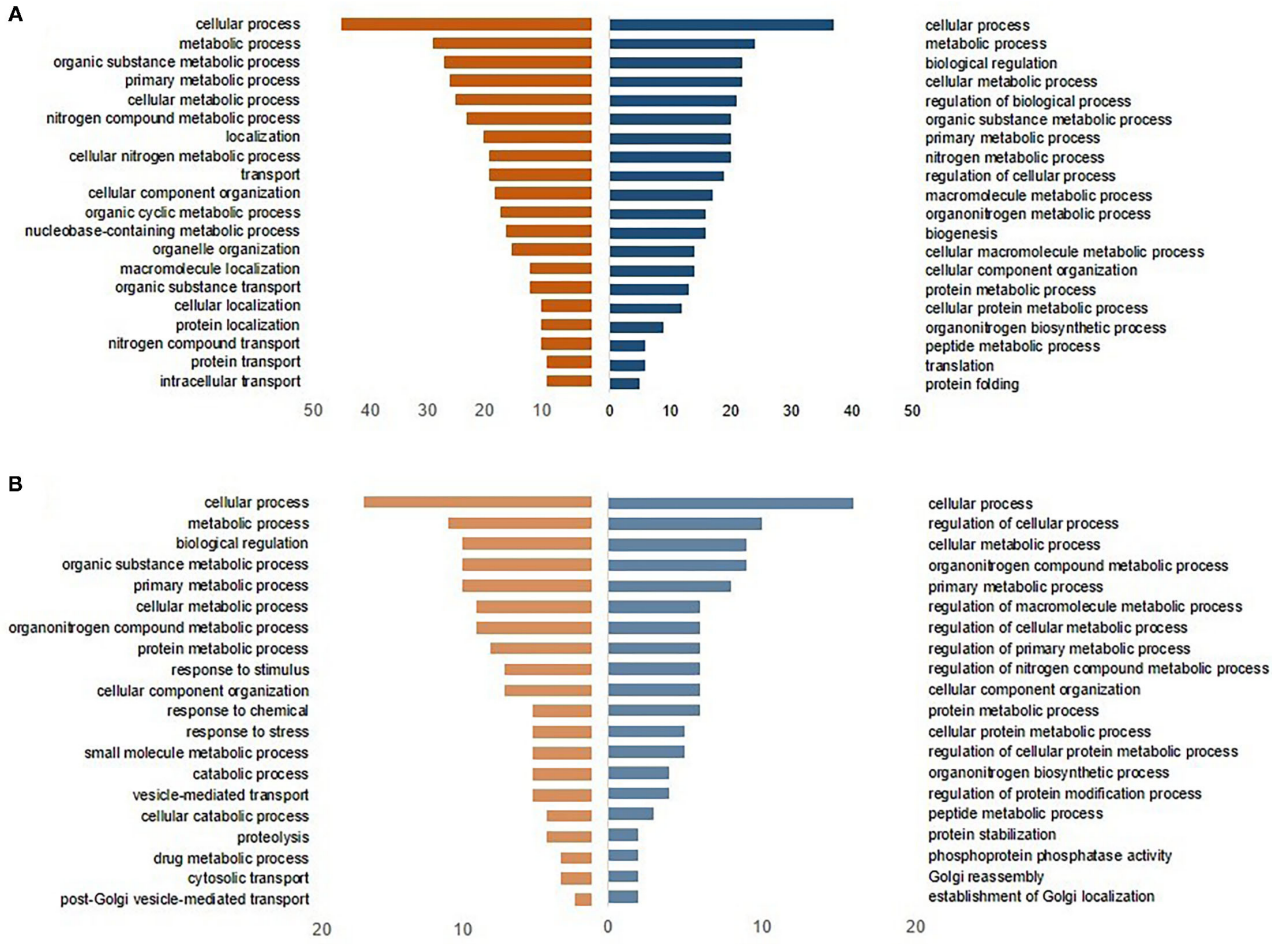

FIGURE 3 | Gene Ontology (GO)-enriched terms of intestinal differentially expressed proteins from broilers exposed to *Citrobacter freundii* (CF) *in ovo* **(A)** at the day of hatch and **(B)** by 10 days of age. The blue bars to the right indicate the most GO-enriched terms by upregulated proteins. The orange bars to the left represent GO terms enriched by downregulated proteins, while the axis at the bottom is the number of proteins in each biological process.

analyses to understand the impact of different early life intestinal colonizers on innate and adaptive immune processes in chicks. It was also the focus of this research to identify candidate protein markers for monitoring immune biological functions in broilers. The results of this study provide a significant comprehension of how early exposure to beneficial bacteria may affect immune programming in broilers.

Our previous work also addressed the *in ovo* technique as an experimental model to study how early intestinal colonization can shape the development and persistence of microbiome in chicks (Rodrigues et al., 2020a; Wilson et al., 2020). In those studies, it was shown that different bacterial isolates provided *in ovo* resulted in distinct microbiome profiles on DOH and by 10 days of age. Notably, inoculation of L resulted in increased Lactobacilli populations at DOH, which may have influenced the establishment of butyrate-producing bacteria and segmented filamentous bacterium (SFB) in young broilers. The poultry GIT microbiota undergoes a period of heavy changes during the first days of life. Some reports have shown that the microbiota composition of post-hatched chicks is primarily Proteobacteria derived from opportunistic environmental communities (Ballou et al., 2016; Donaldson et al., 2017; Rodrigues et al., 2020a; Wilson et al., 2020). Concomitant with this process occurs the development, expansion, and education of the mucosal immune system (Chow et al., 2010; Gensollen et al., 2016; Zheng et al., 2020). The adaptive immune functions of newly hatched chicks develop only toward the end of the first week post-hatch (Barshira and Friedman, 2006). Therefore, maternal antibodies and the innate immune system are the main apparatus for dealing with any early pathogenic assault.

From that perspective, early exposures are significant determinants for programming innate immune functions. We found that L treatment enriched the key pathways of the innate immune signaling as heterophil degranulation and antimicrobial peptides at DOH (**Figure 5**). One of the mechanisms displayed by the innate immune system of the host in reaction to microbial stimulation or into the pathogen-containing phagosome is heterophil degranulation, which releases granule substances

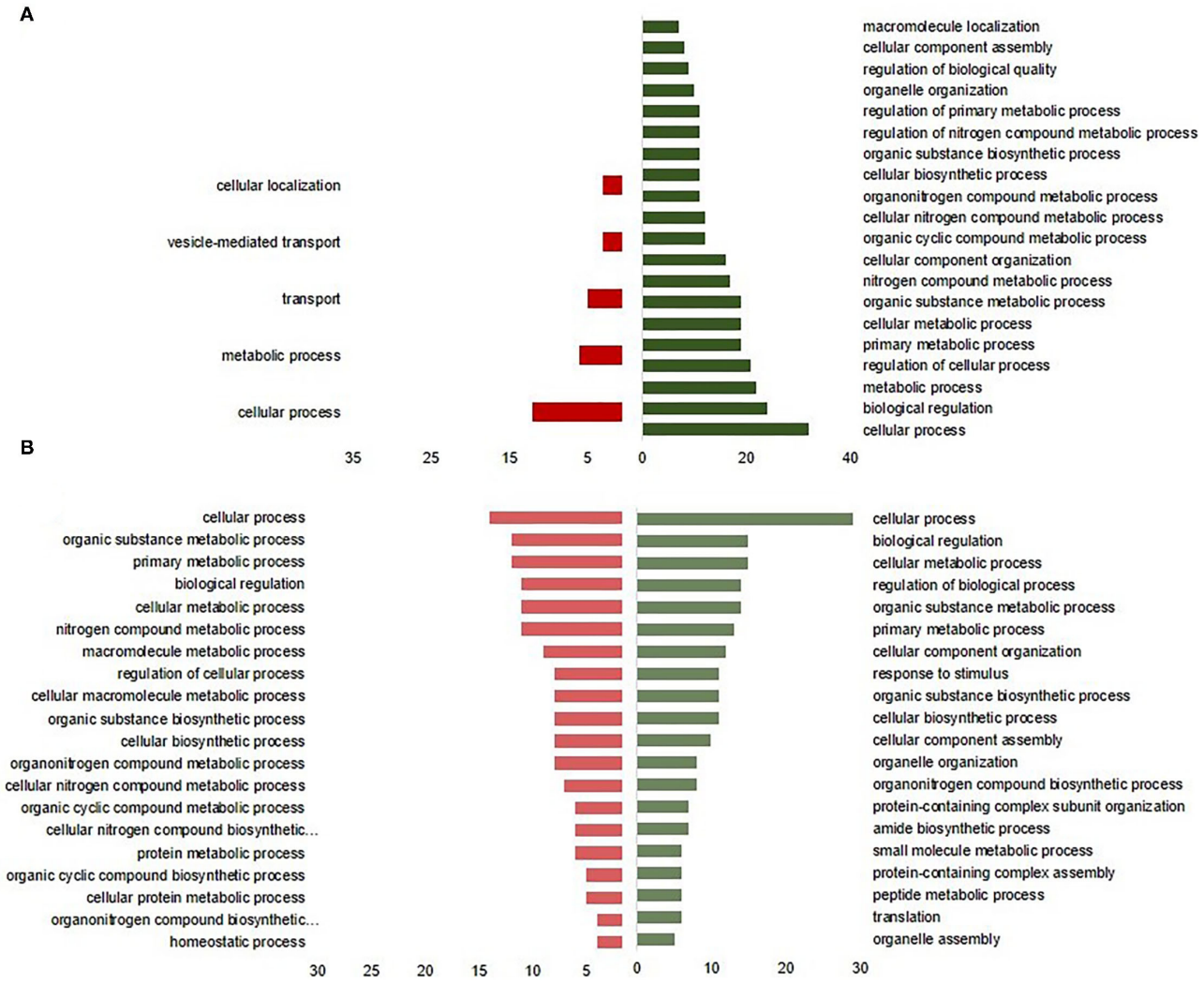

FIGURE 4 | Enrichment of biological process of intestinal differentially expressed proteins from broilers exposed to *Citrobacter* spp. (C2) *in ovo* **(A)** at the day of hatch and **(B)** by 10 days of age. The green bars to the right indicate the most GO-enriched terms by upregulated proteins. The red bars to the right represent GO terms enriched by downregulated proteins, while the axis at the bottom is the number of proteins attributed to each GO term.

including antimicrobial peptides into the external environment (Kogut et al., 2001; Genovese et al., 2013). In agreement with our study, Farnell and Donoghue (2006) reported that oral administration of probiotics could stimulate heterophil oxidative bursting and degranulation in the poultry intestine. Likewise, the upregulated DEPs were predominantly associated with cellular response to stress and external stimuli pathways. Part of cellular responses to external stimuli is carried out by macroautophagy that is considered a cytoprotective host defense mechanism against damaged organelles, cytosolic proteins, and invasive microbes (Feng et al., 2014; Delorme-Axford and Klionsky, 2018). Macroautophagy assists both innate and adaptive immunity releasing lysosomes for the degradation of foreign substances, including pathogenic proteins within cells (Gannagé and Münz, 2009; Bel and Hooper, 2018). Following this process, the products of lysosomal degradation are presented for MHC class II molecules and recognized by CD4+ T cells (Schmid et al., 2007). Correspondingly, there was an enrichment of the MHC class II antigen presentation pathway in L by 10 days of age.

In contrast, these results revealed that the introduction of CF *in ovo* downregulated the MHC class II antigen presentation pathway. In addition, the downregulated DEPs in CF treatment were allied to transport, localization, and response to the stimulus at an early and later age, indicating that the functions associated with the trafficking of immune cells, detection, and response to a biotic and abiotic stimulus may have been impaired. Nedjic et al. (2008) have shown that a change in the MHC class II system by a genetic interference of autophagy in the thymus of mice resulted in severe colitis and multi-organ inflammation. Comparably, our recent research has indicated that pre-hatch *Enterobacteriaceae* colonization perturbed the initial microbial establishment and promoted intestinal proteomic changes

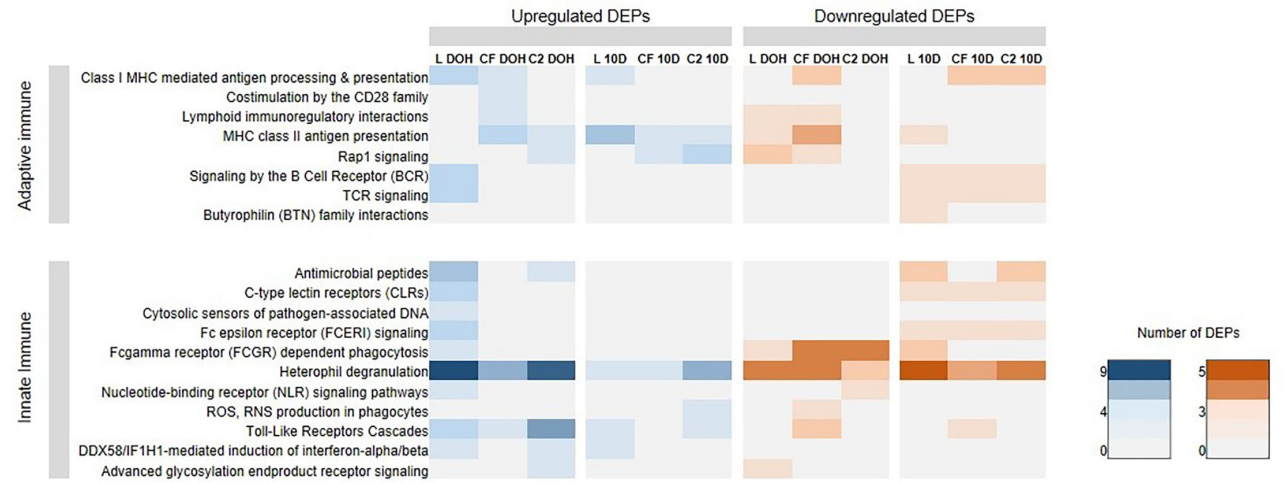

FIGURE 5 | Reactome Pathways analyses, at the day of hatch (DOH) and 10 days of age (10d), related to immune response based on differentially expressed proteins (DEPs) in intestinal samples of broilers treated with lactic acid bacteria (L), *Citrobacter freundii* (CF) or *Citrobacter* spp. (C2).

Protein	Name	Role in immune system
LECT2	Leukocyte cell-derived chemotaxin-2	Response to antibiotic; chemotaxis
AVD	Avidin	Antibacterial humoral response
HMGB1	High mobility group protein B1	Promotes host inflammatory response to sterile and infectious signals
ALCAM	Activated leukocyte cell adhesion molecule	Promotes T-cell activation and proliferation via its interactions with CD6
CATHL2	Cathelicidin-2 also called CAMP	Lipopolysaccharide binding, defense response to bacterium
LYZ	Lysozyme C	Bacteriolytic function; enhance the activity of immunoagents

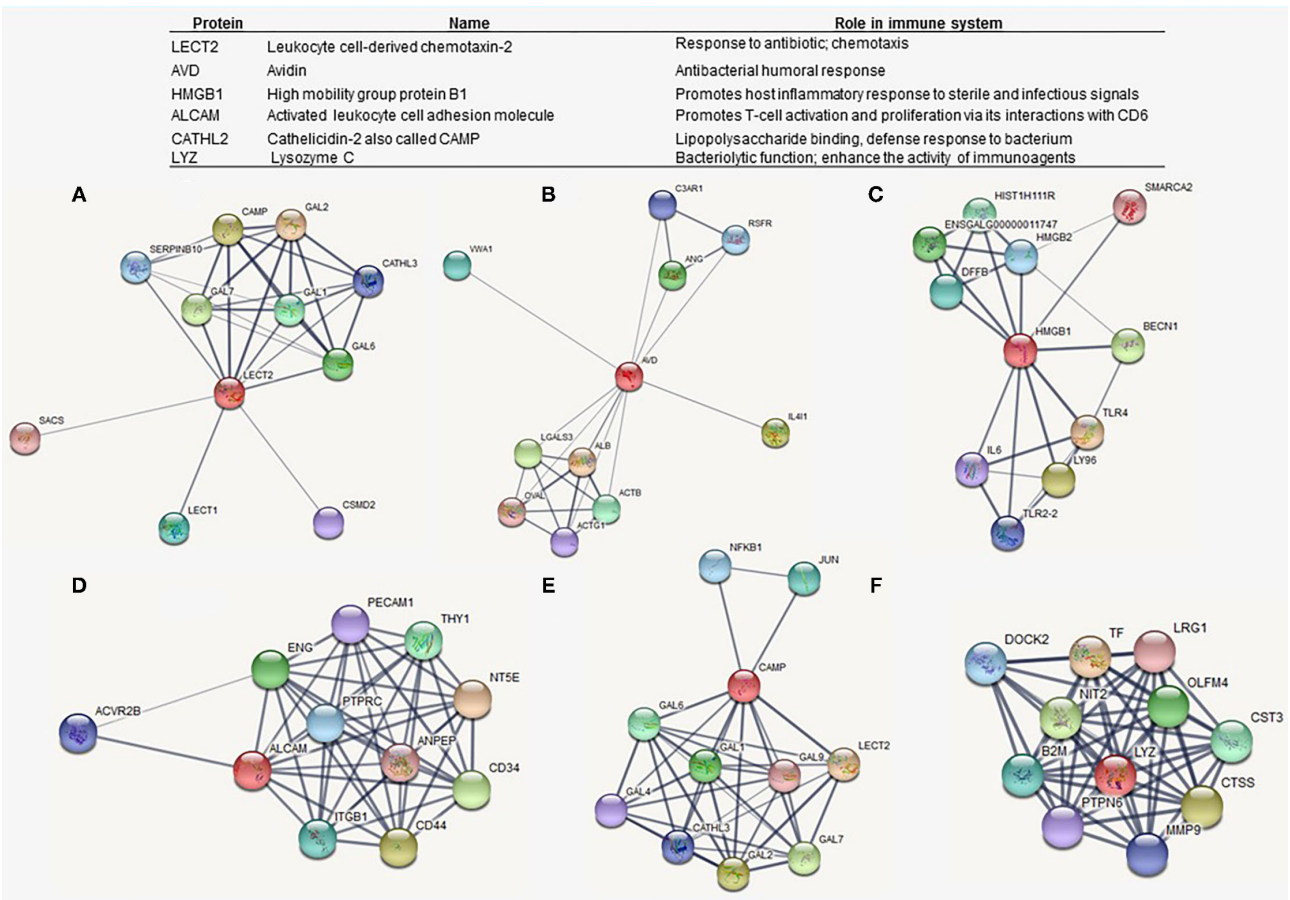

FIGURE 6 | Protein-protein interaction (PPI) network analyses of selected differentially expressed proteins associated with immunomodulatory functions. Red nodes denote the selected immune-related protein, and edges show the biological relationship between nodes. **(A)** Leukocyte cell-derived chemotaxin-2 (LECT2). **(B)** Avidin (AVD). **(C)** High mobility group protein B1 (HMGB1). **(D)** Activated leukocyte cell adhesion molecule (ALCAM). **(E)** Cathelicidin-2 also (CAMP). **(F)** Lysozyme C (LYZ).

TABLE 1 | The five most significant pathways based on the upregulated proteins expressed in intestinal samples of broilers treated with lactic acid bacteria (L), *Citrobacter freundii* (CF) or *Citrobacter* spp. (C2) in relation to control treatment at the day of the hatch (DOH) and 10 days of age (10d).

Pathway name	DOH			10d		
	Ratio	*P*	FDR	Ratio	*P*	FDR
L treatment						
Amyloid fiber formation	0.006	4.54e−06	0.003	–	–	–
Glutathione conjugation	0.005	1.93e−05	0.007	–	–	–
Cellular responses to stress	0.036	3.27e−05	0.008	–	–	–
HSF1 activation	0.003	5.94e−05	0.011	–	–	–
Cellular responses to external stimuli	0.044	1.75e−04	0.025	–	–	–
Muscle contraction	–	–	–	0.018	1.60e−04	0.022
Smooth muscle contraction	–	–	–	0.04	1.99e−04	0.022
Kinesins	–	–	–	0.05	3.69e−04	0.027
Axon guidance	–	–	–	0.041	0.001	0.056
OPI-dependent Golgi-to-ER retrograde traffic	–	–	–	0.008	0.001	0.06
CF treatment						
Syndecan interactions	0.002	1.08e−05	0.006	–	–	–
Integrin cell surface interactions	0.006	5.05e−05	0.013	–	–	–
Assembly and cell surface presentation of NMDA receptors	0.003	7.59e−05	0.013	–	–	–
Cellular responses to external stimuli	0.044	1.15e−04	0.014	–	–	–
Extracellular matrix organization	0.023	1.32e−04	0.016	–	–	–
P75NTR negatively regulates the cycle via SC1	–	–	–	4.24e−04	5.80e−05	0.009
Phase II conjugation of compounds	–	–	–	0.018	9.61e−05	0.009
Glutathione conjugation	–	–	–	0.005	2.54e−04	0.016
Acetylation	–	–	–	0.001	4.08e−04	0.019
FOXO-mediated transcription	–	–	–	0.008	0.001	0.038
C2 treatment						
Toll-like receptors (TLRs) by endogenous ligand	0.002	1.54e−5	4.17e−3	–	–	–
Signaling by RAF1 mutants	0.003	5.09e−5	4.17e−3	–	–	–
Paradoxical activation of RAF signaling by Kinase inactive BRAF	0.004	7.39e−5	4.17e−3	–	–	–
Signaling by moderate kinase activity BRAF mutants	0.004	7.39e−5	4.17e−3	–	–	–
Signaling downstream of RAS mutants	0.004	7.39e−5	4.17e−3	–	–	–
Cellular response to starvation	–	–	–	0.012	1.58e−6	2.77e−4
Response of EIF2AK4 (GCN2) to amino acid deficiency	–	–	–	0.008	0.000002	2.77e−4
Eukaryotic translation elongation	–	–	–	0.007	2.05e−5	1.61e−3
GRB2:SOS provides linkage to MAPK signaling for Integrins	–	–	–	0.001	4.05e−5	1.61e−3
L13a-mediated translational silencing of Ceruloplasmin expression	–	–	–	0.008	4.43e−5	1.61e−3

FDR, false discovery rate.

accompanied by inflammation in chicks (Wilson et al., 2020). The failure of immune regulation suggested by these results might be connected with the onset of intestinal chronic inflammation signaling in CF treated broilers at 10 days of age (Rodrigues et al., 2020a,b).

Interestingly, we found that C2 overrepresented the regulation of TLRs by endogenous ligand and TLRs receptors cascade pathways at DOH. As feedback mechanisms during either infection or injury to the organism, pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) indicate a danger alert that activates TLRs (Piccinini and Midwood, 2010). Additionally, there was evidence of other innate mechanism activation by the enrichment of the heterophil degranulation pathway in C2 on DOH (**Figure 5**). Contrarily, the inoculation of C2 *in ovo* has been shown to downregulate the pathway FCGR-dependent phagocytosis at the same age. Phagocytosis is a significant event involving the recognition of invading foreign particles by specific types of phagocytic receptors and the subsequent internalization of the particles (Alberts et al., 2002; Henneke and Golenbock, 2004; Feng et al., 2014). These early events that are mediated by the innate immune system are critical to eliminating the invading infectious agents (Henneke and Golenbock, 2004). The clustering of FCGRs by IgG antibodies on the phagocyte initiates a variety of signals, which lead, through the reorganization of the actin cytoskeleton and membrane remodeling, to the formation of pseudopods and phagosomes (Joshi et al., 2006). As a result of this process,

pathogen-derived molecules can be presented on the surface of the host cell, allowing the induction of pathogen-specific adaptive immunity (Alberts et al., 2002; Kumar et al., 2013). Collectively, the underexpression of DEPs on GO terms related to immune defense and the downregulation of pathways related to phagocytosis suggested that C2-treated chicks may have been able to activate the recognition of potentially harmful microorganisms, despite that their defense mechanisms may have failed in executing bacterial clearance. Although we found links between proteomic signatures and the immune system, this study did not assess the link with the host phenotypes. Future research is warranted to evaluate whether immune response-associated proteomic signature can affect body weight and gut health parameters.

We took our results one step further to identify DEPs directly relevant to biological functions associated with early-age immune response in poultry. The mass spec-based proteomics outcomes have been extensively used to discover potential biomarkers to predict or confirm health disorders in human medicine (Geyer et al., 2017). Despite the lack of information, the expansion of modern technologies is beginning to be approached for this purpose in poultry production (Arsenault et al., 2014; Kuttappan et al., 2017). Here, in this study, we suggest a panel of proteins for evaluating mucosal immune response in broilers (**Figure 6**).

Among the highlighted proteins, LECT2 plays an important role in the immune processes by increasing cytokine expression, inducing chemotaxis, and activating macrophages (Liu et al., 2014; Slowik and Apte, 2017; Jung et al., 2018). Previous work revealed a decreased expression of LECT2 on the proteome of heterophils from the spleen of the chicken in response to *Salmonella* Enteritidis infection. Birds vaccinated against *Salmonella* sp. succeeding a *Salmonella* challenge upregulated granular proteins as CATHL2, also called CAMP, and LECT2, suggesting that these peptides might be considered a positive marker of enhanced immune response to vaccination (Sekelova et al., 2017). **Figure 6** demonstrates a co-expression, with a high score (0.976; see **Supplementary Data**), between LECT2 and CATHL2. Also, LECT2 is strongly related to the GAL1 and other β-defensins, which are a family of peptides with antimicrobial activity and immunomodulatory functions in chickens (Kalenik et al., 2018). In fact, CATHL2 was identified as upregulated in this study along with all treatments, while LECT2 was differentially expressed by L and C2 treatments.

Otherwise, ALCAM and HMGB1 were only upregulated by L treatment, which might have contributed to explaining the activation of immunostimulatory complexes by DOH and 10 days of age, respectively. HMGB1 is actively secreted by innate immune cells in response to PAMPs, where it mediates the activation of innate immune responses, including cytokine release (Yanai et al., 2011; Yang et al., 2013). HMGB1 can also act as a chemotactic mediator by transmitting signals to the cell interior *via* the activation of receptors that include TLR4 (Yang et al., 2013). In relevance of HMGB1 role in cellular and humoral immunity, this protein has been reported as a potential immunological adjuvant in poultry vaccines (Sawant et al., 2015; Yang et al., 2017; Vuong et al., 2020).

Avidin and LYZ are other proteins recently associated with innate antimicrobial activity by preventing direct access of bacteria to the intestinal epithelial surface in newly hatched chicks (Shira and Friedman, 2018). LYZ was found upregulated in L treatment at DOH. Given the potent function of LYZ in limiting the bacterial growth at mucosal surfaces, we speculate that early exposure to L might be a strategy to enhance the innate antimicrobial activity. Finally, further studies are recommended to validate this panel of proteins as potential biological markers of the enhanced immune response in broilers.

Our study indicated that there were predicted biological functions of L-treated chicks associated with the activation and balanced function of the innate and adaptive immune systems. At the same time, the *Enterobacteriaceae* exposed birds presented dysregulated immunological mechanisms or downregulated processes related to immune development. It is expected that the activated response at an early age may manifest the ability of the immune system to recognize, uptake, and destroy foreign microorganisms. Those findings highlighted that a proper immune function was dependent on specific GIT microbiota exposure, in which the prenatal probiotic application may have favored the fitting programming of immune functions in chicks.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data has been deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD015504.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

KW and DR carried out the project. DR performed the analyses, interpreted the results, and wrote the manuscript in consultation with LB. All authors contributed to the experimental design, discussed the results, and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.758183/full#supplementary-material

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The Microbiota-Gut-Brain Axis During Heat Stress in Chickens: A Review

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Heat stress is a global issue for the poultry industries with substantial annual economic losses and threats to bird health and welfare. When chickens are exposed to high ambient temperatures, like other species they undergo multiple physiological alterations, including behavioral changes, such as cessation of feeding, initiation of a stress signaling cascade, and intestinal immune, and inflammatory responses. The brain and gut are connected and participate in bidirectional communication via the nervous and humoral systems, this network collectively known as the gut-brain axis. Moreover, heat stress not only induces hyperthermia and oxidative stress at the gut epithelium, leading to impaired permeability and then susceptibility to infection and inflammation, but also alters the composition and abundance of the microbiome. The gut microflora, primarily via bacterially derived metabolites and hormones and neurotransmitters, also communicate via similar pathways to regulate host metabolic homeostasis, health, and behavior. Thus, it stands to reason that reshaping the composition of the gut microbiota will impact intestinal health and modulate host brain circuits via multiple reinforcing and complementary mechanisms. In this review, we describe the structure and function of the microbiota-gut-brain axis, with an emphasis on physiological changes that occur in heat-stressed poultry.

Keywords: heat stress, microbiota-gut-brain axis, anorexia, immune response, poultry

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INTRODUCTION

The microbiota-gut-brain axis (MGBA) has been widely investigated in human and mammalian species for decades due to its vital role in not only homeostatic maintenance but also the pathology of various neurodevelopmental and neurodegenerative disorders (Cryan et al., 2019). In addition, the importance of this axis in non-mammalian species, such as chickens, has been acknowledged and the potential mechanisms are being investigated. The relationship between the gut microbiome and host is considered to be mutualistic rather than commensal (De Palma et al., 2014). The host provides the microbiome with hospitable niches and undigested food, and in turn, these microorganisms metabolize and produce neuroactive components. These neuroactive molecules, such as serotonin (5-HT), exert a systematic or local effect in regulating host physiological processes, by entering the circulation or interacting with enteric nervous and immune systems, respectively. Factors that act on the central nervous system

(CNS), for instance via vagal afferents, influence host behaviors, whereas others trigger structural and functional changes in the intestine (Villageliũ and Lyte, 2017). The interactive effects between the gastrointestinal microbiota and the host could be benign or detrimental, depending on the type and magnitudes of factors, including but not limited to dietary composition, environmental stimuli, and host genetics and phenotypes (Kers et al., 2018).

Stress, by a simple definition, is the adaptive physiological and psychological response of an organism to restore homeostasis (Glaser and Kiecolt-Glaser, 2005). The intestinal tract is reported to be involved in responses to all kinds of stressors, including heat stress (Rostagno, 2020). Heat stress is a major environmental challenge and occurs when there is an imbalance in the net amount of heat energy produced by and released from an organism (Renaudeau et al., 2012), during which the organism accumulates more heat than it can utilize and release. Heat stress is closely related to changes in the intestine, both structurally and functionally, and in the composition of the gut microbiota (Song et al., 2014; Sohail et al., 2015). Studies on the effects of heat stress on the gut microbiota in humans are lacking (Karl et al., 2018), and a variety of animal models is utilized to thoroughly investigate these effects to provide clinical insights. In this review, we focus on avian models, because heat stress has been widely reported to influence poultry meat and egg production, as well as flock health and wellbeing, through major changes in intestinal physiology and the gut microbiota (Lara and Rostagno, 2013; Rostagno, 2020). However, how heat stress interacts with the chicken's gut microbiota and affects the MGBA is not fully understood and requires further elucidation.

Herein, we review literature related to heat stress-induced alteration in chicken behaviors (such as feeding and social behaviors), physiological processes, intestinal integrity and the microbiota, and the immune system, with an emphasis on the relationships of these alterations to gut microbiota composition. We also review what is known regarding the use of probiotics and prebiotics as preventative and therapeutic interventions in heat-stressed animals, and discuss strategies to ameliorate the detrimental effects of high temperatures on bird behavior and health.

MICROBIOTA-GUT-BRAIN AXIS

Microbiome Composition

Gut microbes consist of different microorganisms, such as bacteria, viruses, yeast, and other fungi, and protozoa. Most research on gut microbiota has focused on evaluating bacteria composition and function (Karl et al., 2018); hence, bacteria being the target of this review. The amount of microbiota varies dramatically between intestinal sites, from about 10^5 colony-forming units (CFU) per gram of digesta in the small intestine to around 10^{11} CFU per gram of digesta in the cecum (Xing et al., 2019; Rychlik, 2020). During the past decade, technological advances in profiling microbiomes within the host, from improvements in laboratory culture techniques to 16S rRNA gene sequencing and metagenomics sequencing, have enabled the study of the composition of the microbiome with greater resolution and depth. However, it is important to note that knowing the microbiome composition does not necessarily facilitate an understanding of their function and physiological consequences. In chickens, Lactobacilli dominate several parts of the upper digestive tract, including the crop, proventriculus, and ventriculus (gizzard), whereas the small intestine is mainly inhabited by *Lactobacillus*, *Enterococcus*, and *Clostridiaceae*. This prevalence of specific species is, to some extent, related to the function of the digestive organs, since the pH of gastric juices is relatively low, which favors domination by Lactobacilli. In the cecal tonsils, where digesta resides the longest time during digestion, and the concentration of short-chain fatty acids (SCFAs) synthesized by bacteria is greater than elsewhere in the gastrointestinal tract (GIT), the most abundant phyla are *Firmicutes*, *Bacteroides*, and *Proteobacteria* (Oakley et al., 2014; Villageliũ and Lyte, 2017; Karl et al., 2018; Rychlik, 2020).

Functions of Microbial Products

Microbial products can serve as an energy source to fuel the host and are capable of interacting with immune or neuroendocrine systems to influence host health and behaviors (Shenderov, 2016). SCFAs, once being taken up, can be used as a metabolic substrate (ATP production) by intestinal cells, particularly intestinal epithelial cells (enterocytes) (Bergman, 1990). Butyrate and propionate, two major SCFAs, interact with specific G-protein-coupled receptors to regulate and maintain energy and immune homeostasis in cells and thus influence their activity, by activating pathways, such as chemotaxis, apoptosis, proliferation, and differentiation, through gene expression programming (Clarke et al., 2014; El Aidy et al., 2016). Acetate and butyrate are reported to participate in the maintenance of GIT barrier intactness, through which bacterial colonization and translocation are prevented (Fukuda et al., 2011; Fachi et al., 2019). Additionally, SCFAs act as signaling molecules and are closely related to the synthesis of a variety of neuroactive molecules, such as leptin, glucagon-like peptide 1, and other hormones, which can be transported through the circulation to several brain regions. Neurons in the arcuate nucleus of hypothalamus, for instance, receive signals through receptors of these neuropeptides and neurotransmitters that are integrated to then regulate the host's appetite (Tolhurst et al., 2012; Clarke et al., 2014; El Aidy et al., 2016).

Bacteria synthesize classic neurotransmitters, such as 5-HT, which can act locally or distantly through the circulation or nervous system, and as such have been referred to in the literature as "mind-altering" (Cryan and Dinan, 2012). In the intestine, host enterochromaffin cells, a type of entero-endocrine cell, produce 5-HT. While most dietary-derived tryptophan is metabolized in the liver via the kynurenine shunt, some is converted into 5-HT. In fact, the majority (> 95%) of 5-HT in the body is synthesized in the gut, occurring via sequential conversion of tryptophan via two enzymatic reactions. Intestinally derived 5-HT, whether of host or bacterial origin, can then

act via the endocrine system or through the vagus nerve. Within the small intestine, most 5-HT is released into the mucosa, and it is estimated that roughly 2% of all enteric neurons are serotonergic (Mawe and Hoffman, 2013). Via a variety of receptors, including the ionotropic 5-HT3 and metabotropic 5-HT1, 2, 4, and 7, 5-HT influences gut motility (peristalsis), secretion of chemicals, such as bicarbonate, during digestion, vasodilation, and neuronal survival and inflammation (Mawe and Hoffman, 2013).

Relationship Between Microbiota and the Host Gut

Under normal and healthy conditions, microbial communities in the host GIT play an overall beneficial role. They assist in competing against pathogenic microbial taxa and maintaining intact intestinal lumen surface structures, ferment undigested polysaccharides into SCFAs, and provide additional vitamins (Oakley et al., 2014). Indeed, coprophagic species, such as rabbits and rodents, recover such vitamins by consuming fecal pellets. There are detrimental effects of the gut microbiota undergoing dysbiosis, which can be initiated by but also exacerbated in response to gastrointestinal environment perturbations (temperature, pH, nutrient composition, toxins, introduction of microflora, etc.), resulting in several acute or chronic diseases in the host (Karl et al., 2018). There is a clear relationship, for example, between intestinal diseases, such as Crohn's disease and inflammatory bowel syndrome, and unbalanced SCFA production and 5-HT availability in the gut (Oligschlaeger et al., 2019). Thus, maintaining a healthy gut microbial community and hospitable mucosal environment is of utmost importance to host health and wellbeing. Generally, a microbiota that is diverse in both composition and genetic content or is dominated by beneficial taxa is characterized as being a healthy community (Karl et al., 2018).

Physiological Connections Between Gut Microbiota and the Host Brain

The gut microbiota and brain have bidirectional connections. On the one hand, gut microbiota themselves are an important source of peripheral neurotransmitters and hormones. These molecules not only modulate gut functions like peristalsis, as described above, but also directly communicate the intestinal state through vagal afferents to the brainstem and higher brain regions. Various stressful stimuli through peripheral and central pathways lead to the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which might further alter gut microbiota composition and activity as well as intestinal epithelial cells' function. Release of corticotropin-releasing factor (CRF) from the hypothalamus stimulates adrenocorticotropic hormone (ACTH) release from the anterior pituitary into the circulation, which then triggers the release of corticosteroids from the adrenal glands, including cortisol by humans and corticosterone by birds from the adrenal cortex into the circulation. Corticosteroids exert a multitude of effects on the GIT via direct interactions with enteric muscle cells and neurons, bacteria, and intestinal immune cells, leading to the release of cytokines, which via the circulation can act on the brain to affect mood, appetite, cognition, and emotion (Cryan and Dinan, 2012). Several environmental factors, such as dietary composition and drugs, can influence activity of the MGBA through one or more pathways that feed into these mutually reinforcing connections. For instance, appetite regulation is mediated mainly in several brain regions like the hypothalamus and brainstem. Nutrients in the gut stimulate the release of satiety factors, such as cholecystokinin, and also directly affect the microbiota, which in turn regulate the concentration of cytokines and neuroactive molecules that modulate brain function (Petra et al., 2015).

HEAT STRESS

The adaptive physiological and behavioral responses of an organism to environmental demands or pressures have been described as stress responses, by which the organism attempts to maintain or restore homeostasis (De Palma et al., 2014; Karl et al., 2018). Stressors or stressful stimuli can vary from acute to chronic and from one time to several repetitive occurrences, and their magnitude can be mild or severe. Additionally, the different capabilities of individuals to perceive stress result in various outcomes (Lucassen et al., 2014). Individuals exposed repeatedly to stressful situations appear to be more vulnerable to gastrointestinal diseases.

There exist various factors that cause changes in the intestinal microbiota of chickens. One major source of these factors is characteristics of the host itself, such as age, type and breed, sex, and sampling site in the GIT. Environmental factors also influence the microbiota composition, including biosecurity level, housing condition, litter, feed, temperature, and location (Kers et al., 2018). Among those environmental factors, a growing amount of evidence indicates that heat stress has significant effects on the intestinal microbiota composition and tissue structure (see these effects via altered concentrations of neural/humoral factors in **Table 1**).

When birds are exposed to stressful factors (such as long-term exposure to sunlight, high ambient temperature and humidity, and poor ventilation), their internal energy homeostasis is disrupted and physiological alterations ensue. The transient or long-term imbalance between heat dissipation to the environment and heat production inside the animal can disturb thermostasis and eventually result in heat stress. The thermoneutral zone is the ambient temperature range where the animal efficiently regulates and maintains a constant body temperature (Pollock et al., 2021). When environmental temperature exceeds the upper critical temperature, which is the upper limit of the thermoneutral zone, animals are considered to be exposed to heat stress (McNab, 2002). In general, thermoneutral zones for broiler chickens are 28 ~ 34, 25 ~ 31, 22 ~ 28, 20 ~ 25, 18 ~ 24, and 18 ~ 24°C for each of the first six weeks of age, respectively (Cassuce et al., 2013).

Core body temperature, when elevated by exposure to high ambient temperature, surprisingly did not dramatically alter the microbiota in the cecal tonsils (Xing et al., 2019). However,

TABLE 1 | The effects of heat stress-induced neuronal/humoral factors on intestinal physiology and gut microbiota.

Factors	Effects	Species	References
HSF, HSP, and TLR	Induced oxidative stress and intestinal barrier breakdown, initiated inflammatory signaling	Chicken	Varasteh et al., 2015
Proinflammatory cytokines (e.g., IL-1β, IL-2, IL-6, and TNF-α)	Damaged tight junction and gut epithelial integrity, activated HPA axis, enhanced successful transmission of pathogens	Chicken	Al-Sadi et al., 2010; Deng et al., 2012; Alhenaky et al., 2017; Jiang et al., 2021
Corticosterone	Altered HPA axis, increased macrophage oxidative burst and decreased numbers of macrophage undergoing phagocytosis, depressed immune response, induced intestinal lesions, altered gut microbial communities	Chicken	Deng et al., 2012; Quinteiro-Filho et al., 2012b; Beckford et al., 2020; Zaytsoff et al., 2020
Monoamines (e.g., 5-HT, NE, E, and DA)	Increased corticosterone and inflammatory cytokines	Chicken, rat	Johnson et al., 2005; Bahry et al., 2017
Appetite-related neuropeptides (e.g., CCK, ghrelin, CRF, and NPY)	Reduced food intake, activated HPA axis and stress response, impaired intestinal structure	Chicken	Lei et al., 2013; Bohler et al., 2020; Wang et al., 2021
Reactive oxygen and/or nitrogen species	Abnormal heat tolerance, injured intestinal barrier, invading pathogens, translocated endotoxins, increased inflammatory cytokines	Rat	Hall et al., 2001

5-HT, 5-hydroxytryptamine; CCK, cholecystokinin; CRF, corticotropin-releasing factor; DA, dopamine; E, epinephrine; HPA axis, hypothalamic-pituitary-adrenal axis; HSF, heat shock factor; HSP, heat shock protein; IL, interleukin; NE, norepinephrine; NPY, neuropeptide Y; TLR, toll-like receptor; and TNF-α, tumor necrosis factor-α.

another study (Alhenaky et al., 2017) found that compared with the thermoneutral condition, both acute and chronic heat stress led to a higher rectal temperature, with the magnitude even higher in the former situation. Rectal temperature peaked during the first two days of heat exposure, then fluctuated until it reached a plateau. After that, individuals under heat stress showed thermo homeostasis during the rest of the observation period. The prevalence of intestinal pathogens (*Salmonella* spp.) was increased in heat-exposed chicks in comparison with the control birds (Alhenaky et al., 2017). These results suggest that core body temperature, despite being temporarily affected by heat exposure, could be adjusted promptly and exert a limited direct effect on the gut microbiota. However, the capacity for adaptation might be compromised if the chickens are exposed to consistent high ambient temperatures after the first few days, which can lead to a severe susceptibility to heat stress.

Thus, the influence of heat stress on the composition of the gut microbiota can occur as a direct consequence of altering body temperature, or indirectly due to an acute or gradual change in the birds' behavior, physiological status, intestinal integrity, and immune system activity (See **Figure 1**), which will be discussed in detail in the following sections.

HEAT STRESS INDUCES APPETITE SUPPRESSION

Our group demonstrated that exposure to high ambient temperatures suppressed food consumption in young broiler chickens, which was associated with changes in the activity of several appetite-regulating peptides, such as orexigenic neuropeptide Y and anorexigenic CRF, which both have peripheral effects associated with the enteric system and HPA axis (Bohler et al., 2020). In another heat stress study, heat-exposed birds ate less, ingested more water, panted more often, and lifted the wings much longer (Mack et al., 2013). Typically, the reduction in food intake is sustained during heat stress and is thought to be a compensatory mechanism to reduce heat production associated with nutrient metabolism, although heat stress is associated with changes in nutrient absorption, particularly amino acids and glucose. For this reason, a multitude of heat stress studies with chickens and other species have employed dietary strategies to mitigate nutrient-induced heat production, including formulating diets to vary in macronutrient composition (Chowdhury et al., 2021). The GIT of chickens consumes about 7% of the energy derived from the diet, so reduced feeding, while offsetting some of the heat production in the animal, could also elicit a fast and dramatic response in the GIT, primarily jeopardizing gut integrity and mucosal immunity, which further compromises nutrient assimilation, triggers systemic inflammation, and impairs production (Thompson and Applegate, 2006; Deng et al., 2012).

In some commercial practices, the distance of the grow-out facility from the brooder house necessitates transporting chicks over long distances after hatching and processing. Although the residual yolk sac provides a reservoir of nutrients that are resorbed into the intestine and used by the chick after hatching, delayed access to food after hatch can impair intestinal development (Lamot et al., 2014; Proszkowiec-Weglarz et al., 2019, 2020; Qu et al., 2021), and also establishment of the microbiota (Flint et al., 2012). Similar influences were observed in response to food withdrawal at a later age (Burkholder et al., 2008). Having no access to food, even for a period of 6 h, allows pathogens, like *Salmonella* (Burkholder et al., 2008), to colonize within the gut and reshape the microbial community (Thompson et al., 2008). Sequencing techniques used to evaluate the taxonomy of the gut microbiota revealed that chickens subjected to food withdrawal had altered populations in the ileum and cecum, with increased *Firmicutes* and diminished *Proteobacteria*. Overall, the major effects of food deficiency on the intestinal microbiome are on the dominant families, such as *Turicibacteraceae*, *Ruminococcaceae*, and *Enterobacteriaceae* (Metzler-Zebeli et al., 2019). In broiler breeders, it is common practice to restrict the amount of food consumed throughout life, in order to meet target body weights to prevent

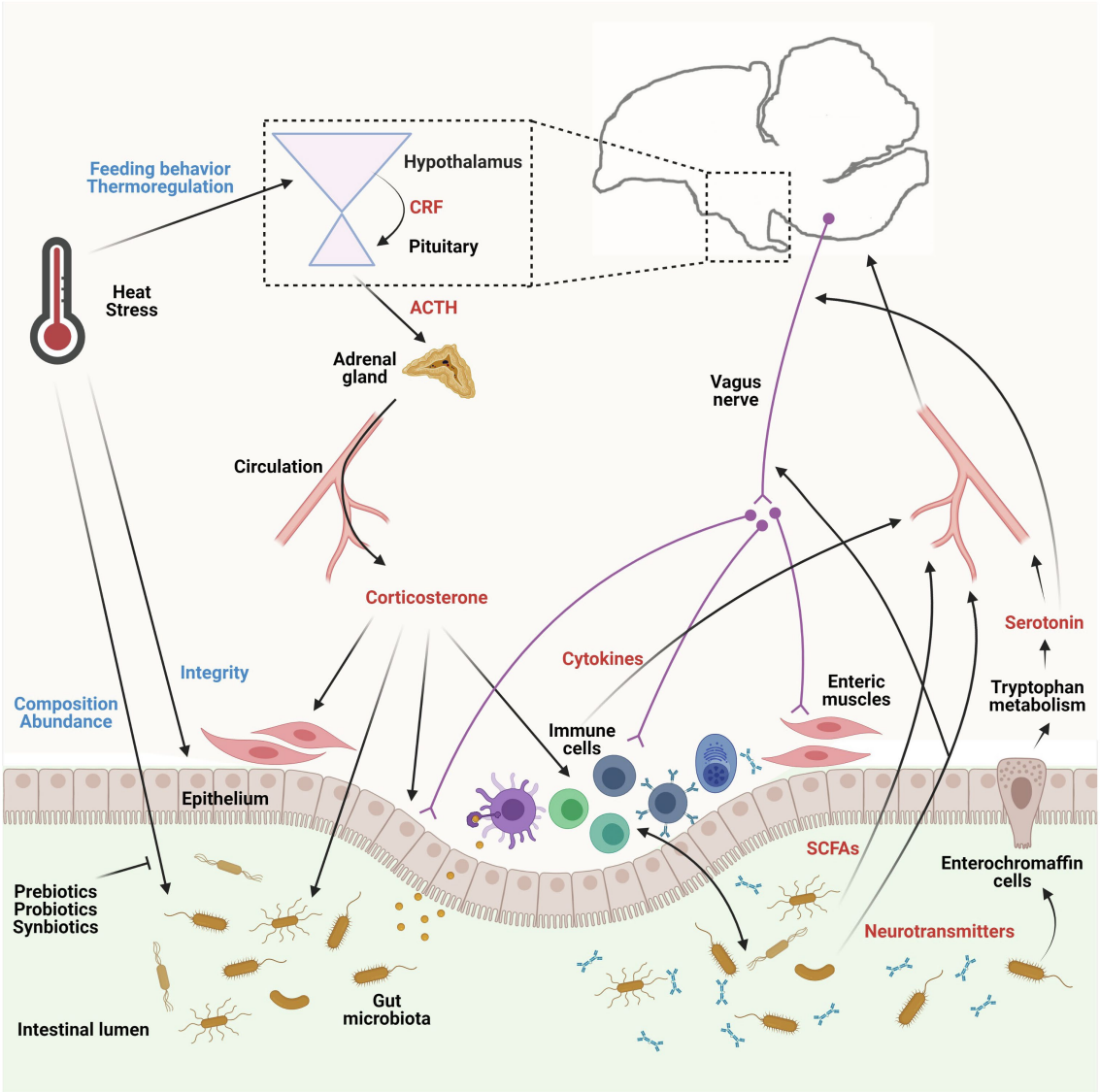

FIGURE 1 | Influences of heat stress on the microbiota-gut-brain axis (MGBA) and the pathways involved in the axis in chicken. The gut microbiota communicates with the gut-brain axis through several pathways, including neural, immune, and endocrine signaling. The gut microbiome produces neurotransmitters, such as serotonin, which can trigger responses of the vagus and enteric nervous system, and short-chain fatty acids (SCFAs), which can nourish the host and regulate the host's brain activity and behaviors. Gut microbiota also stimulates intestinal immune cells to generate and secrete cytokines to induce immune response locally and systemically and modulate the brain. In turn, the brain utilizes the same pathways to alter the gut microbiota, especially their composition and abundance, under circumstances, such as heat stress. Heat stress can influence the MGBA both directly and indirectly. The hypothalamic-pituitary-adrenal axis is activated by heat stress and facilitates the production of corticosterone in birds, which further affects enteric cells and gut microbiota. The utilizations of prebiotics, probiotics, or symbiotics are promising approaches to alleviate the adverse effects of heat stress. ACTH, adrenocorticotropic hormone; CRF, corticotropin-releasing factor. This figure is adapted from previous publications (Cryan and Dinan, 2012; Aoki et al., 2017) and created with BioRender.com.

metabolic disorders and support optimal reproduction. However, as described above, such practices could negatively impact the gut microbiome which in turn could impact health of the chicken. Combined with exposure to high temperatures, restricted access to feed could have major impacts on the bacterial composition of the GIT and thereby affect the bird's whole-body physiology.

Heat stress also negatively affects layer-type chicks, by reducing their food consumption, egg production, and quality, and increasing death rate (Mack et al., 2013; Mignon-Grasteau et al., 2015; Sahin et al., 2018). In laying hens, integrity of the gut mucosa was impaired under heat exposure, resulting in limited nutrient transport across the intestinal mucosal layer (Zhang et al., 2017). Moreover, the intestinal microbiome community was modified in heat-stressed pullets and hens (Burkholder et al., 2008; Song et al., 2014; Zhu et al., 2019). Xing et al. (2019) found that layer chicks displayed an altered microbiome composition rather than species abundance, in response to exposure to a high ambient temperature (29–35°C), and this change was closely associated with less food consumption. Another study involved exposing the layers to a cyclic temperature

of 35°C 7 h per day and found an increased alpha diversity, that is, the present species of the microbiome were elevated in the cecum after 2 weeks of exposure, although they returned back to normal levels after 4 weeks (Hsieh et al., 2017). Additionally, the two most abundant cecal phyla, *Bacteroidetes* and *Firmicutes*, showed different richness by the end of the experiment. This study suggested that heat stress started to reshape the microbiota in layers at 2 weeks but the bacteria adapted to the temperature change later at 4 weeks (Hsieh et al., 2017). Shi et al. (2019) found slightly different results and observed significant changes in the abundance of those two phyla starting at 1 week, although also a loss of significance by 4 weeks. All of these findings suggest that the influence of heat stress on the gut microbiota depends on the magnitude and duration of heat exposure.

In layer chicks under heat exposure, there were elevated numbers of several detrimental genera, including *Escherichia*, *Shigella*, and *Clostridium*, which generate alpha-toxins and contribute to the occurrence of necrotizing enterocolitis. On the other hand, advantageous bacteria, such as *Lactobacillus* and *Ruminococcaceae*, were scarce (Heida et al., 2016). Bacteria in the genus *Lactobacillus* are widely used as probiotics, as their metabolites are capable of regulating the acid-base equilibrium in the intestine, which favors the development of a beneficial but not pathogenic microbiome (Menconi et al., 2011). Some species in the *Lachnospiraceae* group are also inhibited during heat stress (Biddle et al., 2013; Meehan and Beiko, 2014). These species produce a relatively large proportion of butyrate, which helps maintain intestinal health by facilitating epithelial development. Generally, butyrate is less abundant than other SCFA (60% acetate, 25% propionate, and 15% butyrate; at least in humans), although it serves as the major energy source for colonocytes in the large intestine and is known to affect gene expression by acting as a histone deacetylase (HDAC) inhibitor and affects signaling via activation of several G-protein-coupled receptors (Liu et al., 2018). Many studies have demonstrated a beneficial role for butyrate in maintaining intestinal barrier integrity, and in preventing inflammation (Liu et al., 2018). Thus, changes in the numbers of butyrate-producing bacteria could modulate the MGBA via effects of butyrate signaling on the peripheral and CNS (Liu et al., 2018).

HEAT STRESS REDUCES INTESTINAL INTEGRITY

The gut microbiome environment is normally stable under healthy conditions. The intestine provides niches for bacteria to colonize and thrive, and in turn, commensal bacteria compete with pathogenic bacteria for space and nutrients to survive and produce metabolites that boost host intestinal immunity and suppress the growth of pathogens, which collectively protect the gut epithelium. However, stressful stimuli can concurrently impair intestinal barrier integrity and alter the microbiome (Tannock and Savage, 1974; Söderholm et al., 2002; MacDonald, 2005). Once the mucosal layer is penetrated, intestinal pathogens have access to the host circulation and cause diseases and impair the efficiency of nutrient digestion and assimilation (Sansonetti, 2004; Keita and Söderholm, 2010).

There is evidence that the intestinal mucosa, which is susceptible to heat stress and microbiome change-induced damage and inflammation, can also adapt to maximize nutrient assimilation in some circumstances. Heat-treated chicks had decreased plasma thyroid hormone and increased plasma corticosterone, as well as a damaged mucosal layer in the jejunum, but the ability to transport glucose across the jejunal epithelium was enhanced, which may have compensated for the lack of energy due to reduced food consumption (Garriga et al., 2006). In another study, however, when chicks experienced a higher temperature (35°C, 5 degrees higher), their intestinal structures were significantly damaged, with reduced villus heights and functional absorptive surface areas, and elevated levels of blood endotoxins. These adverse impacts were not overcome by host adaptations alone but required exogenous butyrate supply for alleviation of symptoms, further demonstrating a beneficial role for butyrate in maintaining intestinal structure and function (Abdelqader et al., 2017).

The ileum is a unique intestinal niche because of its proximity to the cecum and receipt of end-products of digestion that are not absorbed in the proximal small intestine. It is home to a larger amount of bacteria, even the pathogenic *Salmonella*, than the proximal small intestine and provides a rich source of nutrient substrate for fermentative activity (Fanelli et al., 1971). The intestine stands as the first line of defense against invading pathogens (Fagarasan, 2006). If, for some reason, the chicken small intestinal epithelium is damaged, *Salmonella* adhere at impaired locations and translocate into the host, causing a systemic infection (Burkholder et al., 2008). This was observed in chickens that underwent 24 h of food deficiency or heat stress (McHan et al., 1988; Alhenaky et al., 2017). Treating chicks with high temperature chronically or acutely result in invading *Salmonella*, which are later detected in the liver, spleen, and muscles. The liver and spleen typically handle these exogenous pathogens, which are engulfed by macrophages and transported through the circulation. However, the organs that are primarily targeted by *Salmonella* during a systemic infection have yet to be identified (Chappell et al., 2009).

Two mechanisms were proposed that mediate the effect of heat stress on the intestinal epithelium. The first is that reactive oxygen and/or nitrogen species are produced in response to high environmental temperature and increased oxidative activity, overwhelming the capacity of endogenous antioxidant systems (Hall et al., 2001). When chicks are heat-exposed, the production of these free radical molecules provokes injury to the epithelial cell membranes, resulting in fewer tight junctions (TJ) and less expression of TJ genes. Thus, the intestinal barrier becomes permeable to paracellular entry by bacterial endotoxins. The second mechanism is that heat stress promotes the production of proinflammatory cytokines, which also damage the TJ (Al-Sadi et al., 2010). Among those cytokines, interleukin-2 (IL-2) and tumor necrosis factor-α (TNF-α) are among those whose concentrations in circulation are elevated in heat-stressed chicks. IL-2 is produced by T cells, and once released, it activates other types of cells like macrophages, which secrete

proinflammatory cytokines, such as TNF-α, to initiate inflammation (Hoyer et al., 2008). However, the secretion of IL-2 could also be stimulated by endotoxins (Costalonga and Zell, 2007); thus, this mechanism might be a secondary or indirect effect.

HEAT STRESS ACTIVATES THE HPA AXIS

The HPA axis is an essential system that integrates and mediates an organism's response to intrinsic and/or extrinsic stressors (McEwen, 2000). Its activation is characterized by the activation of hypothalamic CRF, the release of ACTH, and the production of corticosterone in rodents and birds (Iyasere et al., 2017). Chronic and acute high ambient temperature exposure activate the HPA axis, which is usually characterized by elevated blood corticosterone in the animal. Elevations in circulating corticosterone are associated with an array of physiological responses, such as suppressed food intake and growth performance, and aberrant immune and inflammatory responses, to name a few (Quinteiro-Filho et al., 2012a; Beckford et al., 2020).

In addition to corticosterone, activation of the HPA axis is accompanied by the generation of many other hormones, neuroactive molecules, and cytokines. These factors are shared by many systems in the body (such as the CNS, endocrine, and immune systems) and directionally mediate systematic interplay through the binding of ligands to their receptors (Kaiser et al., 2009). For example, the CNS regulates immunity primarily through HPA axis activity and sympathetic outflow (Ziegler, 2002). Hormones involved in the regulation are corticosterone from the HPA axis and catecholamines from sympathetic activity. Two major catecholamines, norepinephrine (NE) and epinephrine (E), could further regulate the synthesis of inflammatory cytokines, with reduced levels of proinflammatory IL-12, TNF-α, and interferon γ, and enhanced expression of anti-inflammatory IL-10 and transforming growth factor β (Johnson et al., 2005). In turn, signals from visceral organs or tissues, particularly the GIT, can be picked up by parasympathetic inflow or sent back to the HPA axis (Calefi et al., 2016). Indeed, intestinal inflammation provides feedback to the HPA axis, which in turn regulates immune defense against pathogens (Karrow, 2006).

Although activation of the HPA axis by heat stress is linked to intestinal immunity and inflammation (Lara and Rostagno, 2013; Galley and Bailey, 2014; Scanes, 2016; Calefi et al., 2017), few have gone so far as to investigate actual changes in the gut microflora and brain activity. Generally, beneficial commensal bacteria were less competitive, whereas pathogenic species, such as *Escherichia coli* and *Salmonella*, flourished in heat-stressed animals due to impaired intestinal integrity and function, and increased permeability (Song et al., 2013). In a study with broiler chickens, heat stress and/or intestinal infection with *Clostridium* and *Eimeria* spp. (bacteria and protozoal species, respectively) led to changes in concentrations of monoamines in key brain regions, including a decrease in 5-HT, NE, and E in the hypothalamus, and dopamine in the mid-brain (Calefi et al., 2019). Authors speculated that these data demonstrated activation of the HPA axis via increased release of cytokines from intestinal immune cells, in response to the pathogen challenge. Monoamine concentrations and cytokine production in the small intestine were not investigated in that study. Future research should focus more on the connection between neurobiology and the gut microbiome in models of heat- and pathogen-induced intestinal dysfunction.

ALLEVIATING THE ADVERSE EFFECTS OF HEAT STRESS

A multitude of strategies have been employed to alleviate heat stress in chickens, from improvements in housing management to nutritional interventions, such as varying macronutrient composition and supplementing prebiotics, probiotics, and their combination known as synbiotics (Lara and Rostagno, 2013).

Probiotics, such as live yeasts and/or *Lactobacillus* and *Bifidobacterium*, are usually the dominant beneficial bacteria in the GIT. Exogenous supplementation contributes to maintenance of a healthy gut via ensuring their continued establishment and proliferation, which in turn affects the HPA axis and chicken behavior or physiology via immunomodulation, metabolic homeostasis, and neuroendocrine loops (Wang et al., 2018). The most effective probiotics are usually commensal bacteria belonging to the host (Dogi and Perdigón, 2006). *Bacillus subtilis*, for example, when supplied in the broiler diet, competed with pathogens (*i.e.*, *Eimeria* spp. and *Clostridium perfringens*) for colonizing sites and nutrients, thus protecting the gut from their colonization and invasion (Lee et al., 2015). *B. subtilis* was reported to inhibit bacterial pathogenic reproduction and promotes feed utilization by increasing microbiota diversity and promoting the proliferation of the beneficial *Lactobacillus* (Knap et al., 2011). Additionally, *B. subtilis* can stimulate the secretion of intestinal digestive enzymes to speed up nutrient metabolism when the activities of those enzymes were suppressed by chronic heat stress (Chen et al., 2009). Longer villi and larger surface areas were observed in probiotic-supplemented chickens and protected the bird against heat exposure-induced gut dysfunction (Deng et al., 2012; Song et al., 2014).

Prebiotics are generally defined as food ingredients, usually a saccharide, that are not digested (or absorbed) by the host but benefit the host by encouraging the growth of certain species of bacteria for which they serve as fermentative substrates. Common examples include fructo-oligosaccarides (FOS), mannan-oligosaccharides (MOS), and inulin. Mannan-oligosaccharide is harvested from the yeast cell wall and is one of the most common prebiotics used in the poultry industry. Synbiotics are synergistic combinations of prebiotics and probiotics (Schrezenmeir and de Vrese, 2001). Both prebiotics and probiotics exert beneficial effects on animal health when supplemented into the diet (Sohail et al., 2012; Sugiharto et al., 2017; Awad et al., 2021). However, their combination as synbiotics may lead to synergistic and additive effects. Synbiotics not only favor the colonization and thriving of commensal microorganisms, but also activate signaling

in the microbiome-gut-brain axis and microbiome-gut-immune axis to mediate systemic and local functions, which further influences host physiology and behavior (Rooks and Garrett, 2016). In one study, broilers were fed a normal diet or synbiotic-supplied diet and exposed to normal or high temperatures. Synbiotic supplementation not only attenuated heat stress-induced anorexia and body weight loss but was also associated with increased preening and decreased panting and wing lifting (Mohammed et al., 2018). Because of the diverse array of probiotic species and prebiotic saccharides and resulting combinatorial possibilities in a synbiotic, synbiotics can have differing effects on the gut microbiome depending on the composition. For instance, when MOS, but not FOS, was used in a synbiotic mixture, different commensal microorganisms were selectively promoted, and MOS was associated with a binding to and elimination of pathogenic bacteria from the GIT (Spring et al., 2000; Sohail et al., 2012).

CONCLUSION AND IMPLICATIONS

In summary, heat stress induces various physiological alterations that directly or indirectly regulate the intestinal microbiome community. These alterations induce changes in environmental and nutritional conditions in the gut, leading to a breach in the intestinal epithelium or barrier integrity, inflammatory states, and activation of the HPA axis and autonomic nervous system. Although growing evidence links heat stress to changes in the host brain (e.g., monoamine concentrations) and gut that are influenced by alterations in the intestinal microbiota, there are still many gaps in knowledge. For example, most studies focused on the association between heat stress and the chicken gut microbiota, but few confirmed the exact compositional changes of microbiota under different heat stress conditions (such as acute or chronic, one time, or repetitive) or in response to different probiotic and prebiotic interventions in different types, breeds, and ages of chickens. In addition, various metabolites (e.g., SCFAs) and neuroactive molecules (e.g., 5-HT) produced by gut microbiota under different heat stress conditions also require consideration and further exploration. Future studies should focus on utilizing more combinations of probiotics and prebiotics to improve chicken performance under heat exposure and to determine effects on microbiome composition. While it is clear that heat stress influences host and microbial physiology, it is unclear the extent to which the former is driven by the latter and vice-versa. Thus, elucidating the mechanisms that shape the physiology of the GIT and microbiome and how the host and microbial cells interact to drive physiology and behavior will facilitate holistic strategies to ameliorate the effects of heat stress in animals and humans.

AUTHOR CONTRIBUTIONS

CC, MC, and EG conceived the idea for the review. CC drafted the manuscript. VC, MC, and EG edited the manuscript. All authors read and approved the final version.

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Effects of Cyclic Chronic Heat Stress on the Expression of Nutrient Transporters in the Jejunum of Modern Broilers and Their Ancestor Wild Jungle Fowl

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Heat stress (HS) has been reported to disrupt nutrient digestion and absorption in broilers. These effects may be more prominent in fast-growing chickens due to their high metabolic activity. However, the underlying molecular mechanisms are not yet fully elucidated. Hence, the current study aimed to evaluate the effect of chronic HS on jejunal nutrient transport in slow- (Athens Canadian Random Bred, ACRB from 1950), moderate- (The 1995 random bred, 95RAN), rapid- (modern broilers, modern random bred, MRB) growing birds and their ancestor wild jungle fowl (JF). One-day male chicks (n = 150/line) were placed by line in environmentally controlled chambers and kept under the same industry-standard environmental conditions until d28. On d29, an 8-h daily cyclic HS (36°C) was applied to half of the chambers, which lasted until d55, while keeping the rest under thermal neutral (TN, 24°C) conditions. Jejunum tissues were collected for morphology assessment and molecular analysis of carbohydrate-, amino acid-, and fatty acid-transporters. MRB exhibited the highest body weight (BW) followed by 95RAN under both conditions. HS decreased feed intake (FI) in MRB and 95RAN, which resulted in lower BW compared to their TN counterparts; however, no effect was observed in ACRB and JF. MRB showed a greater villus height (VH) to crypt depth (CD) ratio under both environmental conditions. Molecular analyses showed that glucose transporter (GLUT) 2, 5, 10, and 11 were upregulated in MRB compared to some of the other populations under TN conditions. HS downregulated GLUT2, 10, 11, and 12 in MRB while it increased the expression of GLUT1, 5, 10, and 11 in JF. GLUT2 protein expression was higher in JF compared to ACRB and MRB under TN conditions. It also showed an increase in ACRB but no effect on 95RAN and MRB under HS conditions. ACRB exhibited greater expression of the EAAT3 gene as compared to the rest of the populations maintained under TN conditions. HS exposure did not alter the gene expression of amino acid transporters in MRB. Gene expression of CD36 and FABP2 was upregulated in HS JF birds. Protein expression of CD36 was downregulated in HS JF while no effect was observed in ACRB, 95RAN, and MRB. Taken together, these

data are the first to show the effect of HS on jejunal expression of nutrient transporters in three broiler populations known to represent 70 years of genetic progress in the poultry industry and a Red Jungle Fowl population representative of the primary ancestor of domestic chickens.

Keywords: broilers, heat stress, glucose transporters, amino acid transporters, fatty acid transporters

INTRODUCTION

Heat stress (HS) is considered one of the main threats to the poultry industry especially under continuous climate changes, including global warming (Perini et al., 2021). The high susceptibility of chickens to HS is attributed to their limited ability to regulate heat loss due to the presence of feathering and the lack of sweat glands (Wasti et al., 2020). On the other hand, the genetic selection of fast-growing meat-type broilers during the last few decades has focused narrowly and intensely on increasing growth rates and meat yield, improving feed efficiency, and decreasing slaughter age (Wasti et al., 2020). Indeed, modern broilers are characterized by higher metabolic activities compared to their ancestors, resulting in increased body heat production (Al-zghoul et al., 2019). Therefore, the positive achievements in terms of growth performance were accompanied by higher sensitivity in the gut to HS (Havenstein et al., 2003; He et al., 2018a), a lack of effective thermoregulation and heightened metabolism, entailing a strenuous adaptation to harsh environments, and difficulties to cope with HS (Perini et al., 2021).

Heat stress occurs when heat load of an animal is greater than its capacity to lose heat and may cast a shadow over the performance and profitability of livestock production. In this sense, a growing body of scientific evidence has documented the detrimental effects of HS on broiler growth, metabolism, and physiology, such as impairing the intestinal morphology by shortening the villus and deepening the crypt (Song et al., 2017; He et al., 2018a), decreasing jejunal weight and length (Garriga et al., 2006), impairing the intestinal integrity, and inducing oxidative stress leading to epithelial damage and inflammatory response (Lian et al., 2020). It also possesses a negative impact on the immune system (Cui et al., 2016) by triggering the expression of heat shock proteins (HSPs), and thereby leading to decreased energy metabolism (Liu et al., 2015), which reduces the growth performance. The impaired poultry productivity by HS was also attributed to the reduced utilization of nutrients, resulting from altering the activities of enzymes, such as amylase, maltase, lipase, trypsin, and chymotrypsin (Song et al., 2017; He et al., 2018a) and modulating the expression of the genes responsible for nutrient transport (Goel et al., 2021). The abovementioned negative effects may depend on both temperature and duration of HS exposure (Goel et al., 2021). In this context, some studies reported a reduced glucose uptake under chronic HS as evidenced by the downregulation of the sodium-dependent glucose transporter (SGLT) gene responsible for the glucose absorption and the glucose transporter 2 (GLUT2) gene, involved in the transfer of up-taken fructose and glucose into portal blood capillaries, in the intestine of broiler chickens (Sun et al., 2015; Habashy et al., 2017a; Al-zghoul et al., 2019). Similarly, protein expressions of SGLT1, GLUT1, and GLUT10 were lowered in the intestine of laying hens subjected to HS for 12 weeks (Orhan et al., 2019). HS has been also shown to impair fatty acid transporters (FATs), especially by reducing the gene expression of fatty acid-binding proteins (FABPs), such as FABP1, irrespective of exposure time and intensity of stress (Sun et al., 2015; Habashy et al., 2017b; Al-zghoul et al., 2019), the gene expression of fatty acid transport proteins (FATPs), and the gene and protein expression of the cluster of differentiation 36 (CD36) (Al-zghoul et al., 2019; Orhan et al., 2019). Research has shown conflicting results regarding the effects of chronic HS on amino acid transport. Although a decrease of PEPT1 has been reported in broilers subjected to 35°C for 19 days (Habashy et al., 2017b) and laying hens exposed to 34 ± 2°C for 8 h/day during 12 weeks (Orhan et al., 2019), other authors have observed no variation of this transporter in broilers exposed to 32°C for 7 days (Sun et al., 2015). In spite of its detrimental effects on nutrient digestibility and absorption, HS was not shown to modulate *SLC7A1* and *SLC7A7* gene expression (Sun et al., 2015; Yi et al., 2016; Song et al., 2017; He et al., 2018b), but it rather enhances amino acid catabolism in broiler intestine (Lara and Rostagno, 2013).

Although the aforementioned studies are seminal and provided valuable insights, they used only modern chickens (broilers or layers). In fact, there is still a lack of clear understanding of how the gastrointestinal tract has changed in response to intensive genetic selection for rapid growth and feed efficiency and how is it affected by HS. The present study was performed to evaluate the effects of chronic HS on the gene and protein expression of carbohydrate, amino acid, and FATs in the jejunum of three broiler populations [slow growing 1950 (Collins et al., 2016), moderate growing 1995 (Harford et al., 2014), and modern fast-growing 2015 (Orlowski et al., 2017)] known to represent 70 years of broiler genetic progress, and the jungle fowl (JF), representative of the ancestral origin of the modern broiler (Orlowski et al., 2017).

MATERIALS AND METHODS

Chicken Populations

The current study involved four research lines that are housed and maintained at the University of Arkansas research farm. The first line represents the commercial broiler chicken of the 1950s (Athens Canadian Random Bred, ACRB) characterized by slow growth (Collins et al., 2016). One thousand nine hundred ninety-five random bred (95RAN), a moderate-growing line consisting of seven parent stock male and six parent stock female lines

commercially available in the 1990s (Harford et al., 2014). The third line is a modern random bred (MRB) population initially established in 2015 at the University of Arkansas that originally consisted of four commercially available broiler packages from three different broiler genetics companies; Cobb MX × Cobb 500, Ross 544 × Ross 308, Ross Yield 1 × Ross 708, and the Hubbard HiY package. The four packages have been blended homogenously after five generations of random mating to create a population representing a commercially available broiler from 2015 (Orlowski et al., 2017). The fourth generation is the South East Asian JF, serving as the common ancestor to the commercial broiler (Orlowski et al., 2017).

All populations are maintained at the University of Arkansas research farm under close care and supervision and are randomly mated each generation with the avoidance of full and half-sibling pairings. The study was conducted in accordance with the recommendations in the guide for the care and use of laboratory animals of the National Institutes of Health and the protocols received prior approval from the University of Arkansas Animal Care and Use Committee under protocols 18,083 and 16,084.

Experimental Design, Environmental Exposure, and Animal Husbandry

Day-old broiler chicks from the four chicken lines hatched at the University of Arkansas were vent-sexed and individually wing-banded with a number and barcode. Male chicks were separated by line and housed in 12 environmentally controlled chambers in the Poultry Environmental Research Laboratory at the University of Arkansas. Each chamber consists of two equally sized pens allowing for triplication of a 4 × 2 factorial design. Twenty-five male chicks of the same line were randomly placed in each pen and kept at an approximate density of 0.5 m^2 per bird in all pens. All birds were allowed *ad libitum* access to feed and fresh water. The lighting program was set to 23L: 1D (L: light; D: darkness) during the first week and 20L: 4D for the remainder period (day 8–56) of the trial. Chickens were given a two-phase feeding program consisting of commercially available starter (day 0–28) and finisher (day 29–55) formulated to meet or exceed National Research Council (NRC) recommendations (National Research Council, 1994).

The brooder temperature was maintained at 32°C during the first 3 days and was then gradually reduced to 31°C on days 4–6, 29°C on days 7–10, 27°C on days 11–14, and 25°C for day 15 through day 28. The environmental treatments began the morning of day 29 by keeping half of the chambers in a TN environment at a constant temperature of 25°C for the remainder of the study, whereas the remaining six chambers were subjected to an 8 h daily cyclic HS (36°C) from 8 a.m. to 4 p.m., until processing (Tabler et al., 2020). This design resulted in three pens (75 birds) per line being subjected to either a TN or HS environment.

Growth Performance Evaluation and Sample Collection

Live BW and FI were recorded weekly. At the end of the experiment, six birds/line/environmental treatments were randomly selected, electrically water bath stunned (11V, 11 mA, 10), and manually cut through the left carotid artery and allowed to completely bleed. Samples from the jejunal tissue were collected and kept in 4% paraformaldehyde in phosphate-buffered saline (PBS) for further analysis of intestinal morphology. Segments from the jejunum were harvested for molecular analyses as described below. The tissues were snap frozen in liquid nitrogen and stored at −80°C until analysis.

Intestinal Morphology Analysis

Jejunum samples collected at 55 days of age were embedded in paraffin using a tissue processor. Sections of 2.5 μm were stained with hematoxylin and eosin before being analyzed with a digital microscope camera (Leica DFC450 C; Leica Microsystems Ltd.) and the ViewPoint Light software version 1.0.0.9628. Images were analyzed by the same person. The morphometric variables measured included VH, CD, and VH to relative CD ratio (VH: CD ratio). Twelve villi were measured for each sample and only complete and vertically oriented villi were evaluated. The mean from 12 villi per sample was used as the mean value for further analysis.

Cell Culture

As no established chicken jejunal cell line currently exists, the non-transformed intestinal porcine epithelial cells from the jejunum (IPEC-J2) were selected as an *in vitro* model in this study and has been used in previous chicken studies. The IPEC-J2 cell line was originally derived and characterized from jejunal epithelia of unsuckled piglets (Schierack et al., 2006). The cells of the present study were a kind gift from Dr. Maxwell C. (University of Arkansas). Cells were cultured in six-well-plates with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA) in Dulbecco's Modified Eagle Medium (DMEM)/F-12/HAM (Life Technologies, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. At ∼80–90% confluence, cells were exposed to HS (45°C) for 2 h. Cells maintained at 37°C were used as controls.

RNA Isolation, Reverse Transcription, and Quantitative Real-Time PCR

One microgram of total RNA was extracted from chicken jejunum tissues or IPEC-J2 cell line by trizol reagent (Thermo Fisher Scientific, Rockford, IL) following the recommendations of the manufacturer. For each sample, total RNA concentration was determined using Take 3 Micro Volume Plate and the Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). RNA quality and integrity were assessed using the ratio of absorbance (260/280) and 1% agarose gel electrophoresis. Afterward, RNAs were treated with DNAse and reverse transcribed *via* qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). The cDNA was then amplified by real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system) with Power SYBR Green Master Mix (Thermo Fisher Scientific, Rockford, IL, USA) as described previously (Greene et al., 2020; Tabler et al., 2020; Emami et al., 2021).

TABLE 1 | Oligonucleotide real-time qPCR primers.

Gene	Accession number[a]	Primer sequence (5′ → 3′)	Orientation	Product size (bp)
GLUT1	NM_205209.1	TCCTGATCAACCGCAATGAG	Forward	60
		TGCCCCGGAGCTTCTTG	Reverse	
GLUT2	NM_207178.1	GAAGGTGGAGGAGGCCAAA	Forward	61
		TTTCATCGGGTCACAGTTTCC	Reverse	
GLUT3	NM_205511.1	TTGGGCGCTTCATTATTGG	Forward	68
		CTCACTGATGTACATGGGAACAAAG	Reverse	
GLUT5	XM_040689119.1	CCTCAGCATAGTGTGTGTCATCATT	Forward	62
		GGATCGGACTGGCTCCAA	Reverse	
GLUT8	XM_040648927.1	GCTGCCTCAGCGTGACTTTT	Forward	58
		AGGGTCCGCCCTTTTGTT	Reverse	
GLUT9	XM_040670183.1	CAGTGGATGAAAGCACCTTGAC	Forward	63
		CACCGATGGCAAAAATGGA	Reverse	
GLUT10	XM_040688610.1	AACGCAGAACAAAGATTCCTGAA	Forward	65
		GTCATTCCACGTGCCAGCTT	Reverse	
GLUT11	NM_001347709.1	CCCTCATCCAGCTCATGATTCT	Forward	67
		CCACGGTCAATCAAGAGGTATCT	Reverse	
GLUT12	XM_040667840.1	TTTGTGGACCTGTTTCGTTCAA	Forward	61
		GCGTGAGCCCTACCAGCAT	Reverse	
GLUT14	XM_040672375.1	TTGGGCGCTTCATTATTGG	Forward	68
		CTCACTGATGTACATGGGAACAAAG	Reverse	
SGLT1	NM_001293240.1	AGCATTTCAGCATGGTGTGTCT	Forward	64
		TGCTCCTATCTCAGGGCAGTTC	Reverse	
SLC1A4 (ASCT1)	XM_040666934.1	CGACTGATGAACAACGCAGAA	Forward	119
		TCGCCAACCTCCGCATT	Reverse	
SLC3A1 (NBAT)	XM_040667709.1	CCTGGGCTGTGAGAAACCAA	Forward	63
		GGCACAAATTGAGTAGGAAGAAGAG	Reverse	
SLC6A14	XM_040670974.1	GCTTCCGTGGTCAGATTGCT	Forward	66
		TCATTTACGAGGCGTGTTTTACTG	Reverse	
SLC6A19	XM_040663289.1	CGCTGGTGTGCCTAGTTTGA	Forward	64
		ACAGCAATTTCTGATGGCTTTG	Reverse	
SLC6A20 (SIT1)	XM_418798.6	GCTGTCAAACCCCAAAACGT	Forward	63
		CCCAGACCCAGTGAGAAGAAGA	Reverse	
SLC7A1 (CAT1)	NM_001145490.1	AAAACTCCAGTAATTGCAACAGTGA	Forward	68
		AAGTCGAAGAGGAAGGCCATAA	Reverse	
SLC7A2 (CAT2)	NM_001199102.1	AGCTCTCCATCCACCATGTTG	Forward	58
		CCAGGCACCGAACAAAGGT	Reverse	
SLC7A6 (Y + LAT2)	XM_040681081.1	CACGTGGGTGGCTTTGC	Forward	61
		GAATTCTCCACGGCTCTGAACT	Reverse	
SLC7A9 (BAT1)	NM_001199133.1	GCTGTGGGTCCTTGTTTAACCA	Forward	60
		TGCACCTAGTGTTGCCAGAACT	Reverse	
SLC7AL	XM_040665181.1	GCTGAGTTGGGAGCATCCA	Forward	66
		ACCAAACGCTTCCAGGATGT	Reverse	
SLC38A2 (SNAT2)	NM_001305439.1	TGGCATCCTGGGACTTTCC	Forward	66
		AGCAGGAGTATCACAAAAAGAGCAA	Reverse	
LAT1	NM_001030579.2	CTGCTGCCGCCTGAGAA	Forward	62
		CGCCGGCAGGAATTCC	Reverse	
EAAT3	XM_424930.7	GGTGAAGGCGGACAGGAA	Forward	68
		TGCTGAGCAGGAGCCAGTT	Reverse	
PepT1	NM_204365.1	GACAACTTTTCTACAGCCATCTACCA	Forward	65
		CCCAGGATGGGCGTCAA	Reverse	

(Continued)

TABLE 1 | Continued

Gene	Accession number[a]	Primer sequence (5′ → 3′)	Orientation	Product size (bp)
PepT2	NM_001319028.1	TGAAAAACCGCTCCCATCA	Forward	61
		TGTTCCGATGCCCAGTCAA	Reverse	
iFABP	NM_001007923.1	CGTACCATCGACATCGAATTCA	Forward	61
		TCCCGTCAGCCAGACTGTATT	Reverse	
CD36	XM_040686380.1	ACTGCGCTTCTTCTCCTCTGA	Forward	68
		TCACGGTCTTACTGGTCTGGTAAA	Reverse	
18s	AF173612	TCCCCTCCCGTTACTTGGAT	Forward	60
		GCGCTCGTCGGCATGTA	Reverse	

GLUT, glucose transporter; qPCR, quantitative PCR.

Oligonucleotide primers specific for chicken nutrient transport-related genes were used as described in **Table 1**. The quantitative PCR (qPCR) cycle parameters comprised the following phases: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of a two-step amplification program (95°C for 15 s and 58°C for 1 min). At the end of the amplification, melting curve analysis was applied using the dissociation protocol from the Sequence Detection system to exclude contamination with unspecific PCR products. Agarose gel was used to confirm the PCR products, which showed only one specific band of the predicted size. For negative controls, no real-time products were used as templates in the qPCR and verified by the absence of gel-detected bands. Relative expressions of target genes were determined by the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). The 18S ribosomal RNA was used as an internal control to which the fold changes in gene expression were normalized. Samples extracted from JF at TN conditions were used as a calibrator.

Western Blot Analysis

Jejunum samples or IPEC-J2 cell lines were homogenized in lysis buffer (10 mmol/L Tris base, pH 7.4; 150 mmol/L NaCl; 1 mmol/L EDTA; 1 mmol/L EGTA; 0.1% Triton X-100; 0.5% Nonidet P-40; and protease and phosphatase inhibitors) and stainless-steel beads, using the Bullet Blender Storm (NextAdvance, Averill Park, NY, USA). Total protein concentrations were determined using a Bradford assay kit (Bio-Rad, Hercules, CA, USA) and run in 4–12% gradient Bis-Tris gels (Life Technologies, Carlsbad, CA, USA) and then transferred to polyvinylidene difluoride membranes. Once transferred, membranes were blocked using a Tris-buffered saline (TBS) with 5% non-fat milk and Tween 20 (TBST) at room temperature for 1 h. The membranes were washed with TBST and then incubated with primary antibodies (dilution 1:1,000) overnight at 4°C. Primary antibodies used were rabbit anti-GLUT1, rabbit anti-GLUT2, rabbit anti-GLUT3, rabbit anti-SLC38A3, and rabbit anti-CD36 (ABClonal, Woburn, MA, USA). After another wash, secondary antibodies diluted to 1:5,000 were added to 5% non-fat milk in TBS and Tween 20 and incubated with the membranes at room temperature for 1 h. The protein signals were visualized using chemiluminescence (ECL Plus; GE Healthcare, Pittsburg, PA, USA), and images were captured using the FluorCHem M MultiFluor System (ProteinSimple, San Jose, CA, USA). Prestained molecular weight marker (precision plus protein dual color) was used as a standard (Bio-Rad, Hercules, CA, USA). Protein loading was assessed by quantification of the universally expressed 70 kDa band from staining with universal protein Ponceau S (PS) stain (G-Biosciences, St. Louis, MO, USA) (Sander et al., 2019). Image acquisition and analysis were performed by AlphaView software (version 3.4.0, 1993–2011; ProteinSimple). One representative blot was shown for each protein.

Immunofluorescence

Immunofluorescence staining was performed as previously described (Dridi et al., 2012; Greene et al., 2020). Briefly, IPEC-J2 cells were grown in chamber slides, exposed to 37 or 54°C as described above and fixed with methanol for 10 min at −20°C before being permeabilized with Triton-X 100. Cells were blocked with serum-free protein block (Dako, Carpinteria, CA, USA) for 1 h at room temperature, then incubated with anti-GLUT1, anti-GLUT2, anti-GLUT3, anti-HSP60 (Santa Cruz Biotechnology, Dallas, TX, USA), or anti-HSP70 (Pierce Thermo Scientific, Rockford, IL, USA). All antibodies were diluted at 1:200, in antibody diluent (Dako, Carpinteria, CA, USA) and incubated overnight at 4°C. Signal was visualized with DyLight 488- or 594-conjugated secondary antibody (Thermo Fisher Scientific, Grand Island, NY, USA). Slides were coverslipped with vectashield with diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA), and images were obtained and analyzed using Zeiss Imager M2 and AxioVision software (Carl Zeiss Microscopy).

Statistical Analysis

Growth performance, histology, gene expression, and protein expression data from the *in vivo* study were analyzed using a two-way ANOVA considering chicken population (JF, ACRB, 95RAN, MRB) and environmental conditions (TN and HS) as the main effects. In case ANOVA showed significant effects, the means were compared by Tukey's multiple range test. Data from the *in vitro* study were analyzed by the Student's *t*-test. Graph Pad Prism software (version 6.00 for Windows, Graph Pad Software, La Jolla, CA, USA) was used, and data are expressed as the

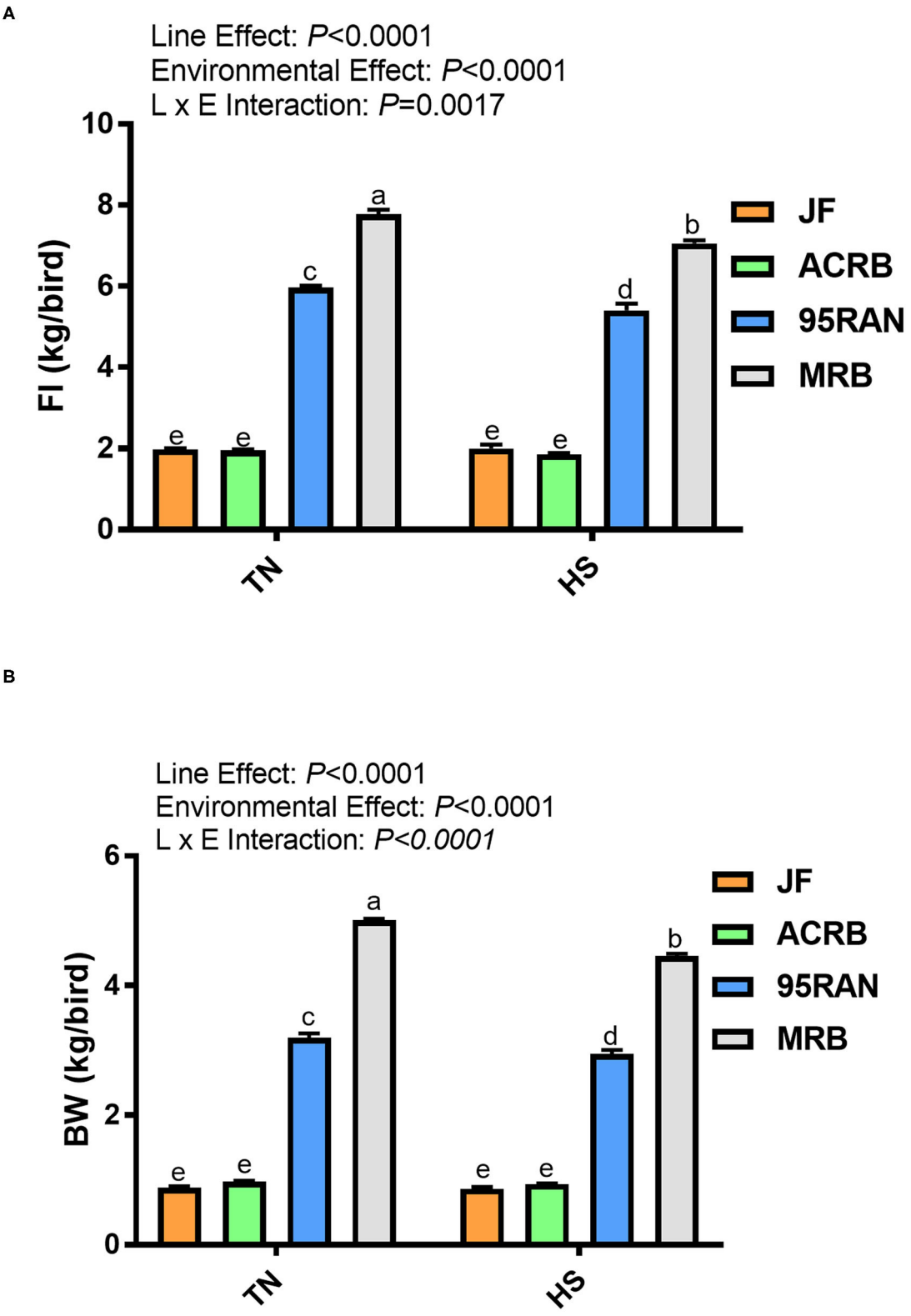

FIGURE 1 | Effect of HS on feed intake **(A)** and body weight **(B)** of JF, ACRB, 95RAN, and MRB birds. Data are mean ± SEM ($n = 3$ pens/group for FI and $n = 75$ birds/group for BW). Different letters indicate a significant difference at $P < 0.05$. BW, body weight; FI, feed intake; HS, heat stress; TN, thermoneutral; JF, jungle fowl; ACRB, Athens Canadian Random Bred; 95RAN, 1995 random bred; MRB, modern random bred.

TABLE 2 | Effect of heat stress on the global feed conversion ratio (FCR through day 54).

	TN	HS
JF	2.349 ± 0.038	2.401 ± 0.099
ACRB	2.080 ± 0.008	2.059 ± 0.033
1995	1.890 ± 0.032	1.855 ± 0.039
2015	1.565 ± 0.022	1.598 ± 005
Main effects		
Environmental treatment	0.8185	
Line	<0.0001	
Interaction	0.7188	

FCR, feed conversion ratio.

mean ± SEM. Means were considered statistically significant at a $P \leq 0.05$.

RESULTS

Effects of HS on Growth Performances

Performance of the four lines within TN and HS conditions was evaluated via comparison of BW, FI, and feed conversion ratio (FCR). Significantly higher BWs were observed for the 95RAN and MRB broilers compared to JF and ACRB in both TN and HS conditions, with the MRB having the highest BW overall (**Figure 1B**). However, BW for the 95RAN and MRB also decreased significantly under HS conditions compared to TN of the same population (**Figure 1B**). FI followed the same pattern of significance with the MRB random bred broiler having the highest FI under both TN and HS conditions (**Figure 1A**). The 95RAN broilers had significantly higher FI compared to JF and ACRB, but the 95RAN had lower FI compared to the MRB, and there was no difference between the JF and ACRB in either TN of HS (**Figure 1A**). Again, under HS conditions, FI significantly decreased in both the 95RAN and the MRB (**Figure 1A**). For FCR, only the main effect of the line was significant ($P < 0.0001$) while environmental treatment and interaction were not significant ($P > 0.05$). Indeed, MRB averaged 33, 52, and 78 points fewer in FCR compared to 95RAN, ACRB, and JF, respectively, confirming the tremendous effect of genetic selection in improving feed efficiency (**Table 2**).

Effects of HS on Jejunum Morphometry

Histological analysis showed that jejunal VH was affected by line ($P = 0.0004$) and by line × environmental interaction ($P = 0.0254$; **Figures 2a–j**). Significant increased VH in MRB was seen compared to all other populations under TN conditions ($P = 0.0030$, **Figures 2a–j**). Under HS conditions, MRB had significantly greater VH compared to JF (**Figures 2a–j**). There were no significant differences in CD between the lines or when comparing environmental conditions. However, the ratio of VH:CD was affected by both line ($P < 0.0001$) and line × environmental effect ($P = 0.0030$), but not temperature ($P = 0.2018$; **Figures 2a–j**). Under TN conditions, the ratio of VH to CD was significantly higher in MRB compared to all other lines (**Figure 2k**). Under HS conditions, MRB still retained a higher ratio of VH:CD when compared to JF and 95RAN (**Figure 2k**). The ratio of VH to CD significantly increased from TN to HS in ACRB (**Figure 2k**).

Effects of HS on the Expression of Jejunal Carbohydrate Transporters

Heat stress significantly upregulated the jejunal expression of *GLUT1*, *GLUT5*, *GLUT10*, and *GLUT11* mRNA in JF, but it downregulated that of *GLUT2*, *GLUT10*, *GLUT11*, and *GLUT12* in MRB and *GLUT6* in ACRB compared to their TN counterparts (**Figures 3E,F**, **4A,B,E–G**). The immunoblot analysis showed that jejunal GLUT1 protein levels were significantly increased in heat-stressed ACRB and 95RAN and decreased in heat-stressed JF compared to their TN counterparts (**Figures 3A,B**). GLUT2 protein levels were significantly increased in the jejunum of heat-stressed ACRB and decreased in heat-stressed JF compared to their TN counterparts (**Figures 3A,C**). Jejunal *GLUT3* mRNA abundances and protein levels remained unchanged between all chicken populations under both environmental conditions (**Figures 3A,D**).

Under TN conditions, the highest mRNA abundances of *GLUT2*, *GLUT5*, *GLUT10*, *GLUT11*, and *GLUT12* were found in the jejunum of the MRB population (**Figures 3A,C**, **4A,E–G**). 95RAN broilers exhibited the lowest mRNA levels of *GLUT6*, *GLUT8*, and *GLUT9* (**Figures 4B–D**). Under HS conditions, the greatest expression of *GLUT1*, *GLUT5*, *GLUT8*, *GLUT9*, *GLUT10*, *GLUT14*, and *SGLT1* was found in the JF jejunum (**Figures 3E**, **4A,C–E,H,I**).

in vitro study using IPEC-J2 cell lines showed that HS exposure induced the expression of HSP60 and HSP90 as demonstrated by immunofluorescence staining (**Figure 5D**), confirming that the cells are stressed. Both immunoblot analysis and immunofluorescence staining showed that HS increased GLUT1 and decreased GLUT3 protein levels in the IPEC-J2 cells (**Figures 5A–D**).

Effects of HS on the Expression of Jejunal Amino Acid Transporters

Western Blot analysis of amino acid transporter showed that HS significantly decreased SLC38A3 protein levels in the jejunum of JF but not in the other bird populations (**Figures 6A,B**). Under TN conditions, jejunal expression of SLC38A3 protein is higher in JF compared to the other populations (**Figures 6A,B**). Under HS conditions, however, the levels of SLC38A3 protein remained unchanged between all tested birds (**Figures 6A,B**). In IPEC-J2 cells, SLC38A3 protein expression was significantly downregulated by HS exposure (**Figures 6C,D**).

Heat stress significantly increased the mRNA levels of *SLC6A14*, *SLC38A2*, and *PEPT2* gene in the jejunum of JF (**Figures 7B,L,O**), *SLC7A9,* and *EAAT3* genes in 95RAN (**Figures 7F,M**), and significantly decreased that of *EAAT3* gene in ACRB (**Figure 7M**). Under TN conditions, the highest mRNA abundances of *SLC7AL* and *SLC7A6* were found in the jejunum of JF (**Figures 7C,D**), *SLC6A19* and *EAAT3* in ACRB (**Figures 7G,M**), *SLC7A1* in 95RAN (**Figure 7H**) and *SLC1A4* and *SLC7A2* in MRB (**Figures 7E,K**). Under HS conditions, however, JF jejunum exhibited greater expression of *SLC6A14*, SLC7A6, *SLC6A20*, *PEPT1*, and *PEPT2* (**Figures 7B,D,J,N,O**).

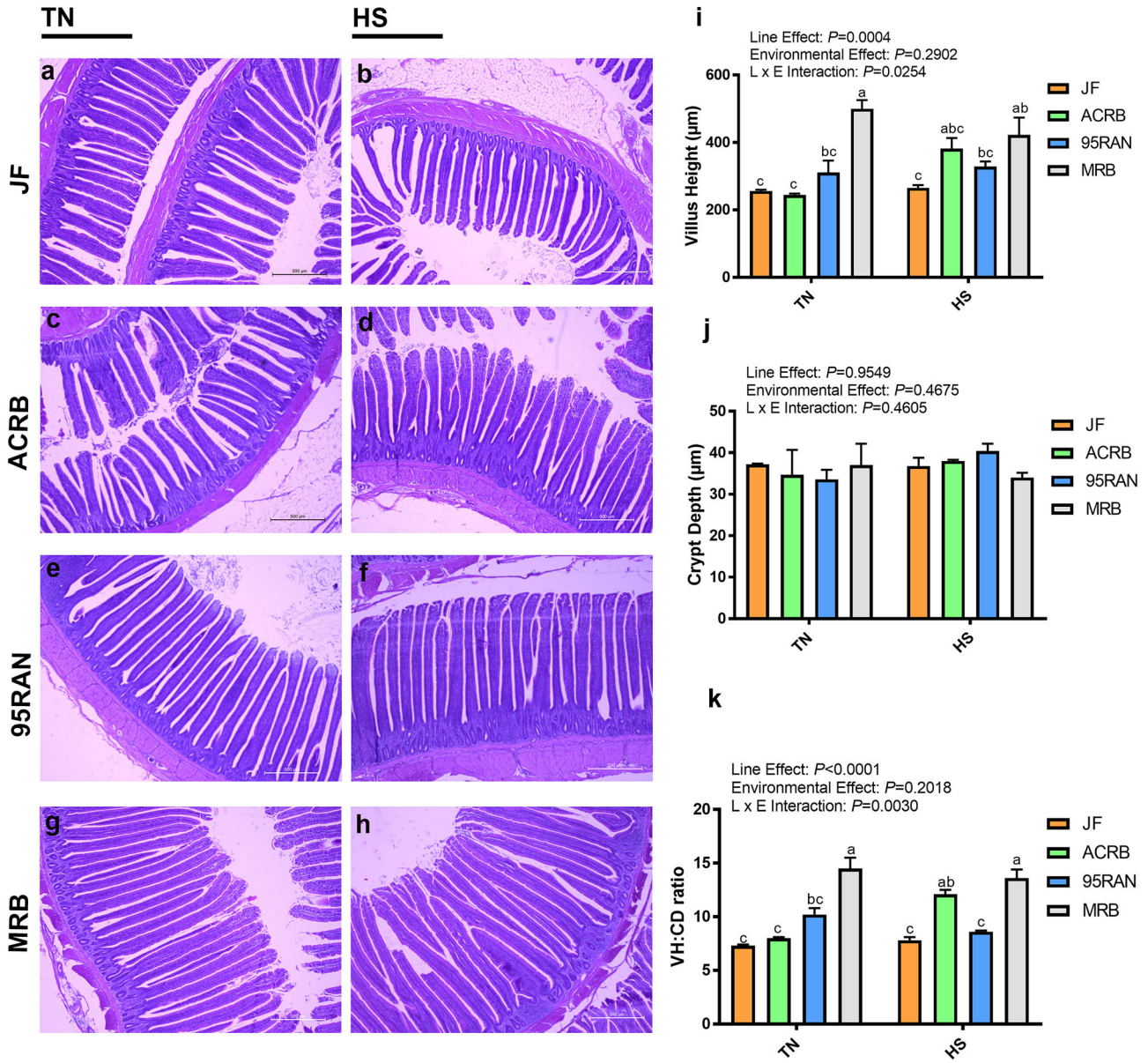

FIGURE 2 | Effect of HS on intestinal morphology of JF **(a,b)**, ACRB **(c,d)**, 95RAN **(e,f)**, and MRB **(g,h)** birds. Tissue from the jejunum of TN **(a,c,e,g)** and HS **(b,d,f,h)** birds were processed and stained for histology. Data are mean ± SEM (n = 2/group). VH **(i)**, CD **(j)**, and VH:CD ratio **(k)** were measured and analyzed. Different letters indicate the significant difference at $P < 0.05$. VH, villus height; CD, crypt depth; HS, heat stress; TN, thermoneutral; JF, jungle fowl; ACRB, Athens Canadian Random Bred; 95RAN, 1995 random bred; MRB, modern random bred.

95RAN birds on the other hand contained a higher expression of jejunal *SLC1A4*, *SLC7A9*, and *EAAT3* genes (**Figures 7E,F,M**). HS significantly down regulated SLC3A1 expression in MRB compared to JF and 95RAN (**Figure 7I**).

Effects of HS on the Expression of Jejunal FATs

Immunoblot analyses showed that HS significantly reduced CD36 protein levels in the jejunum of JF, but not in the other bird populations (**Figures 8A,B**). However, mRNA abundances of both *CD36* and *FABP2* were significantly increased in heat-stressed JF compared to their TN counterparts (**Figures 8C,D**). HS exposure did not elicit any changes to CD36 protein expression in the IPEC-J2 cell line (**Figures 8E,F**).

Under TN conditions, jejunal *CD36* and *FABP2* mRNA abundances remained unchanged between all chicken populations; however, CD36 protein levels were significantly higher in JF (**Figures 8A–D**). Under HS exposure, jejunal CD36 protein expression did not differ between all tested bird populations, however, *CD36* and *FABP2* gene expressions were higher in JF (**Figures 8A–D**).

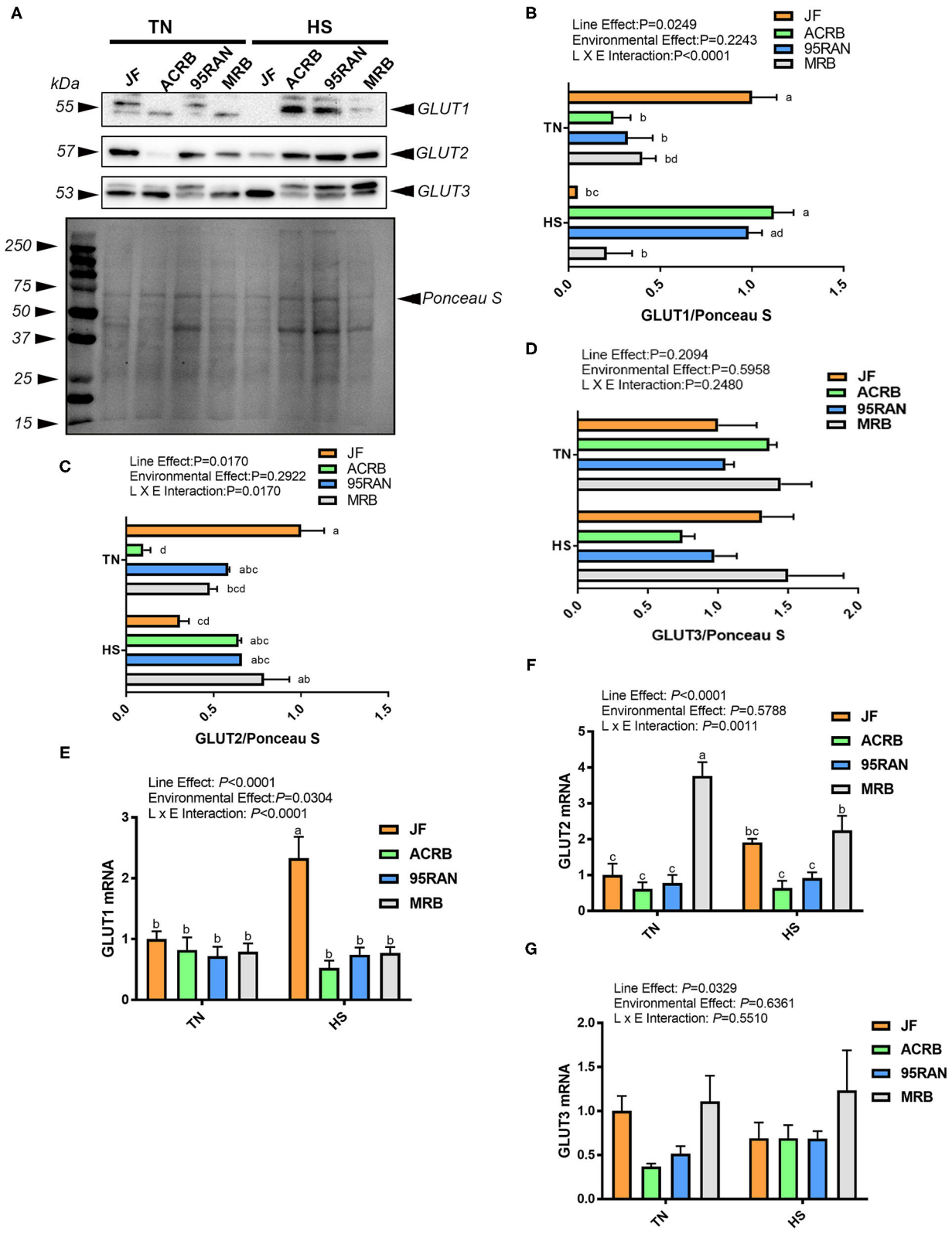

FIGURE 3 | Effect of HS on jejunal expression of GLUT1, GLUT2, and GLUT3 in JF, ACRB, 95RAN, and MRB birds. Protein and RNA were extracted from tissue and analyzed by western blot **(A–D)** and qPCR **(E–G)**, respectively. Gene expression data are mean ± SEM (n = 6/group). Protein expression was normalized to loading via Ponceau stain (PS) **(A)**. Protein was analyzed via AlphaView software and is expressed as mean ± SEM (n = 4/group) with one representative blot shown **(A)**. Different letters indicate the significant difference at $P < 0.05$. GLUT, glucose transporter; HS, heat stress; TN, thermoneutral; JF, jungle fowl; ACRB, Athens Canadian Random Bred; 95RAN, 1995 random bred; MRB, modern random bred.

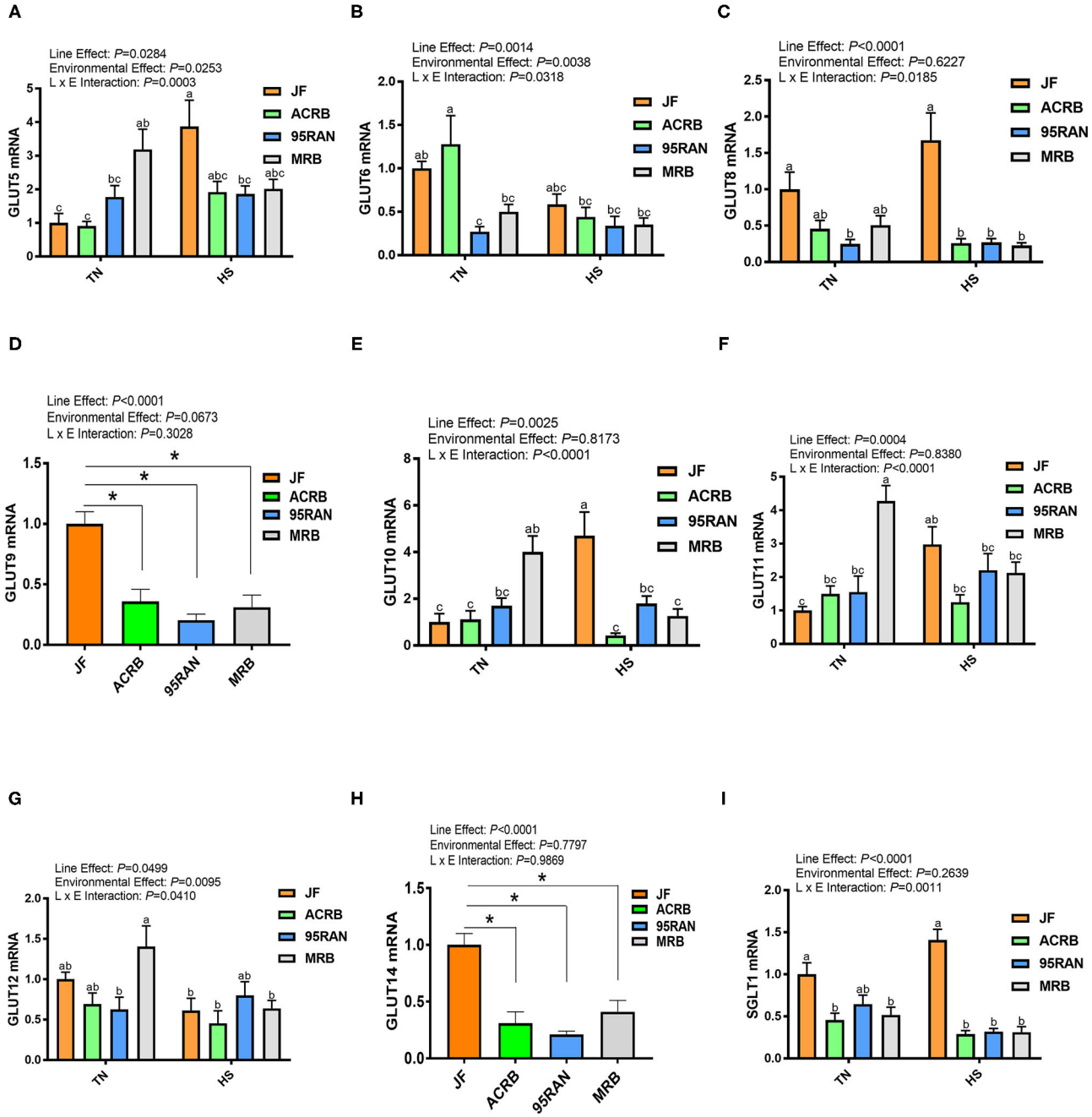

FIGURE 4 | Effect of HS on the carbohydrate transporter gene expression in the jejunum of JF, ACRB, 95RAN, and MRB birds. **(A–I)** RNA was extracted from tissue and analyzed by qPCR. Gene expression data are mean ± SEM ($n = 6$/group). Different letters indicate the significant difference at $P < 0.05$ when the interaction is significant. *indicates a significance compared to JF at $P < 0.05$. GLUT, glucose transporter; HS, heat stress; SGLT1: Sodium/glucose cotransporter 1; TN, thermoneutral; JF, jungle fowl; ACRB, Athens Canadian Random Bred; 95RAN, 1995 random bred; MRB, modern random bred.

DISCUSSION

With the aim to fulfill the increasing demand for poultry meat around the world, genetic selection has led to spectacular achievements in terms of growth rate, feed efficiency, breast yield, and reduction of market age (Thiruvenkadan and Prabakaran, 2017). In the current study, the performance of 54 d-old broilers represented genetics of the 1990s (95RAN) and 2015 (MRB) exhibited a much higher growth rate, and lower FCR compared to the broilers from the 1950s (ACRB) and the ancestral wild-type JF, confirming the aforesaid advancements. However, these signs of progress have been associated with unintended, undesirable

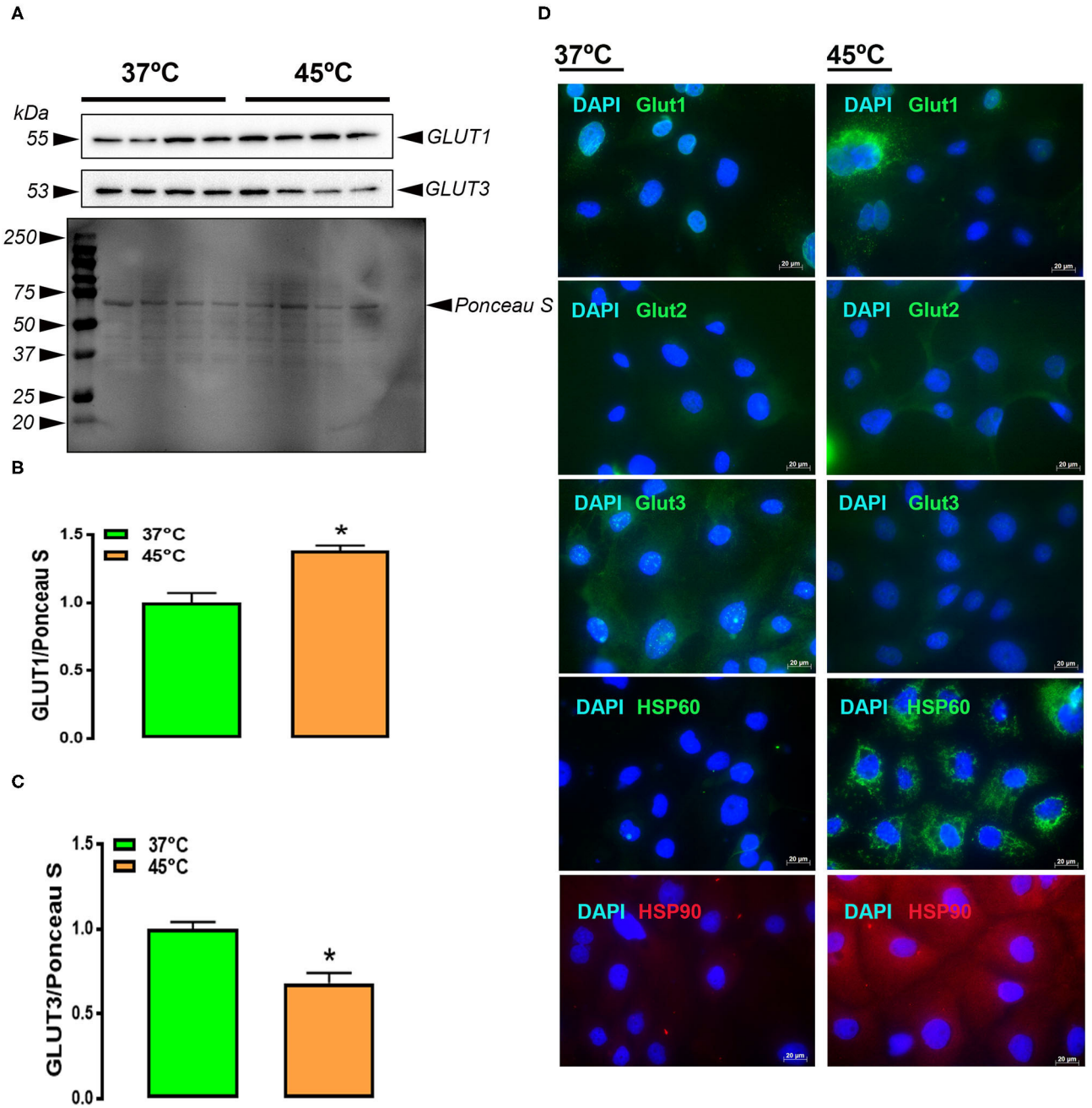

FIGURE 5 | Effect of HS on glucose transporter protein expression in IPEC-J2 cell line. Protein was extracted from cells exposed to HS (45°C) or 37°C (control) and analyzed by western blot **(A–C)** and immunofluorescence **(D)**. Protein expression was normalized to loading via Ponceau stain (PS) **(A)**. Protein was analyzed via AlphaView software and is expressed as mean ± SEM (n = 4/group) with one blot shown **(A)**. *$P < 0.05$. GLUT, glucose transporter; HS, heat stress; TN, thermoneutral; DAPI: diamidino-2-phenylindole.

consequences, including high sensitivity to HS, whose adverse effects on poultry production and welfare are well-documented in the literature. These effects are expected to only get worse in the coming years due to the steady increase in environmental temperature (Wasti et al., 2020; Perini et al., 2021). Indeed, HS has been reported to compromise gut health leading to decreased nutrient absorption in broiler chickens (Goel et al., 2021). In this context, the current study aimed to evaluate the differences in response to HS of four chicken populations representative of eras in the history of a selection of the modern broilers, the JF, the ACRB, the 95RAN, and the MRB. Response to HS was evaluated via analysis of nutrient transport machinery in

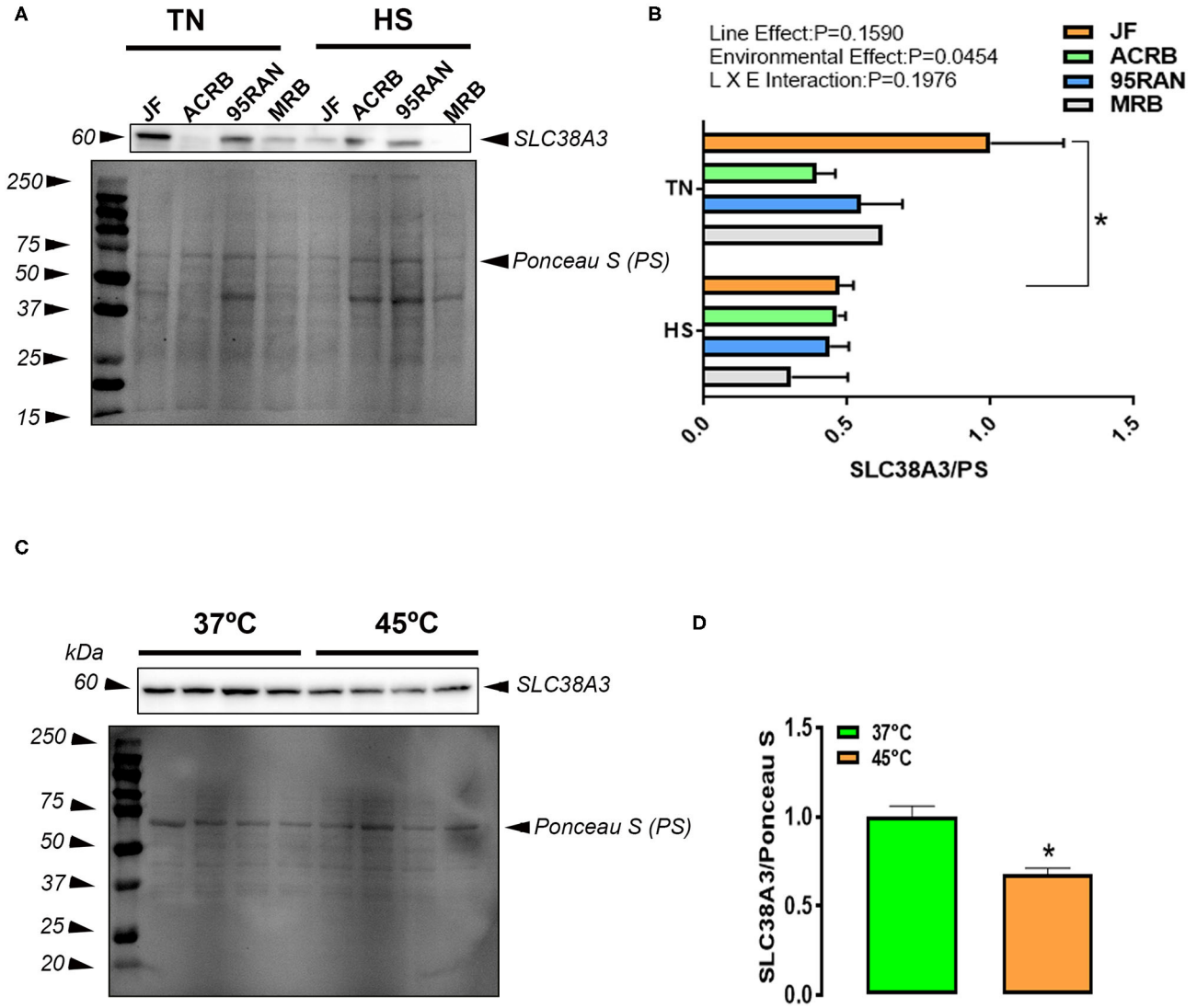

FIGURE 6 | Amino acid transporter protein expression in the jejunum of JF, ACRB, 95RAN, and MRB birds under TN and HS and in IPEC-J2 cell line. Protein was extracted from tissue and cells exposed to HS (45°C) or to 37°C (control) and analyzed by western blot **(A–D)**. Protein expression was normalized to loading via Ponceau stain (PS) **(A,C)**. Protein was analyzed via AlphaView software and is expressed as mean ± SEM (n = 4/group) for IPEC-J2 cells and (n = 4/group) for tissue. *indicate a significant difference compared to TN conditions for bird populations or 37°C for IPEC-J2 cell line at $P < 0.05$. HS, heat stress; TN, thermoneutral.

the jejunum of broiler chickens. To the best of our knowledge, the current study provides the first comparison of the mRNA gene expression and some of the encoded proteins involved in the transport of nutrients (carbohydrates, amino acids, and fatty acids) along with histological evaluation of the jejunum, between the four genetic lines.

The uptake of carbohydrates from the intestinal lumen is crucial to sustaining a constant energy supply and is influenced by both luminal and apical membrane digestion (Sklan et al., 2003). Results from this study suggest that differences in glucose transporter expression in the jejunum depend on line, or the representative genetics over time, and therefore, these differences have the potential to be due to, or the result of, genetic differences. The results also suggest that the expression and presence of glucose transporters in the gut may play a role in HS intolerance or resistance among these different populations. This is evident by the increased gene expression in MRB of *GLUT2*, involved in the transport of carbohydrates, such as glucose, galactose, and fructose across the basolateral membrane, *GLUT5*, a fructose transporter across the brush border membrane (Uldry and Thorens, 2004), *GLUT10*, and *GLUT11* as compared to JF and ACRB under TN conditions. In line with these, a study conducted by Miska and Fetterer (2019) showed *GLUT2* and *GLUT5* mRNA levels to be higher in modern fast-growing Ross as compared to the slow-growing ACRB chickens. The increased gene expression of these transporters in modern birds

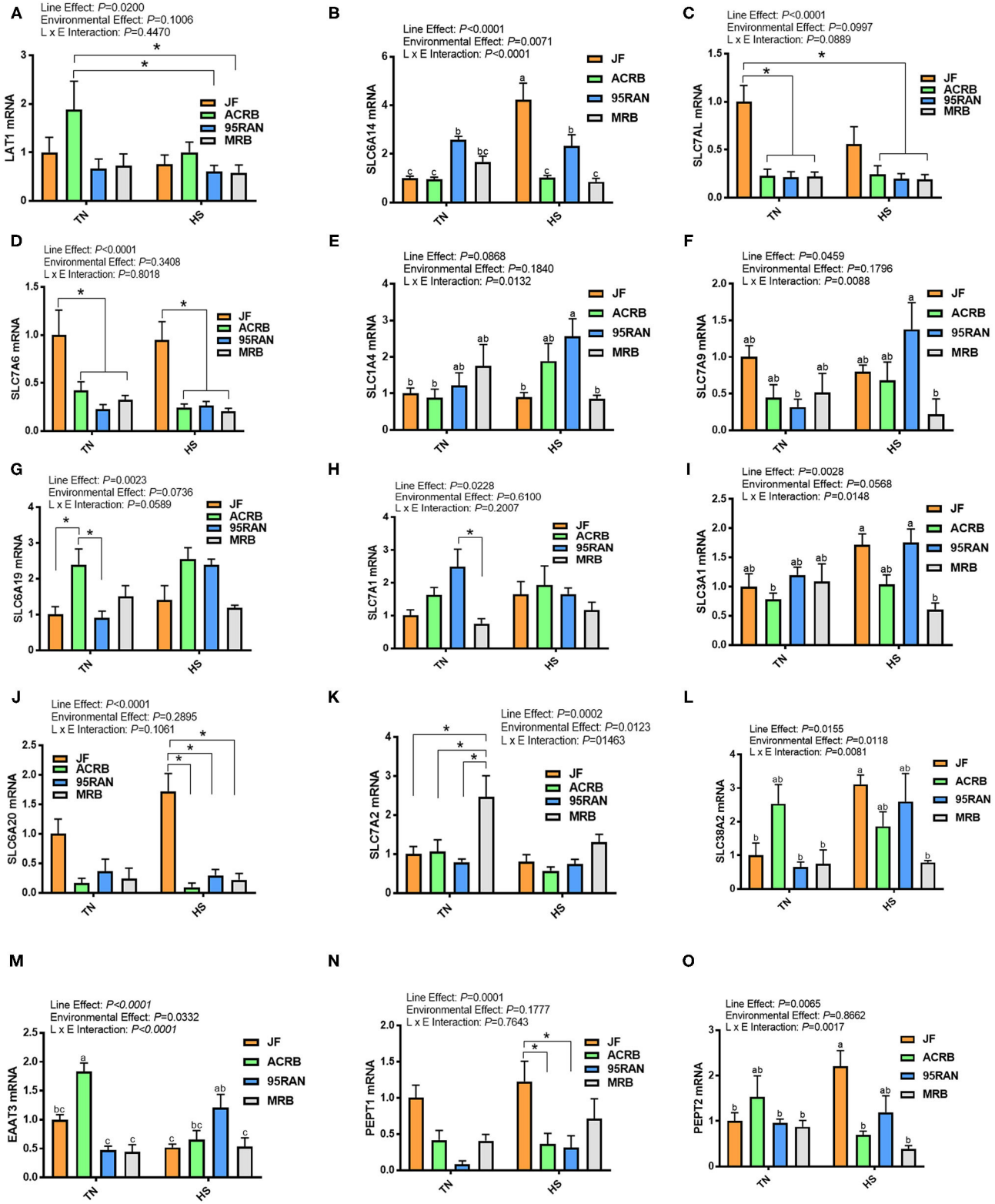

FIGURE 7 | Amino acid transporter gene expression in the jejunum of JF, ACRB, 95RAN, and MRB birds under TN and HS. **(A–O)** RNA was extracted from tissue and analyzed by qPCR. Gene expression data are mean ± SEM ($n = 6$/group). * and different letters indicate a significant difference at $P < 0.05$. LAT1, L-type amino acid transporter 1; EEAT3, excitatory amino acid transporter 3; PEPT, peptide transporter; SLC, Solute carrier family; HS, heat stress; TN, thermoneutral; JF, jungle fowl; ACRB, Athens Canadian Random Bred; 95RAN, 1995 random bred; MRB, modern random bred.

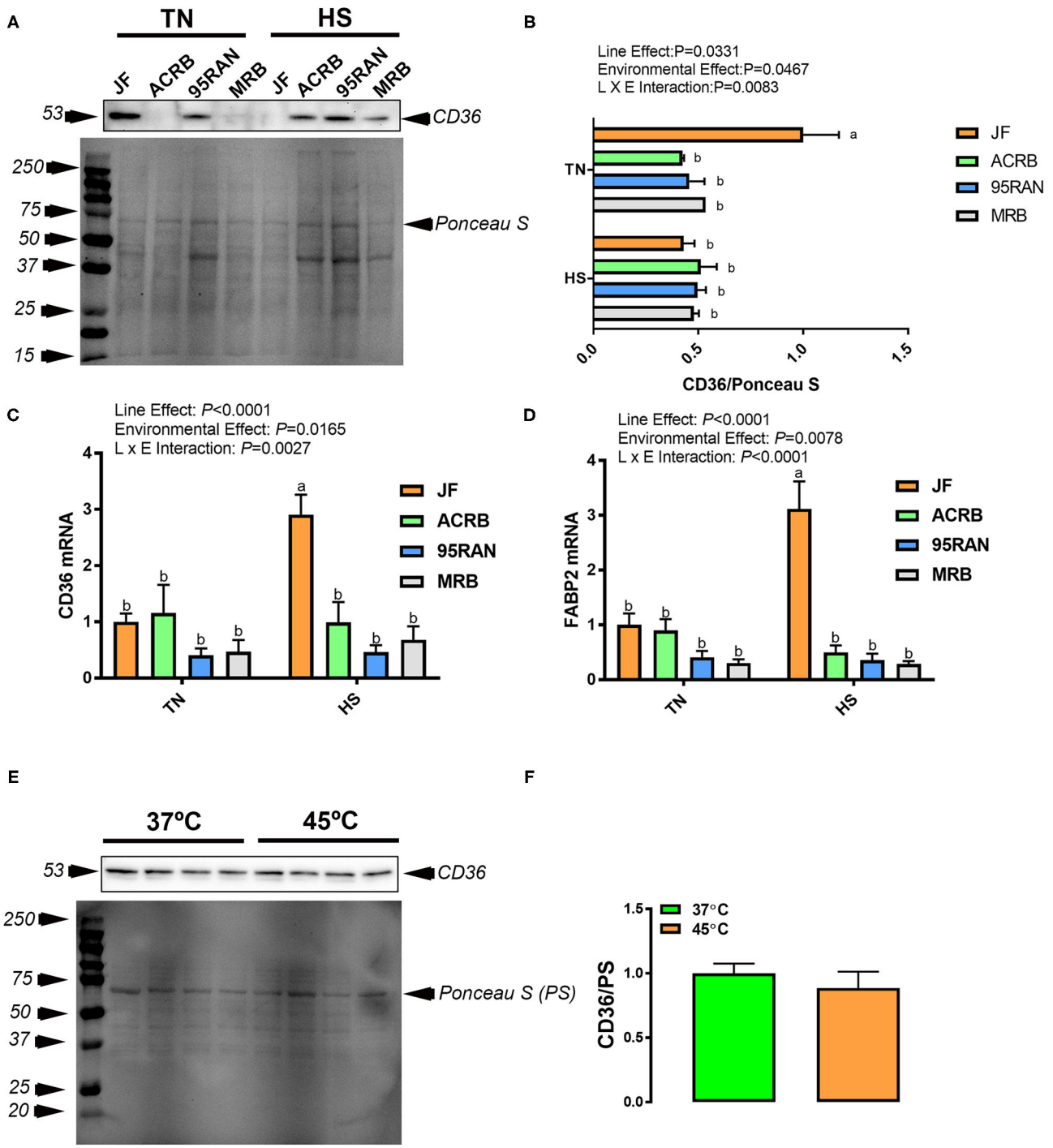

FIGURE 8 | Effect of HS on the expression of fatty acid transporters in the jejunum of JF, ACRB, 95RAN, and MRB birds and in IPEC-J2 cell line. Protein and RNA were extracted from jejunal tissue and analyzed by western blot **(A,B)** and qPCR **(C,D)**, respectively. Protein from cells was also analyzed by immunoblot **(E,F)**. Gene and protein expression data are mean ± SEM (n = 4–6/group). Protein expression was normalized to loading via Ponceau stain (PS). Protein was analyzed via AlphaView software and one representative blot is shown. Different letters indicate a significant difference at $P < 0.05$ when the interaction effect is significant. CD36, the cluster of differentiation 36; FABP, fatty acid-binding protein; HS, heat stress; TN, thermoneutral; JF, jungle fowl; ACRB, Athens Canadian Random Bred; 95RAN, 1995 random bred; MRB, modern random bred.

may emphasize the importance of carbohydrate transporters in the fast-growing chickens providing them with greater capacity to absorb sugars. Conversely, GLUT2 protein expression was shown to be higher in JF compared to ACRB and MRB under TN conditions. GLUT2 significantly decreased in JF under HS conditions while significantly increased in ACRB. The significant increase in protein levels of GLUT2 seen in ACRB and the lack of effect on more modern populations compared to the heat-tolerant JF may be a product of their propensity for better growth performance and also contribute to their intolerance of HS conditions. The heat-stressed MRB chickens also showed a decrease in gene expression of some GLUTs, such as *GLUT10* and *GLUT12* as compared to their TN counterparts, being in line with previous studies reporting a decrease of the jejunum *GLUT2* (Al-zghoul et al., 2019) and ileum *GLUT1* (Habashy et al., 2017b) by applying a chronic HS of 35°C for 7 and 12 days, respectively. These results suggest that the uptake of glucose and galactose was compromised in heat-stressed chickens which may explain the reduction of BW at day 54 in heat-stressed MRB chickens as compared to their TN counterparts. However, the reduction of BW at day 54 was not associated with a decreased VH:CD ratio previously observed in heat-stressed chickens (Song et al., 2017) and considered as a measure of the absorptive capacity of the gut. The lack of effect on intestinal histology may be a sign of recovery or adaptation after 26 days of cyclic HS. On the other hand, heat-stressed JF showed an increase of *GLUT1*, *GLUT5*, *GLUT10*, and *GLUT11* gene expression as compared to their TN counterparts, and greater mRNA levels of *GLUT1*, *GLUT8*, *GLUT9*, *GLUT10*, and *SGLT1* compared to the rest of the lines. It is difficult to make an across study comparative conclusion of how differences in growth capacity among chicken populations affect their response to HS due to the lack of research comparing the ancestor JF with other lines characterized by higher growth rate. However, the results obtained in the current study suggest either JF to be more resistant to HS, or it is nutrient transport machinery to present a more robust response to HS challenging conditions. Granted, this conclusion only bears true if the nutrient transporter machinery responses seen in JF in this study correspond to increased absorption, which was not measured, and if that corresponding absorption in the gut aids in JF resistance to HS.

In 2013, Pearce et al. showed an increased incidence of hyperglycemia and an increase in the expression of ileal GLUTs in heat-stressed pigs. Interestingly, the IPEC-J2 expression of GLUT1 and GLUT3 protein followed the same trends as in chickens (at least the ACRB and 95RAN for GLUT1 and ACRB for GLUT2), indicating that HS had a direct effect on jejunal carbohydrate transporters. Given that the functionality of these transporters is often dependent on their cellular location, further investigation is needed to determine the cellular location and provide a potential explanation for possible trends in expression.

Several amino acid transport systems are expressed on both the apical brush border membrane and basolateral side of the small intestine epithelium (Dave et al., 2004) to bring the amino acids from the gut lumen into the enterocytes, and from inside of the enterocyte to the vascular supply or vice versa, respectively. Results of the current study suggest amino acid transporters (AATs) to be less responsive to HS when compared to carbohydrate transporters. When comparing significant differences seen among amino acid transporters only, the effect on gene expression seems to be more dependent on line than environment for populations other than the MRB. Indeed, as compared to other populations under TN conditions, ACRB birds exhibited greater gene expression of *LAT1* and *SLC38A2*, which are present on the basolateral membrane, and *SLC6A19*, *EAAT3*, and *PEPT2* which encode brush border AATs. However, JF expressed greater *SLC7A6,* present on the basolateral membrane, and *PEPT1*, a brush border AAT, as compared to the rest of TN populations. *PEPT1* and *EAAT3* have been previously reported to be higher in the small intestine of birds selected for low compared to high juvenile BW (Mott et al., 2008). Similarly, *SLC7A1*, *SLC7A4*, *SLC3A1*, *SLC7A6*, and *SLC7A7* gene expression was greater in the intestine of a Chinese slow-growing bird (Wenshi Yellow Feathered Chick; WYFC) as compared to a commercial fast-growing broiler (White Recessive Rock Chick; WRRC) (Zeng et al., 2011). Moreover, a recent study conducted by Miska and Fetterer (2019) showed *SLC1A4*, *SLC6A14*, and *SLC6A19* to be greater in the intestine of ACRB as compared to ROSS broilers. The reason behind this increase is not fully elucidated, but it is possible that AATs of slow-growing lines are less efficient than those of fast-growing lines, such that a higher number of AATs is required to transport a smaller amount of nutrients. As for HS, although its negative effects on nutrient digestibility and absorption are well-described, only a small number of studies have reported HS effects on AATs. In a recent study, Al-zghoul et al. (2019) reported *SLC7A7* to be upregulated and *SLC7A1* to be downregulated by HS; however, this is in contrast with other studies showing no effect on these AATs (Sun et al., 2015; Yi et al., 2016; Song et al., 2017). This discrepancy may be attributed to the heterogeneity of experimental conditions, such as lines, feed, age, duration, and severity of HS. Results of the current study showed differing responses to HS among lines. Although HS decreased *SLC6A14* in MRB and *EAAT3* in ACRB, it increased *SLC6A14* and *SLC38A2* in JF and *SLC7A9*, *SLC6A19*, and *EAAT3* in 95RAN. The jejunum expression of the *EAAT3* gene was also enhanced in ROSS broilers exposed to 35–39°C for 1–5 days (Santos et al., 2019). This increase of *EAAT3* may indicate a greater need for aspartate and glutamate uptake acting as the primary fuel for enterocytes, to maintain intestinal permeability and enterocyte number negatively affected by heat-induced tissue damage (Goel et al., 2021). Another interesting finding in this study was the lack of HS effect on the expression of AAT in MRB. Indeed, a decrease was seen only for *SLC6A14* for MRB while all others remained unchanged. This may suggest that MRB may retain their amino acid absorption regardless of environmental stressors, such as HS, due to the effects of high-performance selection the MRB has gone through over time. However, more research is needed to confirm any direct effects of selection on these AAT and the role HS plays in the dynamics of genotype and environment interactions.

Protein expression of AATs showed a main effect of temperature on decreasing SLC38A3, which seems to be driven

by the effects in JF. Similarly, IPEC-J2 cells subjected to HS conditions also showed significantly decreased SLC38A3 protein. It is possible that an IPEC-J2 model for HS effects on AAT in the gut better represents ancestral JF than more modern populations. However, further analysis is needed to fully characterize IPEC-J2 as a model for JF under HS. These results also point to the potential need for specific *in vitro* cell models per population.

Heat stress did not affect protein expression of fatty acid translocator CD36 in ACRB, 95RAN to MRB; however, JF showed a decrease under HS compared to TN conditions. This lack of response from more modern populations coupled with the JF decreased expression of CD36 under HS may indicate a possible role for decreased fatty acid absorption in resistance to HS and that a lack of response in this translocation may be a result of selection for higher growth performance.

The levels of mRNA for both *FABP2* and *CD36* showed a similar pattern of expression whereby there were no differences among the four populations under TN conditions. HS increased the expression of both FATs in JF compared to the rest of the populations. However, previous studies reported that *FABP*, which mediates the uptake of long-chain fatty acids into enterocytes, was downregulated irrespective of exposure time and intensity of stress. Indeed, *FABP* gene expression was reduced in male Arbor Acres broilers exposed to 32°C from 35 to 42 days of age (Sun et al., 2015), and male Cobb500 subjected to 35°C from 14 to 26 days of age (Habashy et al., 2017b). Similar results were reported with *CD36*, where jejunal expression was shown to be reduced in heat-stressed fast-growing broilers (Sun et al., 2015). The inconsistency between the gene expression results for FATs obtained in the current study and previous ones may be attributed to differences in the temperature, duration of HS, and specific strains of birds used. Moreover, *CD36* gene expression did not show the same pattern as protein expression in the current study. A similar disparity was seen in several other transporters measured and is not surprising, as the correlation between protein concentrations and the corresponding mRNA levels has been previously investigated and established to be 20–40% (Pascal et al., 2008; Zapalska-Sozoniuk et al., 2019) due to several mechanisms such as the rate of mRNA degradation/turnover and efficiency of translation.

CONCLUSION

This study is the first to describe differences in the expression of genes, and some of the encoded proteins, that play a role in jejunal nutrient transport between three broiler populations characterized by different growth (slow, moderate, and rapid) rates and in their ancestor wild JF birds. The obtained results seemingly show that chronic HS alters the jejunal carbohydrate, rather than fatty acid and amino acid, transporters and that differences in these transporters between the populations may be a result of, or mechanism behind, the phenotypic differences that exist. Understanding the molecular mechanisms behind these differences and their potential relationship to selection is of great interest; however, further in-depth investigation is needed and warranted based on these findings.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Arkansas.

AUTHOR CONTRIBUTIONS

SD conceived and designed the study. TT, EG, SO, NBA, and SD conducted the *in vivo* experiments. NA and AR performed the molecular analyses and analyzed the data. LB performed the IF images. NA and AR wrote the first draft. SD edited and corrected the paper with a critical review by all authors.

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Protected Organic Acid and Essential Oils for Broilers Raised Under Field Conditions: Intestinal Health Biomarkers and Cecal Microbiota

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The objective of the present study was to evaluate the effect of protected organic acids (OA) and essential oils (EO) [P(OA + EO)] on the intestinal health of broiler chickens raised under field conditions. The study was conducted on four commercial farms. Each farm consisted of four barns, two barns under a control diet and two tested barns supplemented with P(OA + EO), totaling 16 barns [8 control and 8 under P(OA + EO)]. The control group was supplemented with antibiotic growth promoters [AGP; Bacitracin Methylene Disalicylate (50 g/ton) during starter, grower and finisher 1, and flavomycin (2 g/ton) during finisher 2]. The tested group was supplemented with 636, 636, 454, and 454 g/ton of P(OA + EO) during starter, grower, finisher 1 and 2, respectively. Eighty birds were necropsied (40/treatment; 20/farm; and 5/barn) to collect blood, jejunal tissue, and cecal contents. The data were submitted to analysis of variance (ANOVA) ($P < 0.05$) or Kruskal-Wallis' test and the frequency of antimicrobial resistant (AMR) genes was analyzed by Chi-Square test ($P < 0.05$). It was observed that the supplementation of P(OA + EO) reduced ($P < 0.05$) the histopathology scores, such as the infiltration of inflammatory cells in the epithelium and lamina propria and tended ($P = 0.09$) to reduce the serum concentration of calprotectin (CALP). The supplementation of P(OA + EO) reduced the serum concentration of IL-12 ($P = 0.0001$), IL-16 ($P = 0.001$), and Pentraxin-3 ($P = 0.04$). Additionally, P(OA + EO) maintained a cecal microbiota similar to birds receiving AGP. The substitution of AGP by P(OA + EO) reduced ($P < 0.05$) the frequency of four AMR genes, related to gentamicin (three genes), and aminoglycoside (one gene). Overall, the inclusion of P(OA + EO), and removal of AGP, in the diets of commercially raised broiler chickens beneficially changed the phenotype of the jejunum as shown by the lowered ISI scores which characterizes an improved intestinal health. Furthermore, P(OA + EO) significantly reduced the serum concentration of several inflammatory biomarkers, while maintaining the diversity and composition of the cecal microbiota similar to AGP fed chickens and reducing the prevalence of AMR genes.

Keywords: antimicrobial resistance genes, biomarkers, broiler, essential oils, intestinal inflammation, organic acids

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INTRODUCTION

Optimal intestinal health in animal production is of paramount importance for an animal to achieve its genetic potential and is strongly correlated with overall health and welfare. However, several physiological functions must be studied to define a "healthy intestine" including nutrient digestion and absorption, metabolism and energy generation, a stable microbiome, mucus layer development, barrier function, and mucosal immune responses (Swaggerty et al., 2021)[1]. Organic acids (OA) and essential oils (EO) represent alternatives to antibiotic growth promoters (AGP) used in animal production because they can improve growth performance by different mechanisms. These compounds may have bacteriostatic and bactericidal properties (Ricke, 2003), or direct effects on the host by improving the development of the gastrointestinal tract (GIT) and modulating the immune system. The immunomodulatory effects of OA and EO includes induction of Toll-like receptors, and induction of proliferation and maturation of T-Helper cells (Th-1 and Th-2) to maintain a balance between cellular and humoral immune response (Hashemi and Davoodi, 2012) which may lead to improved growth performance and other health related paraments (Chowdhury et al., 2009; Islam, 2012).

Organic acids and EO are volatile and can evaporate quickly during feed processing or being absorbed in the stomach and proximal small intestine (Michiels et al., 2008). Microencapsulation is a feasible strategy to be used to, among other advantages, improve stability and protect feed additives during processing, reduce the effective dose, delay the absorption of these molecules and to be slowly released throughout the intestine of the animals (Choi et al., 2020). For instance, Choi et al. (2020) showed that 15.5% of microencapsulated thymol was released in the stomach, 41.85% was released in the mid-jejunum section, and 2.21% was recovered in the feces, showing that lipid matrix microparticles can maintain the stability of thymol and allowed a progressive release of thymol in the intestine of pigs. Therefore, microencapsulated OA and EO may have more influence on the distal portions of the GIT when compared to non-encapsulated molecules which may be essential to prevent pathogen proliferation in the lower parts of the intestine, where higher bacterial concentration is found.

The supplementation of OA and EA provide synergistic effects to improve growth performance and gut health of animals (Stefanello et al., 2019; Yang et al., 2019). Yang et al. (2019) demonstrated that a mixture of sorbic acid, fumaric acid, and thymol modulated the microbiota, increased sucrase and maltase activities in the jejunal mucosa, and increased the expression of tight junction protein genes which reflected in a better feed efficiency in broiler chickens. Stefanello et al. (2019) showed that a combination of microencapsulated fumaric, citric, malic, and sorbic acids plus thymol, vanillin, and eugenol increased the expression of tight junction protein genes, improved nutrient digestibility, and intestinal health of broilers, showing that this blend is beneficial for AGP free programs. Furthermore, biomarkers of intestinal health that should be reliable, minimally invasive, and easy to process are constantly being searched (Dal Pont et al., 2021). Among these, calprotectin (CALP), a protein released by heterophils and macrophages during inflammation, has been shown to be a promising biomarker detected in serum or excreta of chickens (Dal Pont et al., 2021). Associated with other approaches, CALP measurement may add essential information when discussing intestinal health of poultry flocks, especially when field samples are analyzed. Therefore, the hypothesis of this study was that the supplementation of protected OA and EO—P(OA + EO)—would improve the intestinal health, modulate the cecal microbiota, and reduce the prevalence of antimicrobial resistant (AMR) genes in the microbiota of broiler chickens. The objective of the present study was to evaluate the dietary supplementation of P(OA + EO) on the jejunum histopathology, serum cytokines and CALP concentrations, microbiota diversity and composition, and frequency of AMR genes in the cecal microbiota of broiler chickens raised under field conditions.

MATERIALS AND METHODS

Birds, Housing, and Treatments

The study was conducted on four commercial farms. Each farm consisted of four barns, two barns under a control diet and two tested barns supplemented with P(OA + EO), totaling 16 barns [8 control and 8 under P(OA + EO)] supplementation. The control group was supplemented with antibiotic growth promoter [AGP; bacitracin methylene disalicylate (BMD; 50 g/ton) during starter, grower and finisher 1, and flavomycin (2 g/ton) during finisher 2]. The tested group was supplemented with 636, 636, 454, and 454 g/ton of P(OA + EO) during starter, grower, finisher 1 and 2, respectively. Both groups were supplemented with narasin (63 and 72 g/ton, for grower and finisher 1, respectively), vaccinated against coccidiosis (ADVENT®) at the hatchery, and followed by a withdrawal period of AGP and narasin at the end of the production cycle. The P(OA + EO) consists of fumaric, citric, malic, and sorbic acids plus thymol, vanillin, and eugenol microencapsulated in a matrix of triglycerides from hydrogenated vegetable oil (Jefo Nutrition Inc., Saint-Hyacinthe, QC, Canada).

Samples Collected

A total of 80 broiler chickens were euthanized by cervical dislocation, and necropsied (40 birds/treatment group, being 20 birds/farm and 5 birds/barn) to collect a section of the jejunum for *I See Inside* (ISI) analysis, blood to determine the serum concentration of cytokines array and CALP, and cecal content to analyze the diversity and composition of the microbiota and the frequency of AMR genes. The sampling was performed on farms 1 (20 birds) and 2 (20 birds) at 28- and 25-days old broiler chickens, and on farms 3 (20 birds) and 4 (20 birds) at 27- and 25-days old broiler chickens, respectively. Since the birds were not sampled on the exact same age because of the date differences in the beginning of the trial, each farm was considered as a block during the statistical analyses.

[1]Swaggerty, C. L., Bortoluzzi, C., Eyng, C., Lee, A., Dal Pont, G. C., and Kogut, M. H. (2021). Development of alternatives to feed antibiotics: interactions at the gut level and their impact on host immunity. *Adv. Exp. Med. Biol.*

I See Inside—Histopathological Analysis

A section of jejunum (∼2 cm) was collected from each bird, rinsed with phosphate buffer solution (PBS), and immersed into formalin 10% for fixation. The samples were then embedded in paraffin following common histological routine and stained with hematoxylin and eosin.

For microscopic evaluation, the *I See Inside* (ISI) methodology was used to determine histologic alterations in the jejunum (Belote et al., 2019), and 20 intact villi/birds were evaluated. Briefly, the ISI methodology is based on a numerical score of alteration. An impact factor (IF) is defined for each alteration in the microscopic analysis, according to the reduction of the organ functionality. The IF ranges from 1 to 3, with three being the worst impacting organ function. The parameters evaluated by the ISI method in the intestine are lamina propria thickness, epithelial thickness, proliferation of enterocytes, inflammatory cell infiltration on the epithelium, inflammatory cell infiltration in the lamina propria, increase of goblet cell, congestion and presence of *Eimeria* oocysts.

In addition, the score of 0–3 is based on the intensity of the observed lesion: score 0 (absence of lesion), score 1 (alteration of up to 25% of the area), score 2 (alteration of 25–50% of the area), and score 3 (alteration of more than 50% of the area). To obtain the final value of the ISI index, the IF of each alteration is multiplied by the respective score number, according to the formula ISI = Σ(IF × S), where IF = impact factor and S = Score.

Serum Calprotectin Concentration

The serum concentration of CALP was determined by an ELISA commercial kit (MBS1601938) following the manufacturer recommendations (MyBiosource Inc., San Diego, CA, United States). Briefly, 40 μL of serum was incubated with anti-CALP antibody and streptavidin-HRP for 60 min at 37°C. The samples were then washed with buffer for five times and incubated in dark room with 50 μL of "solution B" for 10 min at 37°C. Lastly, 50 μL of Stop Solution was added, and the optical density was determined using a microplate reader set to 450 nM. The concentration of each sample was determined based on the standard curve.

Chicken-Specific Cytokine Array Analysis

The cytokines serum concentration was measured by a chicken specific cytokine array (Quantibody® Chicken Cytokine Array 1) following the manufacturer recommendations (RayBiotech, Norcross, GA, United States). The concentration of IFN-gamma, IL-6, IL-10, IL-12, IL-16, pentraxin 3 (PTX3), and chemokine ligand 5 (CCL5) was determined. This sandwich ELISA-based quantitative array platform allows the determination of the concentration of multiple cytokines simultaneously. Briefly, a capture antibody is bound to a glass array surface. After incubation with the sample, the target cytokine is bound on the solid surface. A second biotin-labeled detection antibody is then added, which can recognize a different epitope on the target cytokine. The cytokine-antibody-biotin complex can be visualized by the addition of streptavidin-conjugated Cy3 equivalent dye, using a laser scanner (InnoScan 710 Microarray Scanner; Innopsys Inc., Chicago, IL, United States).

Cecal Microbiota Analysis—16S rRNA Sequencing and Bioinformatics

The caeca from each sampled bird were collected and placed into a Ziploc bag, immediately put in ice, and taken to the lab. The cecal content was gently squeezed into a 10 mL cryotube and frozen at −80°C for further analysis of the cecal microbiota.

The sample preparation was done in accordance with (Bortoluzzi et al., 2018). An amount of 200 μg of the content was used for DNA isolation following the manufacturer recommendations (PowerViral Environmental RNA/DNA Isolation Kit—Mo Bio; Qiagen, Carlsbad, CA, United States). DNA was then quantified using the Qubit™ 4 Fluorometer (Thermo Fisher Scientific). V3–V4 region of the 16S rRNA gene was amplified using the primer set S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 (Klindworth et al., 2013). Polymerase chain reaction (PCR) products were purified with a magnetic bead-based clean-up system (Agencourt AMPure XP; Beckman Coulter, Brea, CA, United States). Indexed libraries were prepared by limited-cycle PCR using Nextera technology and further cleaned up with AMPure XP magnetic beads (Beckman Coulter). Libraries were pooled at equimolar concentrations (4 nM), denatured, diluted, and loaded onto the MiSeq flow cell. Sequencing on Illumina MiSeq platform was performed by using a 2 × 250 bp paired end protocol, according to the manufacturer's instructions (Illumina, San Diego, CA, United States).

Paired-end sequenced reads of samples were analyzed combining PANDAseq2 and the wrapper package Quantitative Insights Into Microbial Ecology (QIIME) v1 (Caporaso et al., 2010; Masella et al., 2012). High-quality reads were binned into operational taxonomic units (OTUs) at a 97% similarity threshold using UCLUST (Edgar, 2010). For bacterial taxonomy assignment, Greengenes database from May 2013 release[2] was used. The chimera filtering was performed discarding singleton OTUs. Subsampling to the number of sequences in the sample with the least coverage was not performed to correct for different sequencing depth of each sample; however, samples that had less than 10,000 reads after Illumina MiSeq sequencing were re-sequenced. The bacterial abundance data were imported into R (version 3.6.2) on Rstudio v1.1.463 where all statistical analysis were performed using R package *phyloseq* (McMurdie and Holmes, 2013; Callahan et al., 2016). Taxa that were present in less than one sample were excluded from the present analysis using the *decontam* R package (Davis et al., 2018).

Frequency of Antimicrobial Resistance Genes

All PCR analysis for characterizing AMR genes was carried out using the following protocol with minor modifications for annealing temperatures of the primers. Briefly, DNA samples were amplified using PCR in multiplex panels to amplify a series of the common AMR genes (Johnson et al., 2008) harbored by Enterobacteriaceae species.

[2]https://www.ncbi.nlm.nih.gov/sra/PRJNA743867

All PCR reactions were prepared in a total volume of 25 μL for each sample. Components for a PCR reaction consisted of 2.5 μL of 10× PCR buffer, 0.4 μL of 10 mM $MgCL_2$, 1.25 μL of (0.2 M) dNTP mixture, 2 μL of TAQ (Dream TAQ, Thermo Fisher Scientific), 1.4 μL of primer pool, 2 μL of DNA, and 15.45 μL of sterile molecular grade water. Positive control strains were included in the analysis for the appropriate genes of interest from previously characterized strains in our lab collection and negative controls included sterile water in place of DNA. Amplification parameters of the thermocycler (Mastercycler X50, Eppendorf, Hamburg, Germany) included an initial denaturing step at 95°C for 10 min, followed by 30 rounds of [94°C for 30 s (various annealing temperatures), for 30 s, 68°C for 3 min], with a final extension of 72°C for 10 min and a final hold of 4°C.

Polymerase chain reaction products generated were subjected to electrophoresis which was performed in a 2% agarose gel (Agarose LE, Lonza, Alpharetta, GA, United States) running at 100 V for 90 min. The gel was stained with ethidium bromide (0.25%) solution for 20 min, visualized using an imager (UVP BioDock-It^2 Imager, Analytik Jena, Jena, Germany) and analyzed for the presence of PCR products of the appropriate size when compared with control strains for the targeted gene.

Statistical Analysis

The ISI data were analyzed by the non-parametric Kruskal-Wallis's test using SAS 9.4 ($P < 0.05$). The serum concentration of CALP and cytokines was checked for normality (Shapiro-Wilk's test) and homogeneity of variances (Bartlett's test), and then submitted to analysis of variance (ANOVA) ($P < 0.05$) using SAS 9.4. Each farm was considered as a block during the analysis. The differences in alpha diversity were evaluated, based on the data distribution of metrics, using ANOVA normally distributed data or Wilcoxon-Mann-Whitney with Holm-Bonferroni correction method for non-normally distributed data. To compare microbial composition between samples, beta-diversity was measured by calculating the weighted or unweighted UniFrac distance matrix. Principal coordinates analysis (PCoA) was applied on the distance matrices to generate bi-dimensional plots on R. The frequency of the main bacterial families observed was submitted to a non-parametric one-way ANOVA (Kruskal-Wallis test). A $P < 0.05$ after false discovery rate (FDR) correction was considered as statistically significant. The prevalence of AMR genes was submitted to a Chi-Square test ($P < 0.05$).

RESULTS

I See Inside—Histopathological Analysis

The results of the ISI analysis are shown in **Figure 1**. It was observed that the supplementation of P(OA + EO) significantly reduced most of the parameters evaluated (**Figure 1A**) such as lamina propria thickness ($P = 0.002$), epithelial thickness ($P = 0.006$), proliferation of immature enterocytes ($P = 0.040$), inflammatory cell infiltration in the epithelium ($P = 0.001$) and in the lamina propria ($P = 0.001$) and the increase of goblet cells ($P = 0.005$). Lastly, the supplementation of P(OA + EO) reduced the total ISI score ($P = 0.001$; **Figure 1B**), compared to the AGP fed birds.

Serum Calprotectin and Chicken-Specific Cytokine Array Analyses

The results of the serum concentration of CALP and the cytokine array are shown in **Table 1**. It was observed that the supplementation of P(OA + EO) tended to reduce ($P = 0.090$) the serum concentration of CALP compared to AGP fed chickens. Moreover, the supplementation of P(OA + EO) reduced the serum concentration of IL-12 ($P = 0.001$), IL-16 ($P = 0.001$), and PTX3 ($P = 0.010$), and tended to reduce IL-10 ($P = 0.070$). Even though not statistically different, it is worth it to note that the supplementation of P(OA + OA) numerically decreased the serum concentration of IFN-gamma.

Cecal Microbiota: Alpha and Beta Diversity

The results of the alpha diversity indices are shown in **Figure 2**. The analysis reported significant differences between groups when comparing the number of Observed Species ($P = 0.048$) and Chao1 ($P = 0.048$) indices. It shows that the bacteria present are similar in the phylogenetic relationships of taxa within each microbiota, in the evenness of taxa distribution (no specific dominance of a bacterial group) across treatments but are slightly different in the richness (total number of a specific bacterial group) of taxa.

Regarding the beta diversity (**Figure 3**), the microbiota of AGP and P(OA + EO) fed birds shared a common dispersion of data, therefore, their variances are homogeneous. To summarize the differences between ecological communities, we applied the Unique Fraction method (UniFrac) to define if qualitative differences (unweighted UniFrac) or quantitative differences (weighted UniFrac) were present among groups based on phylogenetic relationships (UniFrac considers phylogenetic relationships among taxa as central information). The results from this analysis show that both treatments led to a separation of groups in distinct clusters only when applying the unweighted UniFrac method. The permutational multivariate analysis of variance (PERMANOVA) applied to the distance matrix of unweighted UniFrac showed a significant difference of the centroids of the clusters of samples ($P = 0.019$). These results indicate that the differences rely on bacteria presence or absence and not on their relative abundance.

Cecal Microbiota: Composition

The analysis of the composition of the cecal microbiota revealed that the most abundant phylum observed was Firmicutes, followed by Bacteroidetes, Proteobacteria, and Verrucomicrobia, without differences between the treatment groups ($P > 0.05$). At a downstream taxonomic level (**Figure 4A**), it was observed that the microbiota was dominated by members of the family *Ruminococcaceae* (48.8%), *Lachnospiraceae* (20.9%), *Bacteroidaceae* (7.4%), and *Lactobacillaceae* (5.5%), without difference between the treatment groups ($P < 0.05$). The supplementation of P(OA + EO) increased the abundance of

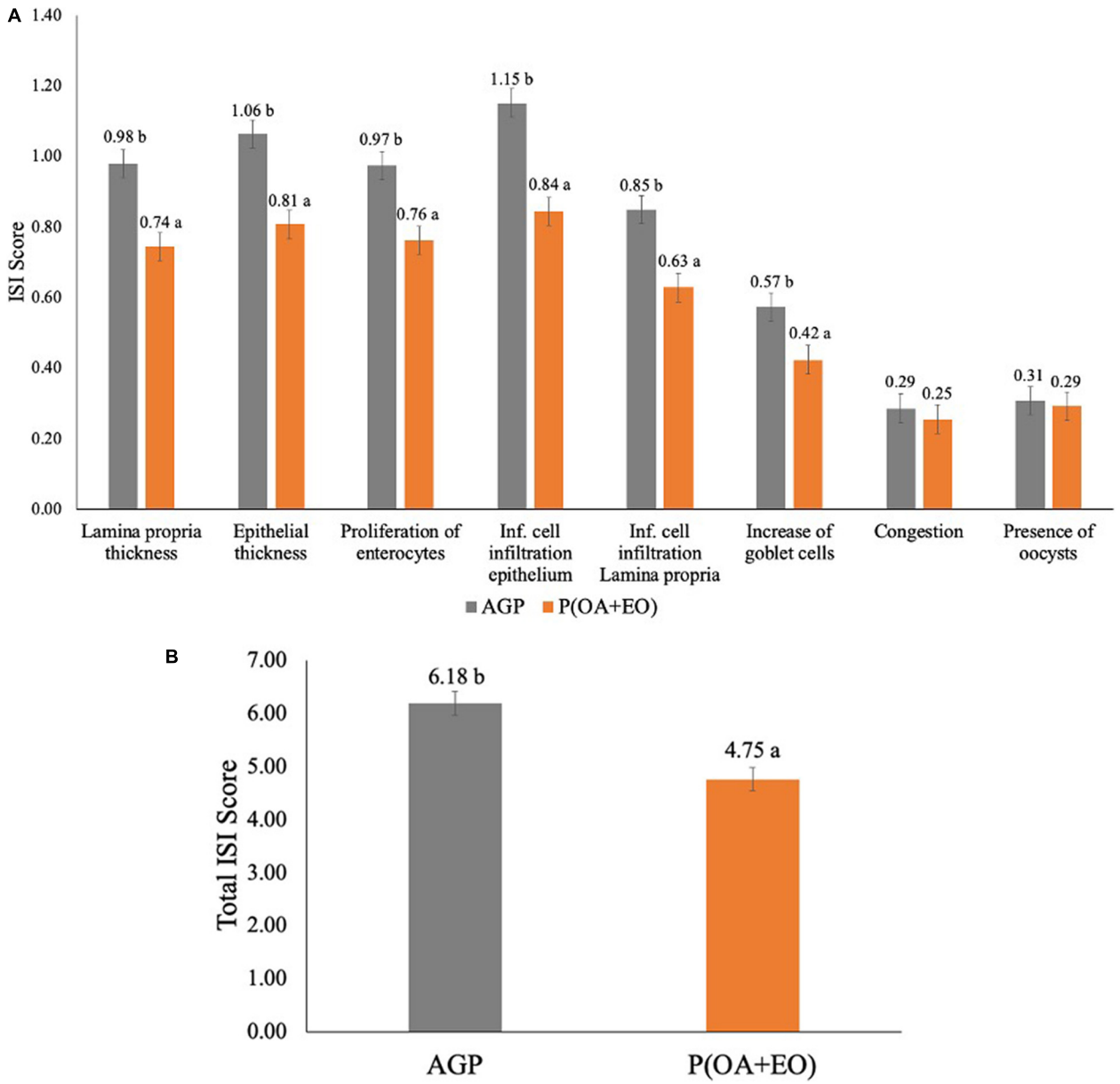

FIGURE 1 | Histopathological analysis by score **(A)** and total **(B)** of the jejunum of broiler chickens supplemented with antibiotic growth promoter (AGP) or protected organic acids (OA) and essential oils (EO) [P(OA + EO)] raised under field conditions. Values are means of 40 replicates. Inf, inflammatory [± standard error of mean (SEM); $^{ab}P < 0.05$].

TABLE 1 | Serum concentration of calprotectin (ng/ml) and cytokines (pg/ml) of broiler chickens supplemented with antibiotic growth promoter (AGP) or protected organic acids (OA) and essential oils (EO) [P(OA + EO)] raised under field conditions.

Treatment	CALP	IFN-g	IL-6	IL-10	IL-12	IL-16	PTX3	CCL5
AGP	30.1	151.6	805.0	160.9	15.3^{b}	57.6^{b}	87.3^{b}	127.4
P(OA + EO)	23.8	118.9	867.6	112.5	5.0^{a}	22.7^{a}	60.5^{a}	122.7
SEM	2.68	19.8	138.0	20.4	2.08	6.9	8.56	13.2
P-value	0.09	0.30	0.81	0.07	0.0007	0.0002	0.01	0.67

Values are means of 40 replicates.
CALP, calprotectin; PTX3, pentraxin 3; CCL5, chemokine ligand 5; SEM, standard error of mean. $^{ab}P < 0.05$.

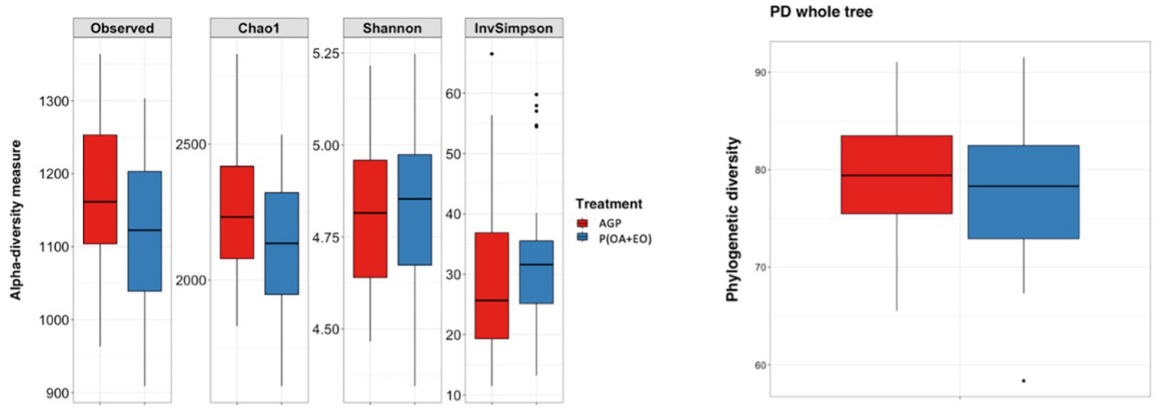

FIGURE 2 | Alpha diversity indices of the cecal microbiota of broiler chickens supplemented with antibiotic growth promoter (AGP) or protected organic acids (OA) and essential oils (EO) [P(OA + EO)] raised under field conditions. Values are means of 40 replicates. PD, phylogenetic diversity (Observed species: $P = 0.048$; Chao1: $P = 0.048$; Shannon, InvSimpson, and PD whole tree: $P > 0.05$).

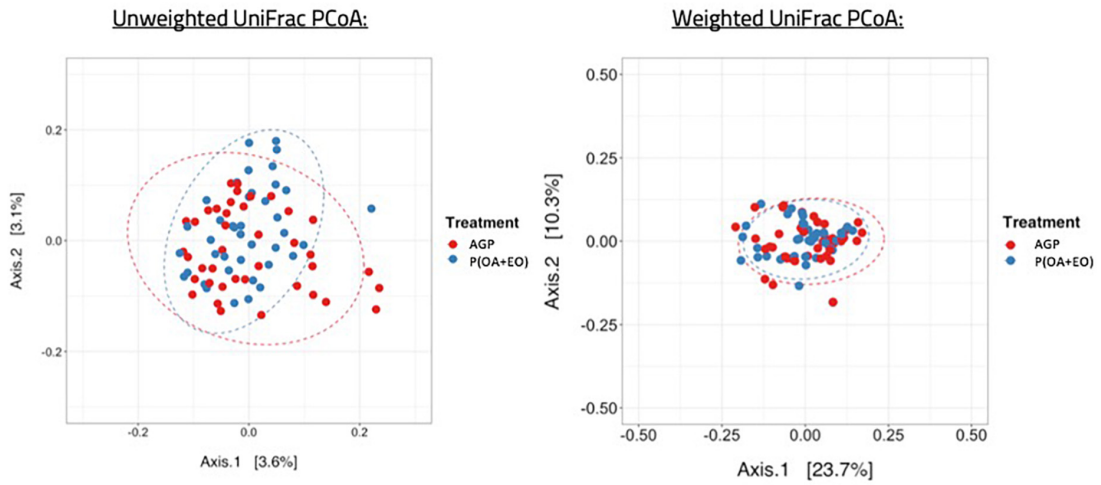

FIGURE 3 | Beta diversity of the cecal microbiota of broiler chickens supplemented with antibiotic growth promoter (AGP) or protected organic acids (OA) and essential oils (EO) [P(OA + EO)] raised under field conditions. Values are means of 40 replicates (Unweighted UniFrac: $P = 0.019$; Weighted UniFrac: $P > 0.05$).

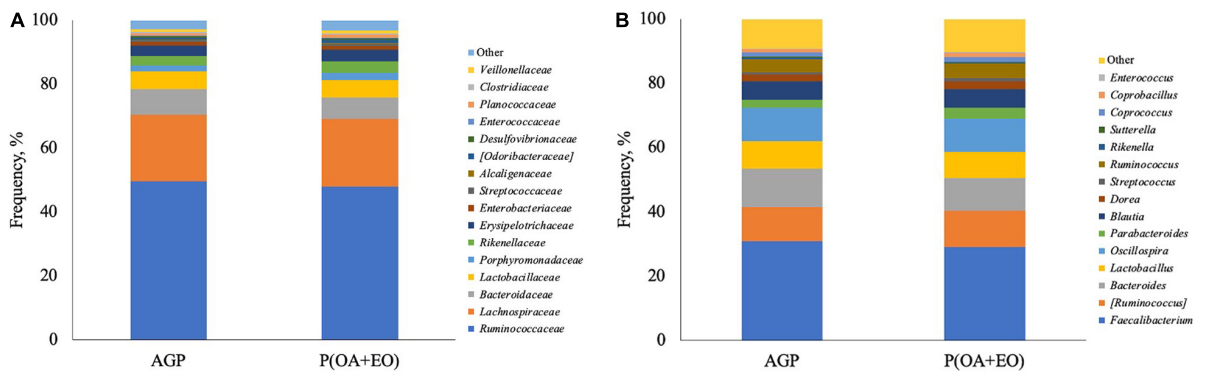

FIGURE 4 | Composition of the cecal microbiota at family **(A)** and genus **(B)** level of broiler chickens supplemented with antibiotic growth promoter (AGP) or protected organic acids (OA) and essential oils (EO) [P(OA + EO)] raised under field conditions. Values are means of 40 replicates.

the family *Rikenellaceae* (3.02 vs. 3.61%; $P = 0.05$) and tended to increase *Porphyromonadaceae* (1.66 vs. 2.34%; $P = 0.08$) and *Anaeroplasmataceae* ($P = 0.07$).

At the genus level (**Figure 4B**), it was observed that the most abundant genera were *Faecalibacterium* (30.0%), *Ruminococcus* (11.0%), *Bacteroides* (11.0%), *Oscillospira* (10.5%), and *Lactobacillus* (8.3%). It was observed that P(OA + EO) supplementation increased the frequency of *Parabacteroides* (2.5 vs. 3.5%; $P = 0.040$), and *Coprobacillus* (1.1 vs. 1.4%; $P = 0.040$) and tended to increase the frequency of the genus *Dorea* (2.1 vs. 2.5%; $P = 0.070$) and decrease *Bacteroides* (12 vs. 10.1%; $P = 0.100$).

Prevalence of Antimicrobial Resistance Genes

The prevalence of AMR genes is shown in **Table 2**. It was observed that the removal of AGP and inclusion of P(OA + EO) reduced ($P < 0.05$) the frequency of four AMR genes, related to gentamicin (three genes), and aminoglycoside (one gene), and tended to reduce ($P = 0.09$) the prevalence of one tetracycline resistance gene.

DISCUSSION

The purpose of the present study was to evaluate the effect of P(OA + EO) to replace AGP in the diets of broiler chickens raised under field conditions. Overall, it was observed that the supplementation of P(OA + EO) improved intestinal health as demonstrated by the histopathological analyses and the additional biomarkers evaluated herein. Furthermore, the supplementation of P(OA + EO) prevented shifts in the microbiota diversity and composition due to the removal of AGP and had positive immunomodulatory effects on the host. Even though the growth performance data was not analyzed, we demonstrated, by using different methodologies, some of the mechanisms by which P(OA + EO) improves the intestinal health of broiler chickens.

It has been widely discussed in the last years that the non-antibiotic anti-inflammatory effects of AGP may explain, at least in part, the beneficial results on the growth performance of broilers (Niewold, 2007). Broom (2017) cited another mechanism of action of AGP that may be responsible for the improvement in growth performance of chickens. The author reported that AGP (sub-inhibitory concentrations) influences the dynamics of the intestinal microbiota which in turn reduces the release of pro-inflammatory molecules, reflecting a change in the immune system-microbiota interface (Broom, 2017). Therefore, one can assume that AGP and its alternatives, such as OA and EO, act directly on the host, or by modulating the intestinal microbiota and its metabolic functions, changing, for example, the production of short-chain fatty acids (SCFA), and antimicrobial peptides, that would exert influence on the immune system of the host. Metagenomics analysis of the intestinal microbiota would be essential to determine the changes of different signaling pathways.

The histopathological assessment used herein allows the study of inflammatory events that may damage the intestine (Kraieski et al., 2016; Belote et al., 2019). Sanches et al. (2020) showed that broiler chickens develop a microscopic basal enteritis, even in the absence of challenge, throughout their life, which is characterized by an increased inflammation of the epithelium and lamina propria, immature enterocytes proliferation, epithelium thickness, congestion and goblet cells proliferation. Additionally, Belote et al. (2019) demonstrated that the ISI histological analysis had a strong correlation with the growth performance, and that the higher the ISI score the worse the performance of broiler chickens. This methodology was also used in the present study as a tool to evaluate the degree of intestinal inflammation. The supplementation of P(OA + EO) reduced most of the parameters evaluated, and the total ISI score, indicating a lessened intestinal inflammatory response associated with its supplementation when compared to AGP supplemented chickens. Furthermore, the reduction of the serum concentration of CALP is another indicator of attenuated intestinal inflammation by P(OA + EO). Dal Pont et al. (2021) showed that broiler chickens with the highest CALP concentration in the blood at 14 days also had the highest total ISI score in the jejunum. At 28 days, the highest CALP concentration in the excreta was positively correlated to the highest ISI total score in the duodenum and ileum (Dal Pont et al., 2021).

Another unique methodology applied in the present study, which has very limited number of publications in chickens, is the chicken-specific cytokine array. With this analysis, we were able to measure the concentration of several cytokines and acute phase proteins (APP) in the serum of broiler chickens raised under field conditions. We demonstrated that the dietary inclusion of P(OA + EO) significantly reduced the serum concentration of IL-12, IL-16, and PTX3 and numerically reduced IFN-gamma. IL-12 is a pro-inflammatory cytokine that induces Th1-type immune response typically associated with the production of IFN-gamma (Degen et al., 2004), and, therefore, its elevated blood concentration may be linked to chronic inflammation. In such scenario, nutrients must be diverted from production parameters to support the immune response, while it can suppress feed intake and nutrient availability for growth and induce catabolism of host tissues (Broom and Kogut, 2018). IL-16 is described as a chemoattractant (Wigley and Kaiser, 2003) which also possess pro-inflammatory characteristics (Saleh and Al-Zghoul, 2019). In agreement to our study, Swaggerty et al. (2020) have demonstrated that heterophils isolated from chickens fed microencapsulated OA and EO showed increased degranulation and oxidative burst response compared to those isolated from chickens fed control diet, showing that they are able to modulate the immune system, and therefore, alter the susceptibility to disease.

Additionally, PTX3 is an indicator of early inflammation recently described in chickens and expressed by a variety of tissues (Burkhardt et al., 2019). It was demonstrated that PTX3 is stimulated by IFN-gamma and is up-regulated by a number of viral and bacterial diseases, such as infectious bursal disease, avian pathogenic *Escherichia coli* (APEC), and Marek's disease (Burkhardt et al., 2019). The authors concluded that PTX3 is

TABLE 2 | Prevalence of antimicrobial resistance genes in the cecal microbiota of broiler chickens supplemented with antibiotic growth promoter (AGP) or protected organic acids (OA) and essential oils (EO) [P(OA + EO)] raised under field conditions.

Treatment	aadA	aac3-VI	aac3-VI	tetB	aph(3)IA	pcoD	SuII	groEL	dfr17
AGP	47.5	10	22.5	25	47.5	42.5	87.5	42.5	55
P(OA + EO)	22.5	5.0	2.5	10.0	25.0	42.5	62.5	47.5	57.5
SEM	0.08	0.08	0.08	0.08	0.06	0.08	0.04	0.07	0.06
P-value	0.02	0.008	0.008	0.09	0.04	0.92	0.74	0.58	0.72

Values are the number of samples positive for the specific gene over the total number of samples (40).
aadA, aminoglycoside; aac3-VI, gentamicin; tetB, tetracycline; aph(3)IA, gentamicin; pcoD, copper; sull, sulfa; groEL, chaperone; drf17, trimethoprim; SEM, standard error of mean.

a potential biomarker to monitor the inflammatory status of poultry flocks. Even though IL-10 was not statistically different between both groups (P = 0.07), it is a regulatory cytokine that suppresses pro-inflammatory cytokines (He et al., 2011). In fact, it has been shown that IL-10 inhibits IFN-gamma synthesis by mitogen-activated lymphocytes (Rothwell et al., 2004). Therefore, the reduction of serum IL-10 concentration, may be due to a lessened inflammatory status, as observed by the reduction of IL-12, IL-16, and PTX3, which clearly shows that the supplementation of P(OA + EO) is beneficial in attenuating chronic inflammation faced by modern strains of broiler chickens.

We observed very slight changes on the makeup of the cecal microbiota following the removal of AGP, which shows that the supplementation of P(OA + EO) maintained the balance of the microbiota similar to AGP fed chickens. One cannot discard all the factors that influence the intestinal microbiota of chickens, including feed, environmental conditions, sex, age, among others (Kers et al., 2018), especially when field samples are analyzed. In terms of bacterial families, *Rikenellaceae* was the only one that was significantly increased when the birds were supplemented with P(OA + EO). *Rikenellaceae* and *Ruminococcaceae* increased in mice fed a high-fat diet, suggesting that the increase in the abundance of these bacteria occurs in obese animals (Kim et al., 2012). Moreover, in humans, this bacterial family has been found to be increased in healthy compared to non-alcoholic fatty liver disease patients (Jiang et al., 2015). On the other hand, *Parabacteroides* and *Coprobacillus* were increased by the P(OA + EO) supplementation. To better understand the core microbiota and its association with the growth performance of antibiotic-free commercial broiler chickens Johnson et al. (2018), using a large dataset, reported that *Parabacteroides* and *Coprobacillus* in the cecum were positively correlated with body weight of 21- to 28-days old broilers. The samples of the present study were collected from 25- to 28-days old birds, which falls within the former range, and shows beneficial effects of the P(OA + EO) supplementation for antibiotic-free chickens. According to a bacterial meta-analysis of the cecal microbiota of chickens, *Coprobacillus*, even though in a small abundance, has been found in most of the analyzed studies (Chica Cardenas et al., 2021).

It has been well-documented in the literature that the indiscriminate use of antimicrobials may accelerate the emergence of AMR genes in bacteria from poultry production, which may also lead to economic losses, derived from the expenditure on ineffective antimicrobial (Nhung et al., 2017). In the present study, we demonstrated that replacing AGP by P(OA + EO) reduced the prevalence of AMR genes in the cecal microbiota, while keeping a balanced microbiota composition and diversity. The possibility of reducing AMR genes by the use of P(OA + EO) must be further studied, but it can be seen as a potential tool to re-establish the antimicrobial sensitivity to improve the action of antibiotics when poultry flocks need to be treated against a disease.

CONCLUSION

Overall, the inclusion of P(OA + EO), and removal of AGP, in the diets of commercially raised broiler chickens beneficially changed the phenotype of the jejunum, as shown by the lowered ISI scores which characterizes an improved intestinal health. Additionally, P(OA + EO) significantly reduced the serum concentration of several inflammatory biomarkers, while keeping the diversity and composition of the cecal microbiota similar to AGP fed chickens. It can be concluded that P(OA + EO) improves the health of the intestinal mucosa by directly acting on the host and maintaining the balance of the microbiota and reducing the frequency of AMR genes, showing its potential to be widely used in field conditions. Further studies should look at the effects of these molecules on the metagenome of the intestinal microbiota.

DATA AVAILABILITY STATEMENT

The data presented in this study were deposited and made publicly available in the NCBI SRA under accession PRJNA743867 (https://www.ncbi.nlm.nih.gov/sra/PRJNA743867; release date: 2022-08-01).

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the samples of the present study were collected from a field study at a commercial farm. Therefore, an ethical review was not necessary. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

CB: conceptualization, sample collection, laboratory analyses, and writing and editing. LL, ES, and MK: conceptualization, data analysis and interpretation, and reviewing and editing. JO and DD: reviewing and editing. CE: samples collection, and reviewing and editing. NB: laboratory analyses, and reviewing and editing. All authors contributed to the article and approved the submitted version.

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Effects of Heat Stress on Production Performance, Redox Status, Intestinal Morphology and Barrier-Related Gene Expression, Cecal Microbiome, and Metabolome in Indigenous Broiler Chickens

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This study was done to evaluate the effects of heat stress (HS) on production performance, redox status, small intestinal barrier-related parameters, cecal microbiota, and metabolome of indigenous broilers. A total of forty female indigenous broilers (56-day-old) were randomly and equally divided into normal treatment group (NT group, 21.3 ± 1.2°C, 24 h/day) and HS group (32.5 ± 1.4°C, 8 h/day) with five replicates of each for 4 weeks feeding trial. The results showed that the body weight gain (BWG) of broilers in HS group was lower than those in NT group during 3–4 weeks and 1–4 weeks ($p < 0.05$). The HS exposure increased the abdominal fat rate ($p < 0.05$) but decreased the thigh muscle rate ($p < 0.01$). Besides, broilers in HS group had higher drip loss of breast muscle than NT group ($p < 0.01$). Broilers exposed to HS had lower total antioxidant capacity (T-AOC) in serum and jejunum, activities of total superoxide dismutase (T-SOD) in the jejunum, glutathione peroxidase (GSH-Px) in the thigh muscle, duodenum, and jejunum; and catalase (CAT) in breast muscle, duodenum, and jejunum ($p < 0.05$). Whereas the malondialdehyde (MDA) contents in breast muscle, duodenum, and jejunum was elevated by HS exposure ($p < 0.05$). Moreover, the relative mRNA expression of *Occludin* and *ZO-1* in the duodenum, *Occludin, Claudin-1, Claudin-4, ZO-1, Mucin-2* in the jejunum, and the *Claudin-4* and *Mucin-2* in the ileum was down-regulated by HS exposure ($p < 0.05$). The 16S rRNA sequencing results showed that the HS group increased the relative abundance of *Anaerovorax* in the cecum at the genus level ($p < 0.05$). Cecal metabolomics analysis indicated 19 differential metabolites between the two groups ($p < 0.10$, VIP >1). The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed that the differential metabolites mainly enriched in 10 signaling pathways such as the Citrate cycle (TCA cycle) ($p < 0.01$). In summary, chronic HS exposure caused a decline of production performance, reduced antioxidant capacity, disrupted intestinal barrier function, and negatively affected cecal microbiota and metabolome in indigenous broilers.

Keywords: antioxidant capacity, cecal microbiota, cecal metabolome, heat stress, intestinal barrier function, slow-growing broilers

INTRODUCTION

With global warming, the ambient temperature has gradually risen worldwide in the past decades. The high temperature has become an environmental hazard with a wide range of detrimental effects on poultry production (Vandana et al., 2021). Heat stress (HS) is the primary harmful consequence caused by high-temperature climates. Because of their strong metabolism, being covered with feathers, and being unable to sweat, broiler chickens are susceptible to HS (Wasti et al., 2020; Goel, 2021). It is well known that HS exposure leads to a reduction in growth performance, which is associated with the loss of appetite and decreased feed intake, also can be ascribed to the deviation of energy resources from production to adaptation pathway in broilers (Gogoi et al., 2021). Besides, under HS situations, systemic disorders of physiology and metabolism appear in broilers, such as the body temperature rises, peripheral blood flow increases, and lipid peroxidation aggravates (Guo et al., 2021a; Guo et al., 2021b; Chauhan et al., 2021). These unfavorable changes in physiological functions have a significant influence on production performances, including but not limited to growth rate, carcass traits, meat yield, and quality (Liu et al., 2019; Emami et al., 2021; Ma et al., 2021), and a detrimental effect on organ health of broilers (Lara and Rostagno, 2013; Liu et al., 2021a).

The gastrointestinal tract has dual functions, which is not only a place for nutrients digestion and absorption but also an innate barrier to maintain homeostasis (Jha et al., 2019; Liu W.-C. et al., 2020). Gut health is particularly vulnerable to HS, which are in line with previous studies showing that HS-induced impairment of intestinal morphology (Song et al., 2013), damage to tight junction structure (Zhang et al., 2017), and imbalance of gut mucosal redox status (Liu G. et al., 2020). These alterations boost intestinal permeability and permit the translocation of pathogens and toxins present in the gut lumen, ultimately leading to intestinal dysfunction (Rostagno, 2020). On the other hand, there is a complex community of microbiota in the intestine, and gut microbiota plays a significant role in nutrient digestion and barrier regulation (Yadav and Jha, 2019). The cecum is a part of the distal intestine and rich in microorganisms among the gut segments, which is also the site for indigestible fiber fermentation (Yadav et al., 2021). The fermentation products produced by cecal microbes positively affect intestinal health, and the numerous cecal metabolites play pivotal roles in maintaining the intestinal barrier function (Jha and Mishra, 2021; Singh et al., 2021). It has been demonstrated that broilers subjected to HS had negative influences on the cecal microbial community and metabolites, but the changes were partially inconsistent (Wang et al., 2018; Liu G. et al., 2020; Wang G. et al., 2021; Wasti et al., 2021a; Wasti et al., 2021b), indicating that further investigations are required.

Indigenous yellow-feathered broilers are slow-growing breeds but have excellent meat quality, and the meat of this type of broilers has become more popular among Chinese consumers in recent years (Wang et al., 2019). Therefore, the production scale of yellow-feathered broilers continues to expand in China. At present, to satisfy the consumer preferences regarding the flavor, the annual production of yellow-feathered broilers is approximately four billion, almost the same as that of fast-growing broilers in China (Wang Y. et al., 2021). Interestingly, indigenous chickens exhibit better heat tolerance than fast-growing broilers, such as Ross/Cobb broilers, due to their slower growth and lower metabolic rate (Xu et al., 2018). However, existing studies mainly focused on fast-growing commercial broilers, and only a few researches revealed that HS has detrimental effects on metabolic status and gut health in Chinese indigenous broilers (Guo et al., 2021a; Liu et al., 2021b). Furthermore, in indigenous broilers, the effect of HS on detailed pathobiology, especially the gut microbiome and metabolome, is not studied well. Therefore, the current study aimed to explore the effects of HS on production performance, redox status, small intestinal barrier parameters, cecal microbiota, and metabolome in native Chinese broiler chickens.

MATERIALS AND METHODS

Birds, Experimental Design, and Management

A total of forty female Huaixiang chickens (Chinese indigenous broiler breed, slow-growing, and yellow-feathered type) at 8 weeks old were obtained from local farms in Zhanjiang, Guangdong, and used in this study. The age selection of broilers was made considering that indigenous broilers enter the growing-finishing stage after 8-weeks of age, and growing-finishing broilers are more sensitive to HS for production (Wasti et al., 2020). The female broilers were only used to exclude the influence of gender on the experiment. The birds with initial average body weight (BW) of 840.75 ± 20.79 g were randomly and equally divided into two treatments in a completely randomized design. The treatments included normal treatment group (NT) (21.3 ± 1.2°C throughout the experimental period, thermoneutral zone) and HS group (32.5 ± 1.4°C, 8 h/day, from 9:00 a.m. to 17:00 p.m.). Each group had five replications with four broilers per replicate and was studied for 4 weeks. The relative humidity of NT and HS were maintained at 55–70%. The temperature of the two groups was controlled by environmental control equipment, including a dehumidifier (SHIMEI, MS-9138BE, Guangzhou, China), humidifier (OROSIN, DRST-03AE, Guangzhou, China), air conditioner (GREE, KF-120LW, Zhuhai, China), and heater (MIDEA, NPS7-15A5, Zhongshan, China). The chickens were kept in three-layer wire-cages of 90 (length) × 70 (width) × 40 (height) cm and were ensured that all chickens had free access to water and feed. The plastic trays were used under each cage to collect the excrements, and the excrements were manually cleaned twice a day. All broilers were fed a corn-soybean meal basal diet to meet the nutrient requirements recommended by the Chinese chicken breeding standard (NY/T33-2004). The composition and nutrient contents of diet were as reported in our previous study (Guo et al., 2021a).

Growth Performance and Carcass Traits Determination

The feed intake was recorded regularly. Briefly, the amount of feed supplied and leftover were recorded daily, and the data

TABLE 1 | Nucleotide sequences of the specific primers used in real-time qPCR analyses.

Genes	Accession No	Sequence
E-cadherin	NM 001039258.2	F: CGACAACATTCCCATCTTCA R: CACCATCCAGGTTCCCAC
Occludin	NM 205128.1	F: CTGCTGTCTGTGGGTTCCT R: CCAGTAGATGTTGGCTTTGC
Claudin-1	NM 001013611.2	F: ATGACCAGGTGAAGAAGATGC R: TGCCCAGCCAATGAAGAG
Claudin-4	AY435420.1	F: AGGACGAGACAGCCAAAGC R: CACGTAGAGCGACGAGCC
ZO-1	XM 413773.4	F: CGTAGTTCTGGCATTATTCGT R: TGGGCACAGCCTCATTCT
Mucin-2	XM 421035	F: TGAGTCAGGCATAAATCGTGT R: CAGGTCTAAGTCGGGAAGTGTA
β-actin	NM 205518.1	F: TTGGTTTGTCAAGCAAGCGG R: CCCCCACATACTGGCACTTT

were used to calculate daily feed intake. Meanwhile, the broilers were weighted in a cage at the start and the end of weeks 2 and 4. The daily feed intake and body weight data were used to calculate the body weight gain (BWG), feed intake, and feed/gain for 1–2, 3–4, and 1–4 weeks of the study period. At the end of week 4, one chicken from each replicate was selected randomly and slaughtered by neck bleeding. Subsequently, the partial carcass traits, including the abdominal fat (%), breast muscle (%), and thigh muscle (%), were determined. The carcass traits were determined as previously reported (Liu et al., 2019).

Measurement of Meat Quality

After the selected broiler from each replicate cage was slaughtered, the left side of breast muscle (pectoralis major) and the left side of thigh muscle (biceps femoris) were collected for the determination of meat quality, including cooking loss (%), drip loss (%), and pH at 45 min and 24 h after slaughter. The meat quality determination was done as in previous studies (Berri et al., 2008; Liu et al., 2019).

Antioxidant Capacity Analysis

To analyze the antioxidant capacity of broilers, 10 g of liver samples, breast and thigh muscle samples from the right side, and mucosal samples of duodenum, jejunum, and ileum were collected from one bird per replicate; 5 ml blood was collected from the wing vein using a vacuum tube without anticoagulant (one chicken per replicate), and the serum samples were obtained by centrifugation at 4,000 rpm (2 min, 4 °C) after blood coagulation. Afterward, the total anti-oxidation capacity (T-AOC), malondialdehyde (MDA) contents, and antioxidant enzymes activity, including the total superoxide dismutase (T-SOD), glutathione peroxidase (GSH-Px), and catalase (CAT), were analyzed using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. The catalog numbers of the kits are as follows: T-AOC, A015-2-1; MDA, A003-1-2; T-SOD, A001-3-2; GSH-Px, A005-1-2; CAT, A007-1-1.

Determination of Intestinal Morphology and Barrier Function Related Genes Expression

For intestinal morphology analysis, approximately 2 cm segments located in the middle of the duodenum, jejunum, and ileum (one broiler from each replicate) were collected, fixed in 4% paraformaldehyde for 48 h. The intestinal segments were mounted with paraffin sections and stained with hematoxylin and eosin (H & E). Subsequently, the sections were observed using an inverted optical microscope (SDPTOP, GD-30RFL, Guangzhou, China) under ×40 magnification. The intestinal morphology parameters, villus height, villus width, and crypt depth were measured using T-Capture Imaging Application 4.3 software, and the villus height/crypt depth ratio was calculated accordingly. The villus surface area was calculated following the formula $(2\pi) \times (\text{villus width}/2) \times \text{villus height}$ (de los et al., 2005).

Total RNA was extracted from the mucosal samples of the duodenum, jejunum, and ileum using RNA extraction kits (catalog No. N066, Jiancheng Bioengineering Institute, Nanjing, China). The reverse transcription of cDNA from RNA using RT reagent kits (catalog No. RR047A, TaKaRa Biotechnology Co., Ltd, Shiga, Japan). Then the quantitative real-time PCR (qPCR) was performed to detect the mRNA expression of intestinal barrier function-related genes, including *E-cadherin, Occludin, Claudin-1, Claudin-4, ZO-1,* and *Mucin-2*. The CFX-96 real-time PCR detection system (BioRad, Irvine, CA, United States) was used for the qPCR reaction. The reaction system and conditions were as in our previous study (Liu et al., 2021c). β-actin was used as an internal reference gene; the primer's information is presented in **Table 1**. Finally, the relative mRNA expression levels of the intestinal barrier function-related genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

TABLE 2 | Effects of heat stress on growth performance and carcass traits in indigenous broilers.

Items	NT Group	HS Group	*p*-Value
Growth performance			
1–2 weeks			
BWG, g	293.00 ± 12.91	255.49 ± 14.56	0.070
Feed intake, g	1341.24 ± 27.19	1243.60 ± 53.51	0.124
Feed/gain	4.64 ± 0.18	4.91 ± 0.10	0.210
3–4 weeks			
BWG, g	292.50 ± 10.09	259.48 ± 10.86	0.039
Feed intake, g	1445.73 ± 30.50	1350.35 ± 42.23	0.084
Feed/gain	4.99 ± 0.17	5.24 ± 0.13	0.250
Overall (1–4 weeks)			
BWG, g	585.45 ± 14.31	514.98 ± 22.44	0.016
Feed intake, g	2,786.77 ± 46.63	2,594.00 ± 92.03	0.078
Feed/gain	4.78 ± 0.12	5.06 ± 0.07	0.057
Carcass traits, %			
Abdominal fat	1.22 ± 0.15	2.89 ± 0.22	<0.001
Breast muscle	9.45 ± 0.62	8.52 ± 0.63	0.322
Thigh muscle	12.47 ± 0.51	8.37 ± 0.74	0.002

NT, normal treatment group, the broilers reared at 21.3 ± 1.2°C throughout the experimental period; HS, the broilers reared at 32.5 ± 1.4 °C for 8 h/day (9:00 a.m. to 17:00 p.m.); BWG, body weight gain. There were 5 replicate cages (20 individuals) per group for determination of growth performance and 5 selected broilers per group for the determination of carcass traits.

TABLE 3 | Effects of heat stress on meat quality in indigenous broilers.

Items	NT Group	HS Group	*p*-Value
Breast muscle			
Cooking loss, %	10.56 ± 1.07	13.79 ± 3.28	0.376
Drip loss, %			
1 day	0.90 ± 0.18	3.74 ± 0.43	<0.001
2 days	1.96 ± 0.30	4.81 ± 0.47	<0.001
3 days	2.98 ± 0.31	5.53 ± 0.51	0.003
pH			
45min	6.84 ± 0.01	6.68 ± 0.06	0.035
24h	6.62 ± 0.09	6.49 ± 0.15	0.475
Thigh muscle			
Cooking loss, %	20.04 ± 4.80	24.61 ± 3.65	0.471
Drip loss, %			
1 day	0.59 ± 0.17	2.17 ± 0.75	0.076
2 days	1.44 ± 0.19	2.33 ± 0.86	0.342
3 days	1.99 ± 0.23	2.93 ± 0.90	0.344
pH			
45min	6.97 ± 0.05	6.69 ± 0.09	0.025
24h	6.90 ± 0.08	6.84 ± 0.07	0.572

NT, normal treatment group, the broilers reared at 21.3 ± 1.2 °C throughout the experimental period; HS, the broilers reared at 32.5 ± 1.4 °C for 8 h/day (9:00 a.m. to 17: 00 p.m.). There were 5 selected broilers per group for the determination of meat quality.

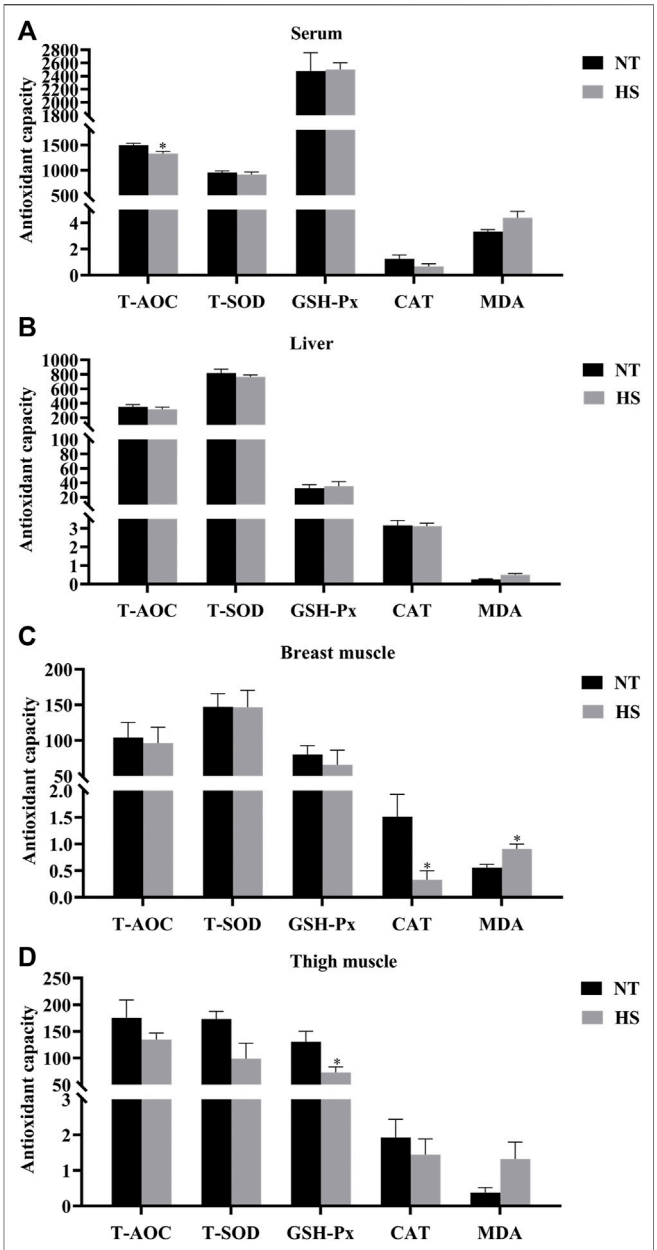

FIGURE 1 | Effects of heat stress on antioxidant capacity of serum **(A)**, liver **(B)**, breast muscle **(C)**, and thigh muscle **(D)** in indigenous broilers (n = 5/group). T-AOC, total antioxidant capacity. T-SOD, total superoxide dismutase. GSH-Px, glutathione peroxidase. CAT, catalase. MDA, malondialdehyde. NT, normal treatment group (21.3 ± 1.2°C, 24 h/day), HS, heat stress group (32.5 ± 1.4°C, 8 h/day). *$p < 0.05$.

Cecal Microbial Community Analysis

After slaughter, approximately 5 g of cecal digesta samples were collected from one chicken per replicate. The microbial DNA of cecal digesta was extracted using QIAamp DNA Stool Mini Kit (catalog No. 51306, QIAGEN, CA, Hamburg, Germany). The V3-V4 regions of the 16S rRNA genes were sequenced on the Illumina MiSeq platform by Personalbio Technology Co., Ltd. (Shanghai, China). The primer pairs were 338F (5′-ACTCCTACGGGAGGCACAG-3′) and 806R (5′- GGACTACHVGGGTWTCTAAT-3′). The raw reads were demultiplexed and quality-filtered using FLASH software (v1.2.7, http://ccb.jhu.edu/software/FLASH/) and QIIME software (v1.8.0 Quantitative Insights Into Microbial Ecology, http://qiime.org/). Then the obtained effective reads were assigned to operational taxonomic units (OTUs) with 97% similarity using UCLUST sequence alignment tool of QIIME software (v1.8.0). The bacterial taxonomic information corresponding to each OTU was obtained from the Greengenes database (Release 13.8, http://greengenes.secondgenome.com/). The diversity, composition, and difference of the cecal microbial community were analyzed using the Gene Cloud Analysis Platform (QIIME 1, https://www.genescloud.cn) of Personalbio Technology Co., Ltd. (Shanghai, China).

Metabolomics Analysis in Cecal Digesta

The 1 g of cecal digesta was used for untargeted metabolomics analysis using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) method and detected by LC-MS/MS system (Personalbio Technology Co., Ltd. Shanghai, China). The experimental procedure included metabolites extraction, LC-MS/MS detection, and data analysis. The metabolites of cecal digesta were extracted using methanol/acetonitrile/water solution (4:4:2, v/v). Then, 2 μl of the supernatant from each sample (one chicken in each replicate) was separated on an Agilent 1290 Infinity LC Ultra High-Performance Liquid Chromatography System (UHPLC) with HILIC column (Agilent Technologies Inc. CA, United States). The conditions of UHPLC were as follows: column temperature 25°C; flow rate 0.3 ml/min; injection volume 2 μl; mobile phase composition A: water +25 mM ammonium acetate +25 mM ammonia water, B: acetonitrile; gradient elution procedure is as follows: 0–1 min, 95% B; 1–14 min, B linearly changes from 95 to 65%; 14–16 min, B linearly changes from 65 to 40%; 16–18 min, B maintained at 40%; 18–18.1 min, B linearly changed from 40 to 95%;

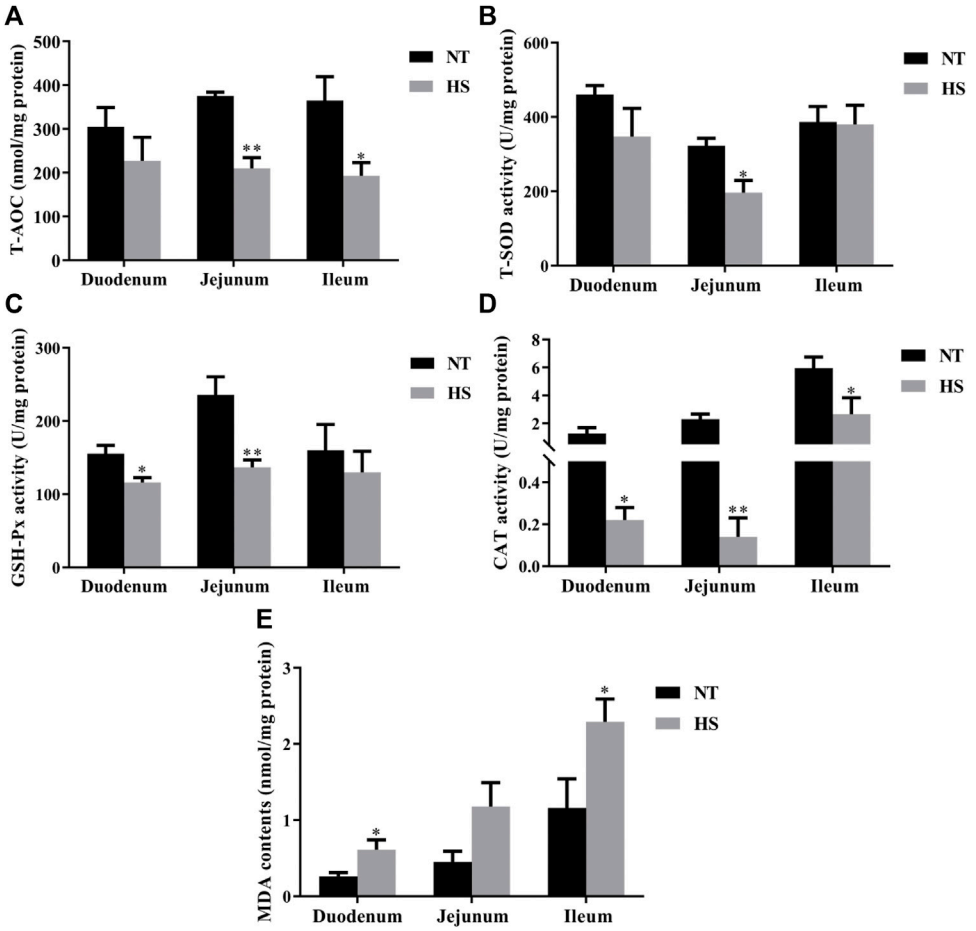

FIGURE 2 | Effects of heat stress on antioxidant capacity of duodenum, jejunum, and ileum in indigenous broilers (n = 5/group). T-AOC, total antioxidant capacity **(A)**. T-SOD, total superoxide dismutase **(B)**. GSH-Px, glutathione peroxidase **(C)**. CAT, catalase **(D)**. MDA, malondialdehyde **(E)**. NT, normal treatment group (21.3 ± 1.2°C, 24 h/day), HS, heat stress group (32.5 ± 1.4°C, 8 h/day). *$p < 0.05$, **$p < 0.01$.

18.1–23 min, B maintained at 95%. The samples were separated by UHPLC and then analyzed by mass spectrometry using a Triple TOF 6600 mass spectrometer (AB SCIEX Inc. MA, United States). The conditions of MS analysis were as follows: Ion Source Gas1 (Gas1): 60, Ion Source Gas2 (Gas2): 60, Curtain gas (CUR): 30, source temperature: 600°C, IonSapary Voltage Floating (ISVF) ±5500 V (plus and minus two mode); TOF MS scan m/z range: 60–1000 Da, Production scan m/z range: 25–1000 Da, TOF MS scan accumulation time 0.20 s/sPectra, Production scan accumulation time 0.05 s/sPectra; Secondary mass spectrometry was acquired using information dependent acquisition (IDA) and used high sensitivity mode, Declustering potential (DP): ±60 V (both positive and negative modes), Collision Energy: 35 ± 15 eV, IDA settings are as follows Exclude isotoPes within 4 Da, Candidate ions to monitor Per cycle: 6. After LC-MS/MS detection, The raw data were converted into mzXML format by ProteoWizard, and the XCMS program was used for peak alignment, retention time correction and extraction of peak areas. The structure identification of metabolites uses accurate mass matching (<25 ppm) and secondary spectrum matching to search the laboratory's self-built database. For data extracted by XCMS, ion peaks with >50% missing values within the group were removed. The software SIMCA-P 14.1 (Umetrics, Umea, Sweden) was used for pattern recognition. After the data was preprocessed by Pareto-scaling, multi-dimensional statistical analysis was performed, including unsupervised principal component analysis (PCA) analysis, and supervised partial least squares discriminant analysis (PLS-DA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA). Statistical analysis included Student's t-test and fold variation analysis. The OPLS-DA model variable importance projection (VIP) > 1 and $p < 0.10$ were used as the criteria to screen significant differential metabolites, and subsequently, cluster analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway analysis were performed on the differential metabolites.

Statistical Analysis

All the statistical analysis of data was by done using SAS 9.4 (SAS, 2013. SAS Institute Inc. Cary, NC, United States). Student's t-test was performed for comparing the differences between the two

groups. Data results were expressed as mean ± standard error. The probability (*P*) value less than 0.05 was considered to be significant, and the *p* value between 0.05–0.10 was considered to be a trend of significance.

RESULTS

Growth Performance and Carcass Traits

The results of growth performance are presented in **Table 2**. During 1–2 weeks, HS exposure tended to reduce the BWG ($p = 0.070$). During 3–4 weeks, HS exposure decreased the BWG ($p < 0.05$) and tended to reduce the feed intake ($p = 0.084$). During the overall study period (1–4 weeks), HS exposure led to a reduction in BWG ($p < 0.05$) and tended to decrease the feed intake ($p = 0.078$) but increased the feed/gain ($p = 0.057$). As shown in **Table 2**, broilers exposed to HS for 4 weeks had a higher abdominal fat compared with NT group ($p < 0.001$), and the broilers under 4 weeks of HS resulted in a lower thigh muscle ($p < 0.01$).

Meat Quality

The meat quality parameters of breast and thigh muscle were presented in **Table 3**. Broilers in HS group had a higher drip loss after 1, 2, 3 days ($p < 0.01$) and lower pH after 45 min ($p < 0.05$) of breast muscle than those in NT group. Compared with NT group, HS exposure caused an increasing trend in drip loss after 1 day ($p = 0.076$) and reduced the pH after 45 min ($p < 0.05$) of the thigh muscle.

Antioxidant Capacity

As illustrated in **Figure 1**, broilers in HS group had lower T-AOC in serum, CAT activity in breast muscle, and GSH-Px in thigh muscle than those in NT group ($p < 0.05$). However, the MDA contents of breast muscle were increased by HS exposure ($p < 0.05$). Besides, the HS exposure reduced the mucosal T-AOC in jejunum (**Figure 2**, $p < 0.01$) and ileum ($p < 0.05$), decreased the mucosal T-SOD activity in jejunum ($p < 0.05$), GSH-Px activity in duodenum ($p < 0.05$) and jejunum ($p < 0.01$), and CAT activity in duodenum ($p < 0.05$), jejunum ($p < 0.01$) and ileum ($p < 0.05$). Compared with NT group, broilers under HS had higher mucosal MDA levels in the duodenum and ileum ($p < 0.05$).

Intestinal Morphology and Barrier Function Related Genes Expression

The results of intestinal morphology are presented in **Table 4**. Broilers in the HS group had a lower villus height of duodenum and ileum than NT group ($p < 0.05$). Additionally, the decreased villus height/crypt depth ratio in the ileum was found in broilers under HS ($p < 0.05$).

The relative mRNA expression levels of intestinal barrier function-related genes are illustrated in **Figure 3**. HS down-regulated the mRNA expression of *Occludin* and *ZO-1* in duodenal mucosa ($p < 0.05$). The mRNA expressions of *Occludin, Claudin-1, Claudin-4, ZO-1* and *Mucin-2* in jejunal mucosa were down-regulated ($p < 0.05$) by HS exposure. Meanwhile, the mRNA expressions of *Claudin-4* and *Mucin-2* in ileal mucosa were down-regulated ($p < 0.05$) in broilers under HS.

Cecal Microbial Community

There were no significant differences ($p > 0.05$) on alpha diversity indexes (Chao 1, ACE, Simpson, and Shannon index) between the NT and HS groups (**Figure 4A**). The beta diversity results showed that the consistency of intra-group samples in each treatment was good (**Figure 4B**). Regarding the relative abundance of cecal microbiota at the phylum level, HS exposure did not significantly affect the relative abundance of microbiota (**Figure 4C**, $p > 0.05$).

TABLE 4 | Effects of heat stress on intestinal morphology in indigenous broilers.

Items	NT Group	HS Group	*p*-Value
Duodenum			
Villus height, μm	1806.82 ± 26.85	1632.74 ± 43.80	0.010
Crypt depth, μm	382.77 ± 46.46	391.69 ± 38.60	0.886
Villus height/crypt depth ratio	5.02 ± 0.61	4.35 ± 0.49	0.422
Villus width, μm	313.15 ± 24.46	279.92 ± 22.63	0.348
Villus surface area, mm^2	1.78 ± 0.14	1.44 ± 0.15	0.143
Jejunum			
Villus height, μm	1289.70 ± 86.92	1163.22 ± 62.97	0.273
Crypt depth, μm	407.81 ± 35.47	354.63 ± 29.89	0.285
Villus height/crypt depth ratio	3.22 ± 0.25	3.34 ± 0.23	0.733
Villus width, μm	300.45 ± 22.43	294.68 ± 26.99	0.873
Villus surface area, mm^2	1.21 ± 0.10	1.08 ± 0.12	0.447
Ileum			
Villus height, μm	900.16 ± 21.28	716.79 ± 73.55	0.044
Crypt depth, μm	206.75 ± 15.93	207.13 ± 10.91	0.985
Villus height/crypt depth ratio	4.44 ± 0.28	3.46 ± 0.29	0.042
Villus width, μm	335.18 ± 47.32	319.28 ± 58.22	0.838
Villus surface area, mm^2	0.94 ± 0.12	0.68 ± 0.09	0.109

NT, normal treatment group, the broilers reared at 21.3 ± 1.2 °C throughout the experimental period; HS, the broilers reared at 32.5 ± 1.4°C for 8 h/day (9:00 a.m. to 17:00 p.m.). There were 5 selected broilers per group for the determination of intestinal morphology.

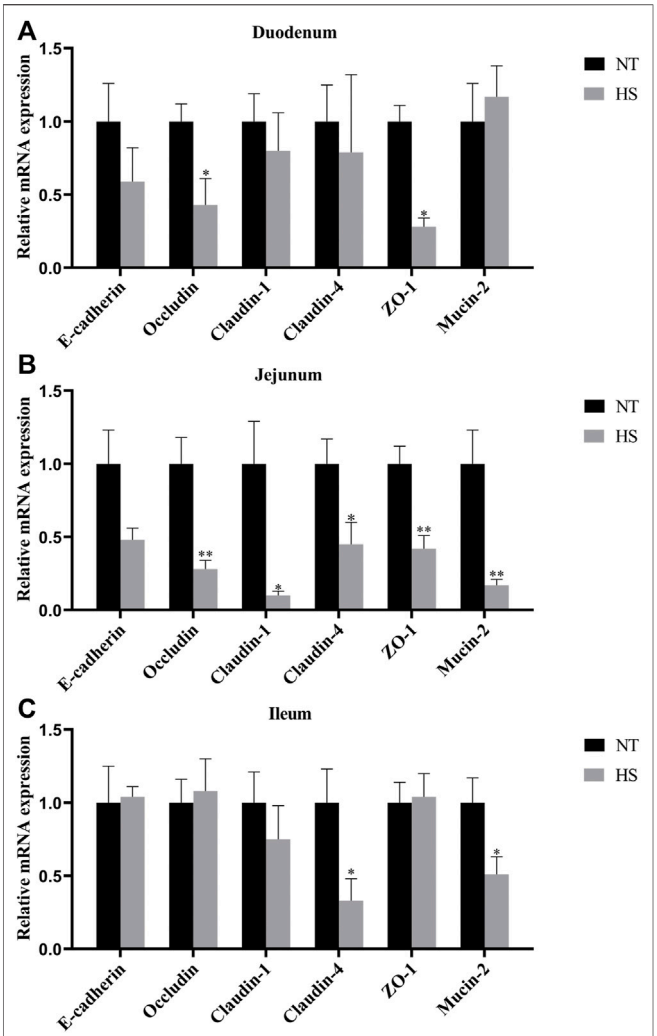

FIGURE 3 | Effects of heat stress on relative mRNA expression of barrier function-related genes of duodenum **(A)**, jejunum **(B)** and ileum **(C)** in indigenous broilers (n = 5/group). NT, normal treatment group (21.3 ± 1.2°C, 24 h/day), HS, heat stress group (32.5 ± 1.4°C, 8 h/day). *$p < 0.05$, **$p < 0.01$.

Notably, the relative abundance of *Firmicutes* in HS broilers' cecum was lower than NT group, and the relative abundance of cecal *Bacteroidetes* in broilers under HS was higher than NT group. Also, the ratio of *Firmicutes/Bacteroidetes* (F/B) was reduced by HS. However, none of these differences reached a significant level (**Figure 4D**, $p > 0.05$). At the genus level, it was found that HS exposure significantly increased the relative abundance of *Anaerovorax* in cecal digesta (**Figures 4E,F**, $p < 0.05$).

Cecal Metabolome

The cecal metabolome results are presented in **Table 5** and **Figure 5**. According to the screening criteria ($p < 0.10$, VIP>1), a total of 19 differential metabolites (containing negative and positive ions mode) were identified between the NT and HS groups. Among the metabolites, five metabolites were significantly down-regulated ($p < 0.05$) by HS exposure, including Cytosine, L-Malic acid, Citrate, Isobutyric acid, and Quinate. In addition, four metabolites were significantly up-regulated ($p < 0.05$) by HS exposure, including all cis-(6,9,12)-Linolenic acid, N1-Acetylspermidine, Sphinganine, and 6k-PGF1alpha-d4. The KEGG metabolic pathways analysis revealed that the differential metabolites mainly enriched ($p < 0.01$) in 10 signaling pathways, including the Citrate cycle (tricarboxylic acid, TCA cycle), biosynthesis of alkaloids derived from histidine and purine, two-component system, carbon fixation pathways in prokaryotes, biosynthesis of alkaloids derived from terpenoid and polyketide, glyoxylate and dicarboxylate metabolism, renal cell carcinoma, pyrimidine metabolism, biosynthesis of alkaloids derived from ornithine, lysine and nicotinic acid, and biosynthesis of plant hormones.

DISCUSSION

In practice, 32°C is the temperature that the indigenous broilers suffer from chronic HS in the environmentally equipped chicken house. Therefore, this study evaluated the changes in various parameters in indigenous broilers exposed to chronic HS at around 32°C for 4 weeks. Under the present thermal conditions, the decline of growth performance could be attributed to a series of adverse effects caused by HS, including electrolyte imbalance, endocrine disorders, energy-substance metabolism dysregulation, and organ injury (Chauhan et al., 2021). According to Lu et al. (2007), the HS exposure at 34°C for 3 weeks reduced the feed intake but had no significant impacts on BWG of slow-growing broilers (Beijing You chicken). Shao et al. (2018) demonstrated that cyclic HS (35°C for 7 days) decreased the BWG, feed intake, and feed/gain ratio of Chinese indigenous broilers (Xueshan broilers). In a study with Thai native chickens, HS exposure also reduced the BWG, but the HS conditions (35°C for 3 weeks) were not consistent with the current study (Malila et al., 2021). A recent meta-analysis study for fast-growing broilers revealed that the feed intake and feed conversion ratio were not significantly affected by 1–21 days of HS (Andretta et al., 2021). Therefore, it was widely accepted that HS results in unfavorable effects on growth performance, but the unequal duration and intensity of HS cause various consequences in growth indicators. The genetic background is also needed to be considered, especially for the indigenous broilers, which have different metabolic and growth rates, resulting in a variety of tolerance to HS (Wasti et al., 2020).

In addition, similar to the results of carcass composition in this study, Lu et al. (2007) demonstrated that the abdominal fat rate was increased in slow-growing Beijing You chickens under HS. Previous studies also reported that the breast and thigh muscle yield was decreased by 12–17% in slow-growing broilers subjected to HS conditions (Shao et al., 2018; Liu et al., 2019). However, these findings contradicted some other reports, which found that HS exposure had no significant effects on abdominal fat and muscle yield of broilers (Humam et al., 2019; Al-Sagan et al., 2020; Malila et al., 2021). The differences could be due to the broiler species and HS intensity. It has been suggested that the lipolysis and lipolytic enzymes activity and lipid metabolism-

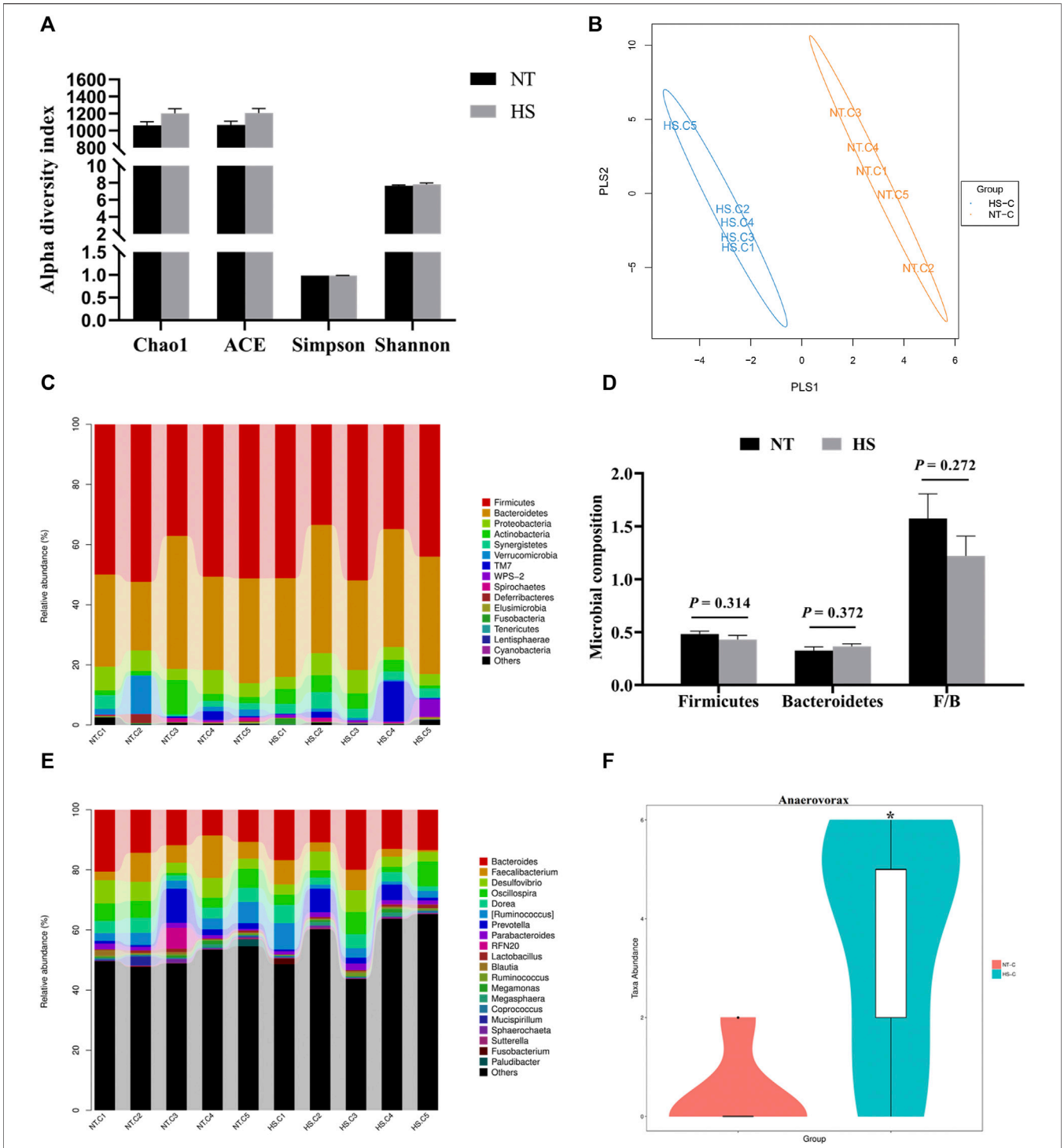

FIGURE 4 | Effects of heat stress on cecal microbial community in indigenous broilers (n = 5/group). **(A)** alpha diversity; **(B)** beta diversity; **(C)** cecal microbial composition at the phylum level; **(D)** impacts of HS on the cecal relative abundance of *Firmicutes*, *Bacteroidetes* and the ratio of *Firmicutes/Bacteroidetes* (F/B) at the phylum level; **(E)** cecal microbial composition at the genus level; **(F)** impacts of HS on the cecal relative abundance of *Anaerovorax* at the genus level; NT, normal treatment group (21.3 ± 1.2°C, 24 h/day), HS, heat stress group (32.5 ± 1.4°C, 8 h/day). *$p < 0.05$.

related pathways were suppressed under HS exposure, which subsequently resulted in abdominal fat deposition in broilers (Luo et al., 2018; Guo et al., 2021a). Furthermore, HS reduced the nutrients utilization and the retention of protein synthesis (Zuo et al., 2015) and caused muscle protein breakdown to an amino acid, thereby providing substrates to hepatic gluconeogenesis

TABLE 5 | Effects of heat stress on cecal differential metabolites in indigenous broilers.

Metabolites	Ratio (HS/NT)	*p*-Value	VIP
NEG			
Ala-Lys	0.451	0.095	1.895
Lys-Pro	0.567	0.093	1.917
Cytosine	0.633	**0.041**	2.164
5-Aminopentanoic acid	0.641	0.078	1.980
Tolazoline	2.145	0.060	1.918
all cis-(6,9,12)-Linolenic acid	2.806	**0.012**	2.471
N1-Acetylspermidine	3.064	**0.032**	2.214
N1, N12-Diacetylspermine	5.933	0.087	1.771
Sphinganine	10.863	**0.014**	2.577
POS			
L-Malic acid	0.262	**0.005**	2.761
Ribitol	0.285	0.064	2.166
Citrate	0.345	**0.045**	2.286
Isobutyric acid	0.473	**0.031**	2.326
Quinate	0.627	**0.027**	2.368
Cytidine	0.671	0.064	2.054
Ribothymidine	0.680	0.091	1.819
Adenine	0.705	0.084	1.827
3-Methylphenylacetic acid	0.762	0.071	2.063
6k-PGF1alpha-d4	1.990	**0.047**	2.001

NT, normal treatment group, the broilers reared at 21.3 ± 1.2 °C throughout the experimental period; HS, the broilers reared at 32.5 ± 1.4 °C for 8 h/day (9:00 a.m. to 17: 00 p.m.); VIP, variable importance projection; NEG, negative ions mode; POS, positive ions mode; Bold values, p *<0.05. There were 5 selected broilers per group for the determination of the cecal metabolome.*

responsible for energy supply, and these changes were through the regulation of the insulin-like growth factor (IGF)/rapamycin signaling pathway (Ma et al., 2018; Ma et al., 2021). Therefore, molecules related to lipid metabolism and proteolysis may have different responses to variable HS intensity, and the expression of the molecules is also affected by genetic background, leading to the differences in carcass traits.

Regarding the meat quality, HS challenge increased the drip loss and reduced the muscle pH in the present study, which is in agreement with previous studies (Lu et al., 2017; Cheng et al., 2018; Wen et al., 2019; Awad et al., 2020). As it is well known, chronic HS induces hypoxia and increases anaerobic metabolism, leading to compensatory metabolic acidosis and a rise in muscle lactic acid concentration (Zaboli et al., 2019). Besides, it was observed that more pyruvate converted to lactic acid in HS broilers' muscle (Gonzalez-Rivas et al., 2020). Therefore, the increased lactic acid content could reduce the pH of meat, and a lower post mortem pH denatures the muscle proteins, which compromises the water-holding capacity and causes an increase in drip loss (Mckee and Sams, 1998). Besides, the muscle mitochondrial dysfunction induced by HS could cause a decrease in the aerobic metabolism of fat and glucose and an increase in glycolysis, and the pH was reduced while the drip loss was increased accordingly (Lu et al., 2017).

Redox balance plays a critical role in maintaining physiological functions. It is widely accepted that HS exposure causes oxidative damage to the tissues and organs (Liu et al., 2021a). The antioxidant enzyme system, including GSH-Px, SOD, and CAT, could protect the body against oxidative damage under normal physiological conditions (Sies, 2015). When broilers were affected by HS at the initial stages, the antioxidant enzyme system was stimulated in response to HS. However, as the HS situation continued, the antioxidant enzyme system was dysregulated, thus leading to oxidative stress in broilers (Chauhan et al., 2021). The current findings further confirmed the theory of chronic HS-induced oxidative stress in broilers, HS exposure reduced the antioxidant enzymes activity and/or elevated MDA levels in serum, muscle, and small intestines. Similar results were also reported by previous studies (Lu et al., 2017; Cheng et al., 2018; Hu et al., 2020; Liu et al., 2021b).

Nowadays, researchers have paid great attention to the gut health of broilers, and growing evidence pointed out that gut health is the main target of HS (Wasti et al., 2020). Under HS conditions, broilers increase peripheral blood flow to accelerate heat dissipation, which in turn redistribute the blood and reduce the blood flow to the intestinal epithelium, thus resulting in gut ischemia-hypoxic injury (Rostagno, 2020). Meanwhile, oxidative stress induced by HS can directly destroy the intestinal epithelial barrier function of broilers (Mishra and Jha, 2019). The physical barrier in the gut is critical to intestinal health, and the tight junctions (TJs) are pivotal components of the physical barrier, which are responsible for regulating paracellular permeability and intestinal homeostasis (Teng et al., 2020). He et al. (2020) reported that the HS impaired small intestinal villus-crypt structure and decreased the expression of gut TJs-related genes in indigenous broilers. Liu et al. (2021c) demonstrated that the genes expression of TJs was down-regulated by HS in yellow-feathered chickens. Liu et al. (2021b) reported that HS exposure decreased the villus height and mRNA expression levels of TJs such as *Occludin* and *ZO-1* in the duodenum of local broilers breed. In agreement with previous studies, the present study found that HS-induced damage to intestinal morphology and TJs. The impairment of morphology and TJs could increase intestinal permeability, cause gut inflammation, reduce nutrients absorption, and consequently decrease BWG (Vandana et al., 2021).

The cecal microbial community was analyzed using 16S rRNA high-throughput sequencing; results showed that HS did not significantly impact the alpha diversity index. Consistently, Liu et al. (2021c) found that the alpha diversity of cecal microbiota was not affected by long-term HS exposure in yellow-feathered broilers. Wasti et al. (2021b) reported that HS had no significant effects on cecal microbial alpha diversity, such as Shannon entropy and Simpson's index in HS Cobb 500 chicks. Prasai et al. (2016) also suggested that the chicken's gut health was generally related to the changes in some specific microbiota rather than the dramatic alterations of the overall microbial-ecological diversity. Regarding the microbial composition, there were no significant differences in the relative abundance of microbiota at the phylum level. This is in accordance with the previous report by Shi et al. (2019), who observed that 28 days of HS did not significantly affect the cecal microbial composition at the phylum level in yellow-feathered broilers. Similar findings of fast-growing broilers were also reported in previous studies (Wang G. et al., 2021; Wasti et al., 2021b). It is worth noting that although there were no significant changes at the phylum level, the F/B ratio was reduced by HS. The ratio of F/B is an important marker of intestinal microbiota homeostasis (Lee and Hase, 2014), implying that HS affected the cecal microbiota balance. To delineate this

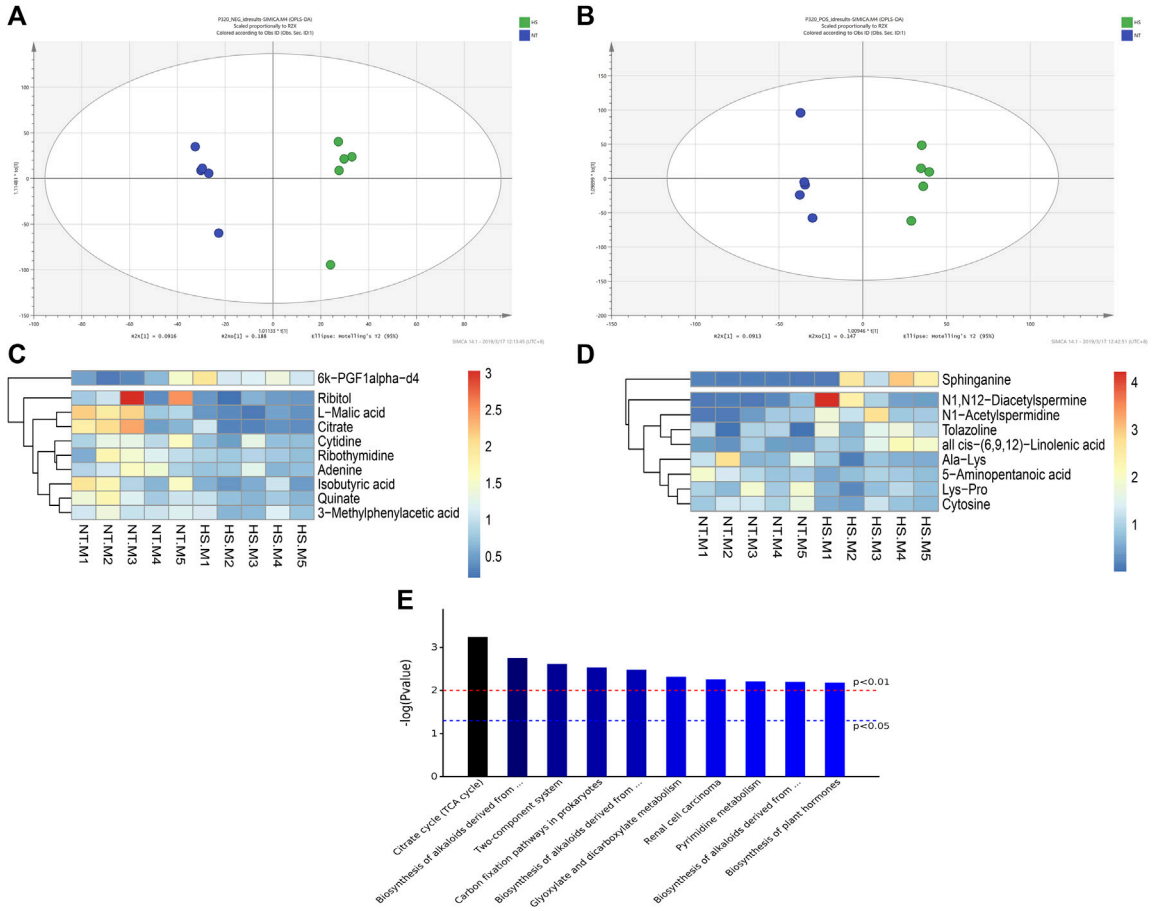

FIGURE 5 | Effects of heat stress on cecal metabolome in indigenous broilers (n = 5/group). **(A)** Orthogonal partial least squares discriminant analysis (OPLS-DA) under negative ions mode (NEG); **(B)** OPLS-DA under positive ions mode (POS); **(C)** Cluster heatmap analysis of samples under positive ions mode (POS); **(D)** Cluster heatmap analysis of samples under negative ions mode (NEG); **(E)** Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways enrichment analysis; NT, normal treatment group (21.3 ± 1.2°C, 24 h/day), HS, heat stress group (32.5 ± 1.4°C, 8 h/day).

effect, we further analyzed the microbial composition at the genus level and found that HS promoted the relative abundance of *Anaerovorax*. Similarly, the fecal *Anaerovorax* level was elevated after the toxins (tributyltin) challenge in rats, and the *Anaerovorax* could reduce the L-glutamic acid contents, thus impairing the intestinal barrier function (Yuan et al., 2020). Therefore, HS-induced injury of intestinal morphology and TJs may be associated with gut microbiome mediation in this study. However, the mechanism by which chronic HS influences intestinal microbiota is intricate and remains elusive. Thus, further works are needed to understand the role of gut dysbiosis in HS response and their correlation with the intestinal barrier function of broilers.

In this study, 19 differential cecal metabolites were identified in broilers after HS challenge based on metabolomics analysis. These mainly include organic acid, amines, and pyrimidine, and the KEGG analysis showed that these metabolites are enriched in TCA cycle, alkaloid synthesis, carbon fixation pathways, pyrimidine metabolism etc. The findings are similar to the study of serum metabolome in broilers under HS (Lu et al., 2018). In particular, the cecal contents of L-malic acid, citrate, and isobutyric acid were decreased by HS, and the L-malic acid was the most obvious of these changes. As an organic acid, L-malic acid has been reported to prevent ischemic injury (Tang et al., 2013), and the antimicrobial effect of L-malic acid was confirmed previously (Coban, 2020). It was found that the dietary L-malic acid displays positive effects on growth performance and gut health in quails (Ocak et al., 2009). Dietary citric acid also showed promotion effects on the gut health of broilers (Nourmohammadi and Afzali, 2013; Kammon et al., 2019). Isobutyric acid is a short-chain fatty acid and is beneficial for gut health (Ye et al., 2020). Accordingly, the impaired intestinal barrier may also be attributed to the reduced L-malic acid, citrate, and isobutyric acid levels in the cecum of broilers under HS. On the other hand, the L-malic acid and citric acid are involved in the TCA cycle (Goldberg et al., 2006). The results suggest that HS profoundly influenced the TCA cycle through metabolites, thus negatively affecting nutrient metabolism. Furthermore, HS reduced the cecal quinate content may deplete the gut antioxidant capacity as the quinic acid is a substrate for antioxidants (Pero et al., 2009). The HS-induced reduction of cytosine has deleterious consequences for nucleic acid synthesis, and an increase in metabolites, such as all cis-(6,9,12)-

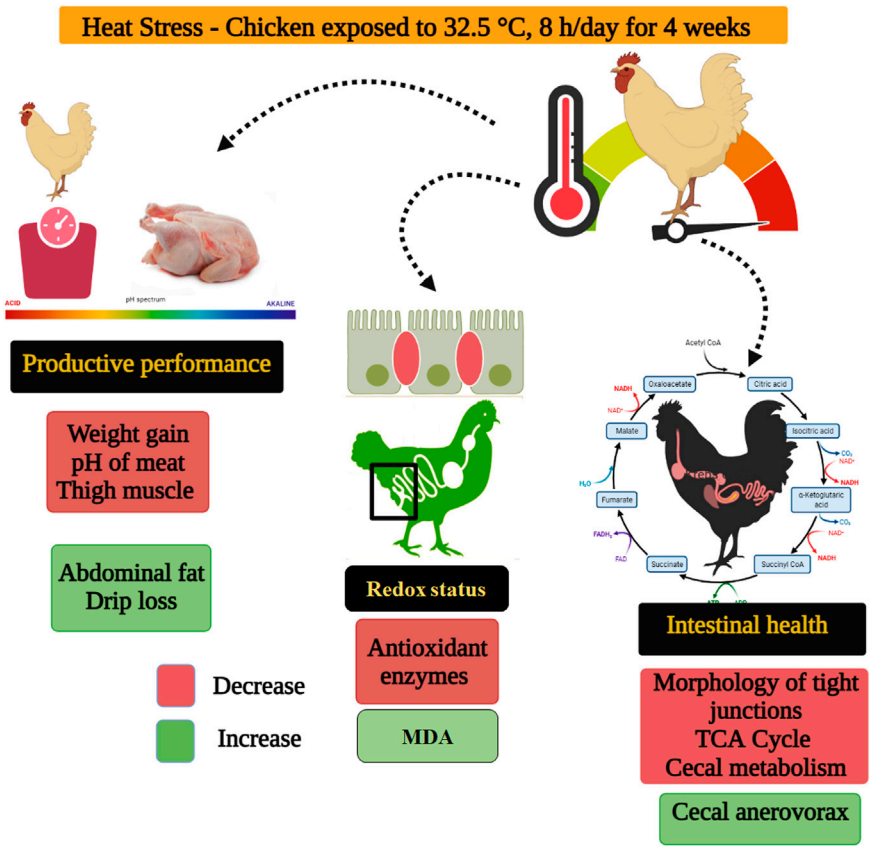

FIGURE 6 | Proposed mechanism of heat stress on indigenous broilers (Huaixiang chickens).

linolenic acid, N1-acetylspermidine, sphinganine, and 6k-PGF1alpha-d4, may be the response molecules to HS and which could be used as potential stress biomarkers (Lu et al., 2018).

Integrated analysis parameters that have been detected in this study (**Figure 6**), HS exposure negatively affected the production (weight gain, carcass and meat quality), redox status, and intestinal health (morphology, TJs, cecal microbiota and metabolome) of indigenous broilers. As oxidative stress causes organ dysfunction and tissue injury (Liu et al., 2021a), the reduced redox balance may be responsible for the deterioration of weight gain, meat quality, and gut health. Furthermore, the decline of weight gain may also be due to the impaired intestinal morphology and TJs function and the disturbances in cecal microbiota and metabolome. The novelty is that the cecal *Anaerovorax* and organic acid metabolites have the potential to be new regulatory targets for gut health in heat-stressed indigenous broilers, especially the L-malic acid. Whether the addition of exogenous L-malic acid alleviates HS-induced intestinal damage in slow-growing broilers, warrants further investigation.

CONCLUSION

Chronic heat stress reduced the body weight gain and the antioxidant capacity, disrupted the intestinal physical barrier such as morphology and tight junctions, increased the relative abundance of *Anaerovorax* in the cecum, and lowered the metabolites related to gut health such as L-malic acid in indigenous broilers. These results could expand the understanding of the effects of heat stress on physiological changes and intestinal health in slow-growing broiler chickens, especially the cecal microbiome and metabolome findings.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI repository, accession number: PRJNA816571.

ETHICS STATEMENT

The animal care, feeding, and sampling involved in the present study were approved by the Department of Animal Science of Guangdong Ocean University (dkx-20181217).

AUTHOR CONTRIBUTIONS

Conceptualization, W-CL, BB, and RJ; Methodology, W-CL, and RJ; Analysis, Z-YP, YZ, YG, S-JQ; Data curation, W-CL,

and Z-YP.; Writing-original draft preparation, W-CL; Writing-review and editing, YZ, BB, and RJ; Supervision, BB and RJ; Project administration, W-CL; Funding acquisition, W-CL.

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Heat Stress Affects Jejunal Immunity of Yellow-Feathered Broilers and Is Potentially Mediated by the Microbiome

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In the perspective of the global climate change leading to increasing temperature, heat stress (HS) has become a severe issue in broiler production, including the indigenous yellow-feathered broilers. The present study aimed to investigate the effects of HS on jejunal immune response, microbiota structure and their correlation in yellow-feathered broilers. A total of forty female broilers (56-days-old) were randomly and equally divided into normal treatment group (NT group, 21.3 ± 1.2°C, 24 h/day) and HS group (32.5 ± 1.4°C, 8 h/day) with five replicates of each for 4 weeks feeding trial. The results showed that HS exposure increased the contents of TNF-α, IL-1β, and IL-6 in jejunal mucosa ($p < 0.05$). The HS exposure up-regulated the relative fold changes of *NF-κB, TNF-α, IL-1β,* and *IL-6* ($p < 0.01$) while down-regulated the relative fold change of *IFN-γ* in jejunal mucosa ($p < 0.05$). Meanwhile, HS had no significant impacts on alpha diversity of jejunal microbiota such as Simpson, Chao1 richness estimator (Chao 1), abundance-based coverage estimators (ACE), and Shannon index ($p > 0.10$). Broilers exposed to HS reduced the jejunal microbial species number at the class and order level ($p < 0.05$). Moreover, HS decreased the relative abundance of *Ruminococcus*, *Bdellovibrio,* and *Serratia* at the genus level in jejunum ($p < 0.05$). At the phylum level, four species of bacteria (*Bacteroidetes, Cyanobacteria, Thermi,* and *TM7*) were significantly associated with immune-related genes expression ($p < 0.05$). At the genus level, ten species of bacteria were significantly correlated with the expression of immune-related genes ($p < 0.05$), including *Caulobacteraceae, Actinomyces, Ruminococcaceae, Thermus, Bdellovibrio, Clostridiales, Sediminibacterium, Bacteroides, Sphingomonadales* and *Ruminococcus*. In particular, the microbial with significantly different abundances, *Ruminococcus* and *Bdellovibrio*, were negatively associated with pro-inflammatory cytokines expression ($p < 0.05$). These findings demonstrated that HS exposure promoted the production of pro-inflammatory cytokines in yellow-feathered broilers' jejunum. The detrimental effects of HS on jejunal immune response might be related to dysbiosis, especially the reduced levels of *Ruminococcus* and *Bdellovibrio*.

Keywords: gene expression, gut microbiota, heat stress, intestinal immunity, yellow-feathered broilers

INTRODUCTION

There is an increasing pursuit of flavor in the huge chicken consumer market in China and almost half of Chinese chicken meat is from locally yellow-feathered broilers because of their good meat quality (Gou et al., 2016). South China is the major production area for yellow-feathered broilers (Wang Y. et al., 2021). However, South China is in tropical and subtropical regions, and heat stress (HS) has become a great challenge for yellow-feathered broiler production (Liu et al., 2019). The HS exposure results in detrimental impacts on growth performance and has been well characterized, such as reduced feed intake, poor feed efficiency and decreased growth rate (Chauhan et al., 2021). Along with reduced growth rate, HS induces multiple deleterious consequences on physiological homeostasis, systemic immune function, metabolism status, and gut health (Guo et al., 2021a; Guo et al., 2021b; Liu et al., 2021a; Wasti et al., 2021a). Hence, HS has been one of the most harmful environmental stressors in broiler production, including the indigenous broilers, which causes large economic losses annually (Lara and Rostagno, 2013; Liu et al., 2019).

The gastrointestinal tract (GIT) is particularly vulnerable to HS, because the vasoconstriction in broiler's GIT is increased to redistribute blood to the peripheral circulation, to maximize heat dissipation under HS situation (Vandana et al., 2021). Thus, HS leads to ischemia-hypoxic injury of the intestinal epithelial barrier and compromises gut integrity (Quinteiro-Filho et al., 2010). Intestinal mucosal immunity has attracted extensive attention for its effectiveness in strengthening the gut barrier function and protecting against pathogenic infection (Kogut and Arsenault, 2016). It has been found that HS-induced gut damage is partly attributed to the inflammatory response, such as an increase of pro-inflammatory cytokines production in broiler's intestines (Song et al., 2017; Wu et al., 2018). On the other hand, the gut microbiota has a variety of biological functions, which is not only involved in the digestion of nutrients but also plays a vital role in maintaining intestinal barrier integrity and modulating the gut immune function of broilers (Yadav and Jha, 2019). With the development of high-throughput sequencing technology, emerging evidence demonstrated that HS disrupted the community structure and composition of hindgut (ileal and cecal) microbiota in broilers (Wang et al., 2018; Shi et al., 2019; Wang G. et al., 2021); but limited information is available about the effects of HS on fore-midgut microbiota in broilers. As the middle segment of the small intestine, the immune and microbial barrier of jejunum is critical for gut health in broilers (Rajput et al., 2017; Li et al., 2019). Furthermore, compared to the fast-growing chickens, including Ross and Cobb broilers, although the yellow-feathered broilers have better heat tolerance due to their slower growth and lower metabolic rate; it has been confirmed that HS also has negative impacts on gut health in this type of broilers (Gou et al., 2016; Liu et al., 2021a). However, there is a lack of information on changes in jejunal immunity, and the microbiome of yellow-feather broilers under HS. In this context, the present study was done to evaluate the effects of HS on jejunal immune response, microbial community, and their correlation in yellow-feathered broilers. Thus, it can provide novel insight into the interaction between the jejunal microbiome and immune response in slow-growing broilers under HS.

MATERIALS AND METHODS

Animals, Experimental Design, and Management

A total of forty female Huaixiang chickens (eight-week-old, slow-growing type Chinese yellow-feathered broilers) were used in a 4 weeks HS trial (9–12 weeks of age during the study). The birds with an initial average body weight (BW) of 840.75 ± 20.79 g were sourced from a local supplier (Zhanjiang, Guangdong, China). The age selection of broilers was made considering the indigenous broilers entering the growing-finishing stage after 8-weeks of age, and growing-finishing broilers are more prone to HS for production (Wasti et al., 2020). The female broilers were only used to exclude the influence of gender on the study. It was also because the local people prefer to consume female yellow-feather broilers (Lin, 2008). Chickens were randomly and equally divided into two treatments in a completely randomized design. The treatments included the normal treatment group (NT) (21.3 ± 1.2°C throughout the experimental period, thermoneutral zone) and HS group (32.5 ± 1.4°C, 8 h/day, from 9:00 am to 17:00 pm). The ambient temperature for the rest of the time of the HS group was the same as the NT group. The relative humidity of NT and HS were maintained at 55–70%. Each group had five replications with four broilers per replicate, and one cage was used as one replicate. The chickens were kept in three-layer wire cages (one cage on top, middle, and bottom, with 20 cm gap between each layer for collecting and cleaning excreta). The size of each cage was 90 (length) × 70 (width) × 40 (height) cm. Plastic trays were used under each cage to collect the excreta, and the excreta were manually cleaned twice a day. The birds were ensured that all chickens had free access to water and feed. All broilers were fed corn-soybean meal basal diet to meet the nutrient requirements recommended by the Chinese chicken breeding standard (NY/T33-2004). The environmental control equipment, diet's composition, and nutrient levels were as reported in our previous study (Guo et al., 2021a).

Sample Collection

At the end of the HS trial, one chicken was randomly selected from each replicate and sacrificed by neck bloodletting (n = 5/group). The jejunum was rapidly separated, and then approximately 2 g of jejunal digesta samples were collected and preserved in the liquid nitrogen and subsequently used for 16S rRNA sequencing. Afterward, the jejunum was opened, and the digesta was washed with PBS at 4°C; the jejunal mucosa was scraped off by glass slides and frozen in liquid nitrogen and then stored at −80°C for further detection of cytokines content and immune-related gene expression.

Determination of Jejunal Cytokines Concentration

The tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin (IL)-1β, IL-2, IL-4, IL-6, and IL-10 concentrations

in jejunal mucosa samples, were analyzed using commercial cytokines ELISA kits of chickens (Catalog numbers: TNF-α, MM-0938O2; IFN-γ, MM-0520O1; IL-1β, MM-36910O2; IL-2, MM-0528O2; IL-4, MM-0527O2; IL-6, MM-0521O2; IL-10 MM-1145O2; Jiangsu Enzyme Immunology Co., Ltd., Suzhou, China) following the manufacturer's instructions.

Detection of Jejunal Immune-Related Genes Expression

Total RNA was extracted from the jejunal mucosa samples using RNA extraction kits (Catalog No. N066, Jiancheng Bioengineering Institute, Nanjing, China). The reverse transcription of cDNA from RNA using RT reagent kits (Catalog No. RR047A, TaKaRa Biotechnology Co., Ltd, Dalian, China). Then the quantitative real-time PCR (qPCR) was performed to detect the mRNA expression of immune-related genes, including *NF-κB, TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-6, and IL-10*. The CFX-96 real-time PCR detection system (BioRad, Irvine, CA, United States) was used for the qPCR reaction. The specific primers, reaction system, and conditions were similar to our previous study (Liu et al., 2021b). β-actin was used as an internal reference gene. The mRNA expression levels of the jejunal immune-related genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and the data were expressed as a relative fold change to the average value of the NT group.

Jejunal Microbiome Analysis

The total microbial DNA of jejunal digesta was extracted using QIAamp DNA Stool Mini Kit (Code No. 51306, QIAGEN, CA, Hamburg, Germany). This study used the Illumina Miseq Platform of Personalbio Technology Co., Ltd. (Shanghai, China) to perform paired-end sequencing of V3-V4 region of 16S rRNA gene. The primer pairs were 338F (5'-ACTCCTACGGGAGGCACAG-3') and 806R (5'- GGACTACHVGGGTWTCTAAT-3'). The processing and analysis of sequencing data was performed using QIIME2 (Quantitative Insights Into Microbial Ecology 2) 2019.4 and according to the official tutorials (https://docs.qiime2.org/2019.4/tutorials/). Briefly, using cutadapt (v2.3) to excise the primer fragments of the raw sequencing reads and to remove the unmatched sequences with the primers. The fastq_mergepairs module of Vsearch (v2.13.4) was used to assemble the sequences; using the fastq_filter module of Vsearch (v2.13.4) to perform quality control on the assembled sequences; the derep_fulllength module of Vsearch (v2.13.4) was used to remove the duplicates sequences, and the uchime_denovo module of Vsearch (v2.13.4) was conducted to remove the chimeras, thereby resulting in high-quality sequences. Subsequently, the high-quality sequences are clustered at the 97% similarity level using the cluster_size module of Vsearch (v2.13.4), and the operational taxonomic units (OTUs) were obtained accordingly. The bacterial taxonomic information corresponding to each OTU was obtained based on the Greengenes database (Release 13.8, http://greengenes.secondgenome.com/).

Statistical Analysis

The data on jejunal cytokines levels, immune-related genes expression, and the difference in the jejunal microbiota were analyzed using SAS 9.4 (SAS Institute Inc., Cary, NC). Student's t-test was performed to compare the differences between the two groups. Results are expressed as mean ± standard error. The alpha diversity, beta diversity, and the composition of jejunal microbiota were analyzed using Gene Cloud Analysis Platform (based on the kernel of QIIME 2, https://www.genescloud.cn) of Personalbio Technology Co., Ltd. (Shanghai, China). The Spearman correlation analysis was performed to determine the association between the jejunal microbiota (at the phylum and genus level) and immune-related genes expression using online software (based on the kernel of QIIME 2, https://www.genescloud.cn) of Personalbio Technology Co., Ltd. (Shanghai, China). The probability (P) < 0.05 was considered to be significant, and $0.05 \leq p < 0.10$ was considered to be a trend of significance.

TABLE 1 | Effects of heat stress on cytokines levels in jejunal mucosa of yellow-feathered broilers (n = 5).

Items	NT group	HS group	p-value
TNF-α, pg/mg protein	136.52 ± 8.76	188.87 ± 10.09	0.005
INF-γ, pg/mg protein	388.10 ± 8.59	376.65 ± 15.51	0.533
IL-1β, pg/mg protein	430.44 ± 7.36	481.72 ± 14.92	0.015
IL-2, ng/mg protein	4.76 ± 0.19	4.37 ± 0.38	0.392
IL-4, ng/mg protein	79.60 ± 4.98	87.14 ± 5.32	0.331
IL-6, pg/mg protein	59.76 ± 6.41	79.47 ± 4.77	0.039
IL-10, ng/mg protein	420.21 ± 8.89	437.65 ± 4.62	0.121

NT, normal treatment group, the broilers reared at 21.3 ± 1.2°C throughout the experimental period; HS, the broilers reared at 32.5 ± 1.4 C for 8 h/day (9:00 am to 17:00 pm); the abbreviations for the detected parameters (TNF-α, IFN-γ and IL) are tumor necrosis factor-α, interferon-γ and interleukin, respectively.

RESULTS

Jejunal Cytokines Content

The jejunal cytokines concentration treatment groups are presented in **Table 1**. Compared to NT group, broilers in HS group had higher levels of TNF-α, IL-1β, and IL-6 in jejunal mucosa ($p < 0.05$). There were no significant differences between NT and HS groups in jejunal INF-γ, IL-2, IL-4, and IL-10 contents ($p > 0.10$).

Jejunal Immune-Related Genes Expression

As illustrated in **Figure 1**, broilers exposed to HS up-regulated the relative fold changes of *NF-κB, TNF-α, IL-1β,* and *IL-6* ($p < 0.01$) while down-regulated the relative fold changes of *IFN-γ* in jejunal mucosa ($p < 0.05$). In addition, the HS exposure had no significant impact on jejunal relative fold changes of *IL-2, IL-4, and IL-10* ($p > 0.10$).

Microbial Diversity and Composition in Jejunum

As shown in **Table 2**, the HS exposure had no significant effects on the alpha diversity of jejunal microbiota, including Simpson, Chao1 richness estimator (Chao 1), abundance-based coverage estimators (ACE), and Shannon index ($p >$

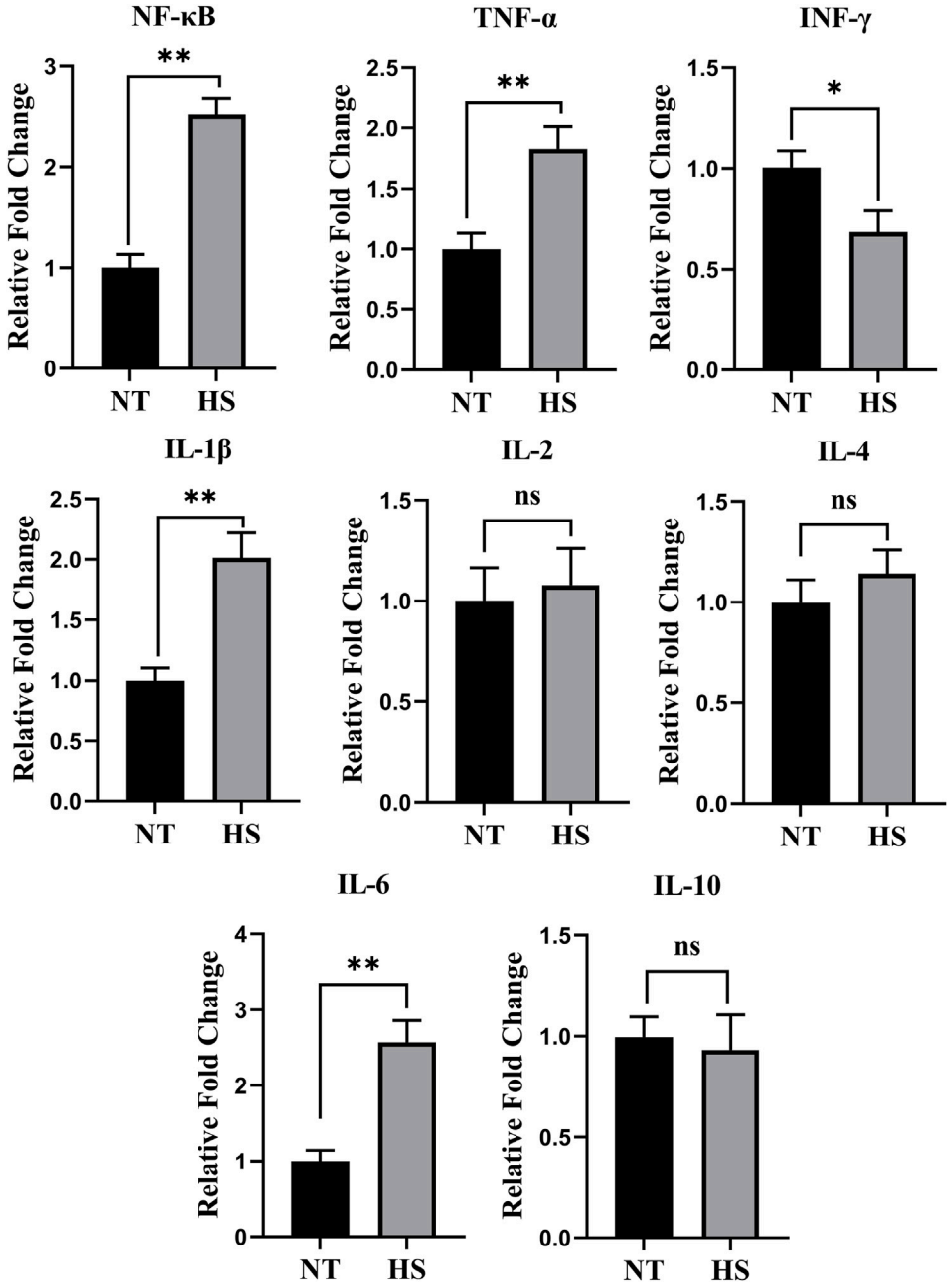

FIGURE 1 | Effects of heat stress on relative fold change of jejunal immune-related genes in yellow-feathered broilers (n = 5). NT, normal treatment group (21.3 ± 1.2°C, 24 h/day); HS, heat stress group (32.5 ± 1.4°C, 8 h/day); the abbreviations for the detected parameters (NF-κB, TNF-α, IFN-γ and IL) are nuclear factor-kappa B, tumor necrosis factor-α, interferon-γ and interleukin, respectively. *p < 0.05, **p < 0.01, ^{ns}no significant difference.

0.10). However, as presented in **Table 3** and **Figure 2**, the HS exposure reduced the jejunal microbial species number at the class and order level (p < 0.05) and had a significant trend to decrease the jejunal microbial species number at the phylum and family level (p < 0.10).

The results of the jejunal microbial community are presented in **Figures 3**, **4**. There were 884 commonly owned OTUs between the NT and HS groups, 243 OTUs of jejunal microbiota were unique in the NT group, and 270 OTUs of jejunal microbiota were unique in the HS group. The beta diversity revealed that the samples in NT and HS groups have different clusters. Besides, there were no significant differences in the relative abundance of each microbial at the phylum, class, order, and family level (p > 0.10). However, the HS exposure significantly reduced the jejunal relative abundance of *Ruminococcus*, *Bdellovibrio,* and *Serratia* at the genus level (p < 0.05).

TABLE 2 | Effects of heat stress on alpha diversity of jejunal microbiota in yellow-feathered broilers (n = 5).

Items	NT group	HS group	*p*-value
Simpson	0.85 ± 0.43	0.88 ± 0.03	0.568
Chao 1	523.07 ± 38.30	552.37 ± 59.17	0.688
ACE	544.31 ± 41.30	573.16 ± 60.74	0.705
Shannon	4.71 ± 0.41	5.00 ± 0.32	0.594

NT, normal treatment group, the broilers reared at 21.3 ± 1.2 C throughout the experimental period; HS, the broilers reared at 32.5 ± 1.4 C for 8 h/day (9:00 am to 17:00 pm); Chao1, Chao1 richness estimator; ACE, abundance-based coverage estimators.

TABLE 3 | Effects of heat stress on jejunal microbial species number at each classification level in yellow-feathered broilers (n = 5).

Items	NT group	HS group	*p*-value
Phylum	7.80 ± 0.58	6.6 ± 0.25	0.094
Class	14.80 ± 1.07	11.80 ± 0.37	0.029
Order	24.60 ± 0.87	20.00 ± 1.05	0.010
Family	38.60 ± 2.62	31.00 ± 2.74	0.080
Genus	44.40 ± 4.76	32.60 ± 4.80	0.119
Species	19.20 ± 2.48	13.40 ± 2.52	0.140

NT, normal treatment group, the broilers reared at 21.3 ± 1.2 C throughout the experimental period; HS, the broilers reared at 32.5 ± 1.4 C for 8 h/day (9:00 am to 17:00 pm).

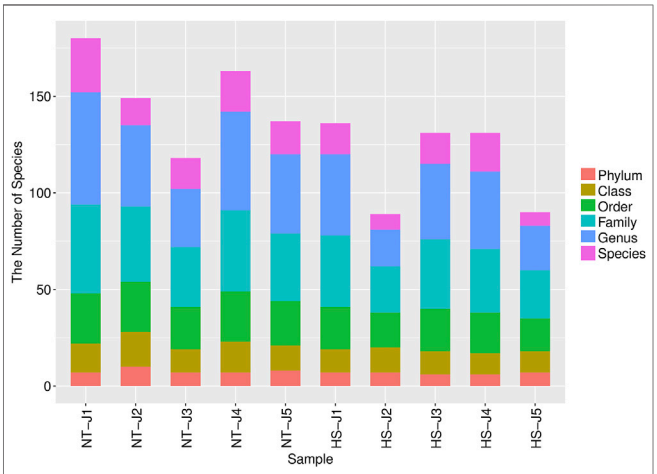

FIGURE 2 | Effects of heat stress on distribution of jejunal microbial species number at each classification level in yellow-feathered broilers (n = 5). NT, normal treatment group (21.3 ± 1.2°C, 24 h/day); HS, heat stress group (32.5 ± 1.4°C, 8 h/day).

Correlation Between Jejunal Microbiome and Immune-Related Genes Expression

The results of Spearman correlation analysis are showed in **Figure 5**. At the phylum level, *Cyanobacteria* was positively correlated with *IL-10* expression ($p < 0.05$). *Bacteroidetes* was negatively correlated with *IL-6* expression ($p < 0.05$). *Thermi* was negatively correlated with *TNF-α* and *IL-6* expression ($p < 0.05$), while positively correlated with *IL-10* expression ($p < 0.01$). *TM7* was negatively correlated with *NF-κB* expression ($p < 0.05$), but positively correlated with *IFN-γ* expression ($p < 0.01$). At the genus level, *Caulobacteraceae* was negatively correlated with *TNF-α* and *IL-6* expression ($p < 0.05$), whereas positively associated with *IL-10* expression ($p < 0.05$). *Actinomyces* and *Sediminibacterium* were positively related with *IFN-γ* expression ($p < 0.01$). *Ruminococcaceae* was negatively correlated with *NF-κB, IL-1β* and *IL-6* expression ($p < 0.05$). *Thermus* was negatively correlated with *TNF-α* and *IL-6* expression ($p < 0.05$), but positively correlated with *IL-10* expression ($p < 0.01$). *Bdellovibrio* was negatively correlated with *TNF-α* ($p < 0.05$) and *IL-6* ($p < 0.01$) expression. *Clostridiales* was negatively related with *NF-κB* ($p < 0.05$), *TNF-α* ($p < 0.01$), *IL-1β* ($p < 0.01$) and *IL-6* ($p < 0.01$) expression. *Bacteroides* was negatively associated with *IL-1β* expression ($p < 0.01$). *Sphingomonadales* was negatively correlated with *NF-κB* expression ($p < 0.05$), but positively related with *IFN-γ* expression ($p < 0.01$). *Ruminococcus* was negatively related with *NF-κB* ($p < 0.05$), *TNF-α* ($p < 0.05$), *IL-1β* ($p < 0.05$) and *IL-6* ($p < 0.01$) expression.

DISCUSSION

Due to the global climate change, high ambient temperature (around 32°C) often occurs in chicken houses during summer, thus impairing the health and productivity of broilers and resulting in significant economic losses to producers (Wasti et al., 2020; Greene et al., 2021). Although the yellow-feathered broilers have better thermal tolerance than fast-growing broilers due to their slower growth and lower metabolic rate, 32°C is not in the thermoneutral zone of slow-growing broilers, which also causes a series of deleterious consequences for yellow-feathered broilers (Shao et al., 2018; Guo et al., 2021a; Liu et al., 2021b). The gut is considered the main target of HS (Chauhan et al., 2021). The jejunum is the middle intestine and is related to most of the nutrients' digestion and absorption in broilers; accordingly, the jejunum is more susceptible to HS (Song et al., 2013). Furthermore, as a representative segment of the small intestine, the jejunum is always selected for intestinal research, and jejunal immunity plays a crucial role in maintaining gut health (Song et al., 2014; Abdelli et al., 2021; Wang C. et al., 2021). However, very limited data is available regarding HS exposure's effects on jejunal immune response in yellow-feathered broilers. In the present research, the ELISA and mRNA results indicated that HS exposure elevated the production of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in jejunal mucosa. In accordance with the studies of fast-growing broilers, Song et al. (2017) found that the HS has increased the mRNA expression levels of pro-inflammatory cytokines such as IL-1β and IL-6 in the jejunum of Arbor Acres broilers. Siddiqui et al. (2020) demonstrated that Ross broilers exposed to HS had high relative mRNA expression levels of jejunal pro-inflammatory cytokine genes (*IL-6* and *TNF-α*). At the same time, Al-Zghoul and Saleh (2020) observed that HS exposure up-regulated the jejunal mRNA expression of *TNF-α*, *IL-1β*, and *IL-6*. Furthermore, this study found that HS promoted the jejunal mRNA expression levels of *NF-κB*. The *NF-κB* is widely reported to activate gut inflammatory responses

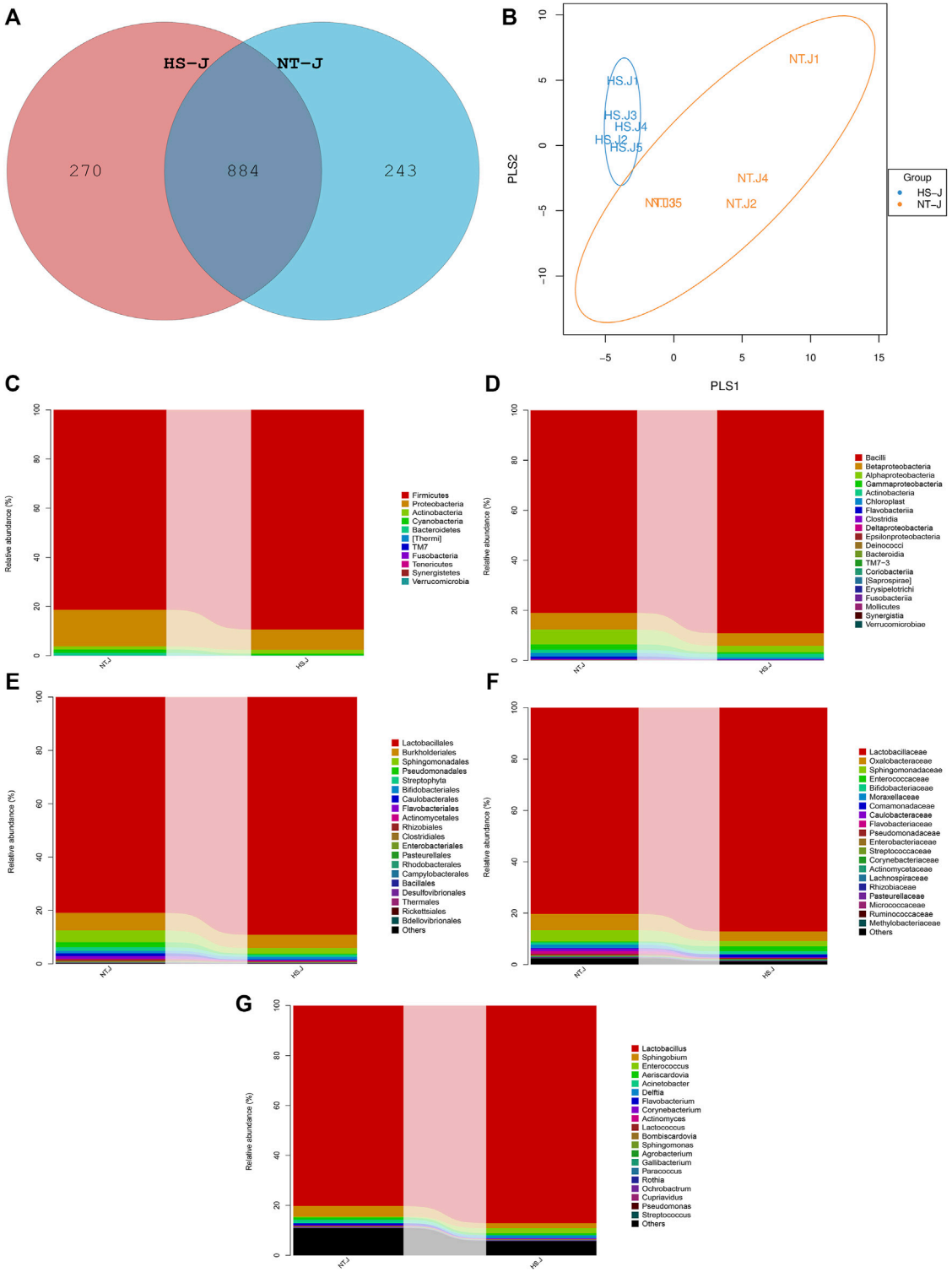

FIGURE 3 | Effects of heat stress on jejunal microbial community in yellow-feathered broilers ($n = 5$). NT, normal treatment group (21.3 ± 1.2°C, 24 h/day); HS, heat stress group (32.5 ± 1.4°C, 8 h/day); **(A)**, operational taxonomic units (OTUs) venn diagram of jejunal microbiota between NT and HS groups; **(B)**, beta diversity of jejunal microbiota between NT and HS groups; **(C)**, microbial compositions at the phylum level; **(D)**, microbial compositions at the class level; **(E)**, microbial compositions at the order level; **(F)**, microbial compositions at the family level; **(G)**, microbial compositions at the genus level.

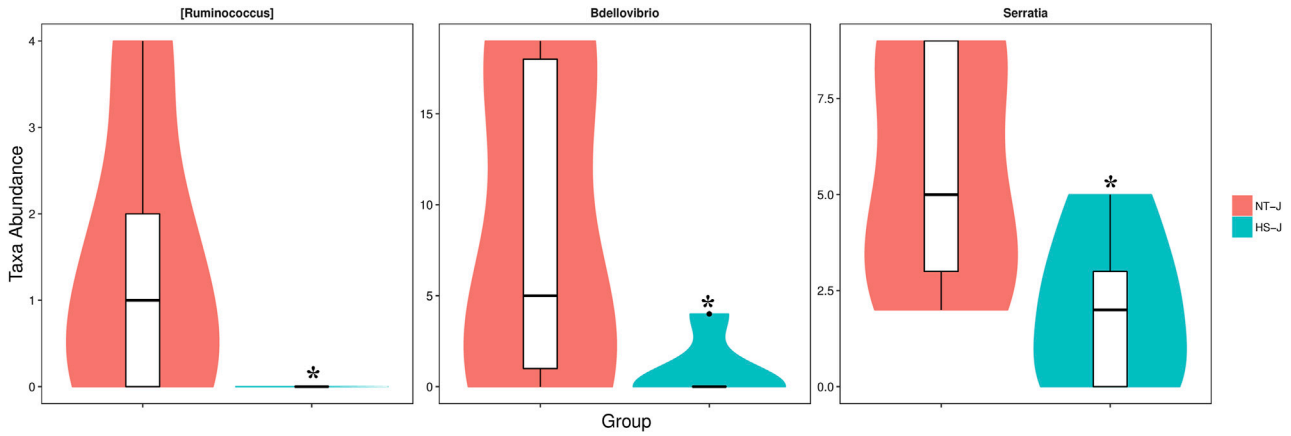

FIGURE 4 | Microbials with significantly differential abundance at the genus level as affected by heat stress in yellow-feathered broilers ($n = 5$). NT, normal treatment group (21.3 ± 1.2°C, 24 h/day); HS, heat stress group (32.5 ± 1.4°C, 8 h/day) *$p < 0.05$.

(Sahin, 2015; Liu et al., 2016). Therefore, the findings of this study further confirmed the theory that HS exposure triggers an intestinal inflammatory response in both fast- and slow-growing broilers, which is probably involved in the activation of *NF-κB* signaling.

Gut microbiota homeostasis is critical for eliminating pathogens, maintaining intestinal epithelial integrity, and regulating mucosal immunity in the GIT (Cao et al., 2021). Existing studies have primarily focused on the effect of HS on the hindgut microbiota of broilers and analyzing the role of microbiota in mediating small intestinal health (Patra and Kar, 2021; Yadav et al., 2021). However, the microbiota colonized in the jejunum can directly crosstalk with the barrier integrity and immune function (Gong et al., 2020). Thus, this study detected the jejunal microbiota structure and community using 16S rRNA sequencing. The alpha diversity of jejunal microbiota was not affected by HS exposure. Similar findings from hindgut microbiota were obtained previously. For instance, Liu et al. (2021a) reported that HS exposure had no significant impact on the alpha diversity index of cecal microbiota in yellow-feathered broilers. Wasti et al. (2021b) suggested that there were no obvious changes in cecal microbial alpha diversity such as Shannon entropy and Simpson's index of Cobb broilers under HS. Moreover, in this study, the jejunal microbial species number was decreased by HS, suggesting that although HS exposure did not alter the alpha diversity, it reduced the species richness of jejunal microbiota in yellow-feathered broilers. Regarding the microbial composition, HS had no significant effects on the relative abundance of microbiota at the phylum, class, order, and family level. Partly consistent with our results, Shi et al. (2019) found that 28 days of HS did not change the cecal microbial abundance at the phylum level. According to Wang G. et al. (2021), there were no significantly different abundances of cecal bacteria at the phylum level in Arbor Acres broilers under HS. On the contrary, in the study of Liu et al. (2021a), there were cecal bacteria with significantly different abundances at the phylum, class, order, and family level in yellow-feathered broilers exposed to HS. Many factors could contribute to the regulation of gut microbiota composition, such as diet, host genetic background, stress intensity, and intestinal segments (Xia et al., 2022), which might explain the inconsistencies. Notably, HS exposure significantly reduced the relative abundance of *Ruminococcus*, *Bdellovibrio,* and *Serratia* at the genus level. *Ruminococcus* can break down the cellulose and ferment glucose, xylose, and polysaccharides, producing beneficial metabolites such as acetate, propionate, and butyrate, which have anti-inflammatory functions in the gut (La Reau and Suen, 2018; Jha and Mishra, 2021). It has been found that the abundance of *Ruminococcus* was reduced in the intestinal inflammatory process of ulcerative colitis and revealed its probiotic and gut health-promoting property (Li et al., 2020). *Bdellovibrio* has bacteriophage-like action to control harmful bacteria (Sockett and Lambert, 2004) and plays a probiotic role in improving gut health (Dwidar et al., 2012). A previous study reported that *Bdellovibrio* enters the intestine and releases peptidoglycan and immune substances through glycanase and peptidase, thereby exerting immunomodulatory activity (Chen et al., 2018). Additionally, *Serratia* is a type of intestinal commensal microbial in animals; the prodigiosin, a metabolite from *Serratia,* which exhibits anticancer and antibacterial functions (Soenens and Imperial, 2020). However, some strains of *Serratia* are pathogenic (Abreo and Altier, 2019); the studies on *Serratia,* including the beneficial and pathogenic strains in poultry intestines, are limited. It is necessary to further isolate and identify the *Serratia* species in the GIT of broilers and clarify their roles in HS response. Based on the potential immunomodulatory and antibacterial effects of the microbiota with significantly different abundances, it could be speculated that HS-induced jejunal immune dysfunction might be related to the reduced levels of *Ruminococcus*, *Bdellovibrio,* and *Serratia*, but the specific interaction analysis is required.

Emerging evidence linking HS to intestinal damage suggests that the gut microbiota might be an under-appreciated mediator of the inflammatory response (Wen et al., 2021). To delineate the interaction of jejunal microbiota and immunity in yellow-

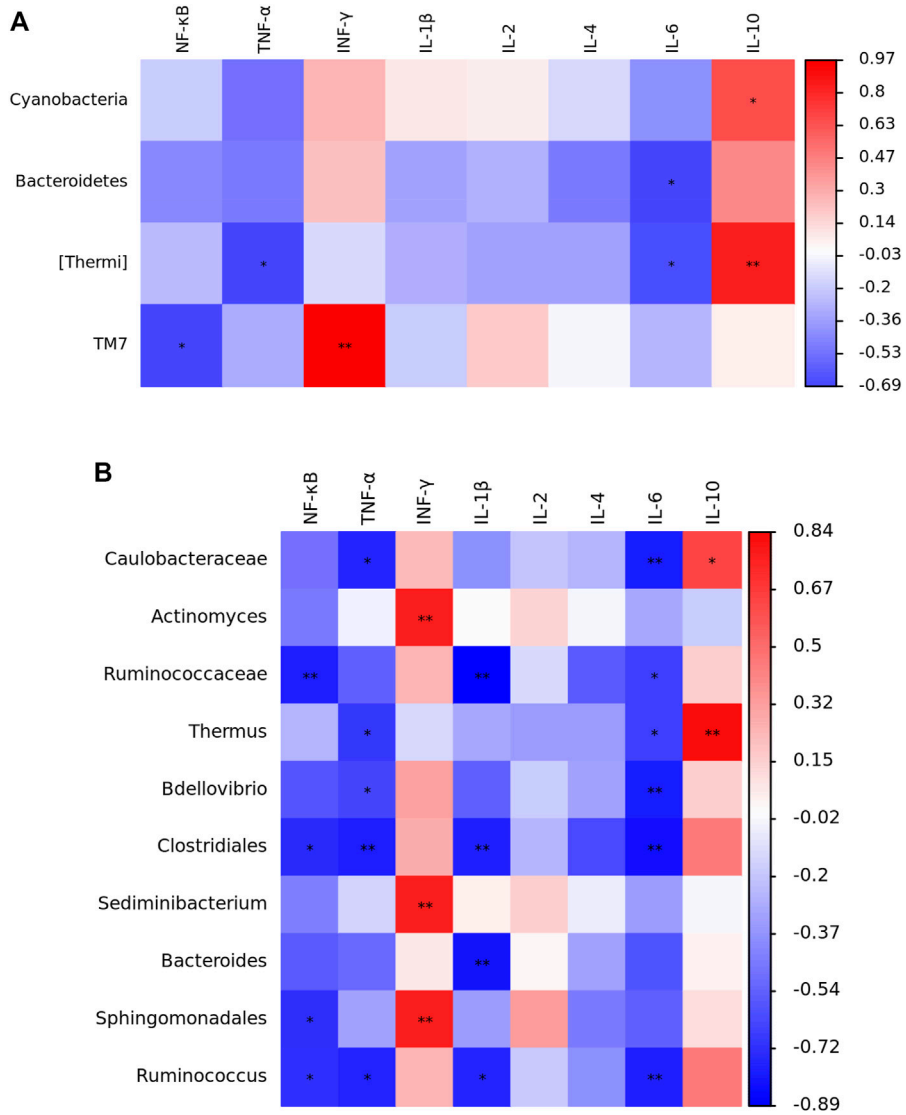

FIGURE 5 | Spearman correlation analysis between the jejunal microbials **(A)**, phylum; **(B)**, genus level and immune-related genes expression in yellow-feathered broilers under heat stress (n = 5). NT, normal treatment group (21.3 ± 1.2°C, 24 h/day), HS, heat stress group (32.5 ± 1.4°C, 8 h/day); NF-κB, nuclear factor-kappa B; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL, interleukin. *p < 0.05, **p < 0.01.

feathered broilers, Spearman correlation analysis was performed in this study. At the phylum level, four species of bacteria were significantly associated with immune genes expression, which were positively correlated with anti-inflammatory cytokines and negatively correlated with pro-inflammatory cytokines. *Bacteroidetes* is a common parasitic microbial in the gut; it also showed a negative correlation with intestinal pro-inflammatory cytokines in pigs (Xia et al., 2022). There are few studies on the relationship between *Cyanobacteria*, *Thermi*, *TM7,* and immune response; further research is needed to elucidate this correlation (Whitton and Potts, 2012; Rinttilä and Apajalahti, 2013; Chen et al., 2021). At the genus level, ten species of bacteria were significantly related to the expression of immune genes. Specifically, the abundances of *Ruminococcus* and *Bdellovibrio* were negatively associated with the pro-inflammatory cytokine expression and *NF-κB* signaling; this may be due to the anti-inflammatory and immunomodulatory activities of the metabolites produced by *Ruminococcus* and *Bdellovibrio* (Chen et al., 2018; Li et al., 2020). Besides, *Actinomyces* has been reported to regulate the immune system, especially cellular immunity, and positively correlated with the production of INF-γ (Hötte et al., 2019); this is consistent with our results. The correlation result of *Ruminococcaceae* was similar to the *Ruminococcus.* A previous study also suggested that the *Ruminococcaceae* was involved in regulating inflammatory bowel disease through secondary bile acids (Guo et al., 2020). *Clostridiales* exhibited a significant negative correlation with pro-inflammatory cytokines and *NF-κB* signaling. The *Clostridium butyricum* of *Clostridiales* is a probiotic and has anti-inflammatory properties

in broilers (Zhang et al., 2014). Conversely, the *Clostridiales* also contain some pathogenic bacteria species (Paredes-Sabja et al., 2011). Therefore, the role of *Clostridiales* in modulating gut immunity is subject to further verification. Furthermore, the *Bacteroides* showed a similar correlation trend with *Bacteroidetes* at the phylum level. However, other microbiota related to immune genes expression, such as *Caulobacteraceae, Thermus, Sediminibacterium,* and *Sphingomonadales*, their function with immunity is still unclear and needs to be elucidated. Together, integrating the bacterial abundances and correlation analysis, it can be noted that *Ruminococcus* and *Bdellovibrio* may be the key microbially mediated elements of the jejunal inflammatory response during HS. And, dietary supplementation of *Ruminococcus* and *Bdellovibrio* as potential probiotics may alleviate HS-induced intestinal inflammation in yellow-feathered broilers; further validation of this hypothesis would be valuable.

CONCLUSION

Four weeks of heat stress at 32.5°C adversely affected jejunal immunity via stimulating the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 and activating the *NF-κB* signaling. Heat stress had no significant impact on alpha diversity. The relative abundance of *Ruminococcus*, *Bdellovibrio,* and *Serratia* in the jejunum was decreased by heat stress. Furthermore, *Ruminococcus* and *Bdellovibrio* showed a significant negative correlation with pro-inflammatory cytokines expression. The findings not only provide novel insights into the interaction of jejunal immune response and microbiota, but also contribute to the development of potential nutritional mitigation strategies (probiotics intervention) for the regulation of intestinal immunity in yellow-feathered broilers under heat stress situations.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA816650.

ETHICS STATEMENT

The animal study was reviewed and approved by Department of Animal Science of Guangdong Ocean University (dkx-20181217).

AUTHOR CONTRIBUTIONS

Conceptualization, W-CL, BB, and RJ. Methodology, W-CL and RJ. Analysis, M-YH. Data curation, W-CL and M-YH. Writing-original draft preparation, W-CL. Writing-review and editing, BB and RJ. Supervision, BB and RJ. Project administration, W-CL. Funding acquisition, W-CL.

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METHODS
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A Poultry Subclinical Necrotic Enteritis Disease Model Based on Natural *Clostridium perfringens* Uptake

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Necrotic enteritis (NE) in poultry is an opportunistic infection caused by *Clostridium perfringens*. Well-known as a multifactorial disease, NE development is under the influence of a wide range of environmental risk factors that promote the proliferation of pathogenic *C. perfringens* at the expense of nonpathogenic strains. Current *in vivo* NE challenge models typically incorporate pre-exposure to disease risk factors, in combination with exogenous *C. perfringens* inoculation. Our goal was to enhance current models using a natural uptake of *C. perfringens* from the barn environment to produce a subclinical infection. We incorporated access to litter, coccidial exposure (either 10× or 15× of the manufacturer-recommended Coccivac B52 *Eimeria* vaccine challenge; provided unspecified doses of *E. acervulina, E. mivati, E. tenella*, and two strains of *E. maxima*), feed composition, and feed withdrawal stress, and achieved the commonly observed NE infection peak at 3 weeks post-hatch. NE severity was evaluated based on gut lesion pathology, clinical signs, and mortality rate. Under cage-reared conditions, 15× coccidial vaccine-challenged birds showed overall NE lesion prevalence that was 8-fold higher than 10× coccidial vaccine-challenged birds. NE-associated mortality was observed only in a floor-reared flock after a 15× coccidial vaccine challenge.

Keywords: broiler chicken, *Clostridium perfringens*, environment, natural infection model, necrotic enteritis

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1 INTRODUCTION

Necrotic enteritis (NE) is an economically important infectious disease for the global poultry sector, causing an annual loss of US $6 billion worldwide (Wade and Keyburn, 2015). This is largely attributable to the costs of prophylactic and therapeutic medications and compromised growth performance. The causative bacterium, *Clostridium perfringens*, is ubiquitously distributed and comprises part of the gut microbiota of healthy chickens, with a high diversity of strains representing the total *C. perfringens* population (Engström et al., 2012; Yang et al., 2018; Kiu et al., 2019). The NE-causing strains are characterized by a capacity to produce necrotic enteritis toxin B (NetB) and possession of genes that function to enhance their proliferation, maintenance, and virulence, including antibiotic resistance genes, adhesins, catabolic enzymes, toxins, and bacteriocins (Bannam et al., 2011; Parreira et al., 2012; Freedman et al., 2015; Keyburn et al., 2008).

One key step in NE pathogenesis is the dominance of pathogenic *C. perfringens* in the gut flora over nonpathogenic strains, followed by profound expression of virulence factors. A number of risk factors promote pathogenic *C. perfringens* development, and hence NE infection. Exposure to coccidial parasites remains one of the best-studied factors due to the strong link between coccidiosis

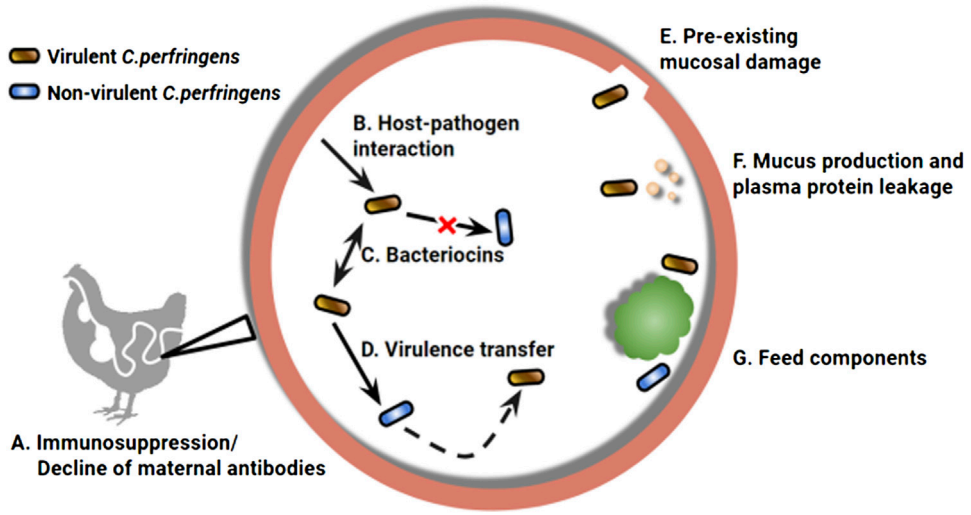

FIGURE 1 | A wide range of environmental factors determines the manifestation of necrotic enteritis (NE) caused by *Clostridium perfringens*. Chickens are at higher risk to develop NE infection with impaired immune status (A), which can result from declining maternal antibodies and/or pre-exposure to immunosuppressive factors (Lee et al., 2011). The intestine environment also plays a role in virulence modulation of *C. perfringens*, the first example can be seen in the upregulated toxin expression when in close contact with epithelial cells (B) (Vidal et al., 2009). A complex microbiota environment, compared to a culture medium, can differentially regulate the virulence phenotype of the bacterium *via* inter and intra strain interactions through bacteriocin production (C) (Ohtani and Shimizu, 2015). Virulent strains are capable of producing bacteriocins that strongly inhibit non-virulent strains, as a mechanism in pathogen-commensal competition (Barbara et al., 2008; Timbermont et al., 2009). Meanwhile, non-virulent strains can inherit the virulence genes horizontally (D), leading to the emergence of new strains that are capable of causing NE (Lacey et al., 2017). Preexisting mucosal damage that exposes epithelial extracellular matrix (E), increased plasma protein leakage and mucus production (F) can provide an extra advantage for pathogenic *C perfringens*, which possess a stronger binding ability and mucolytic activity (Collier et al., 2008; Martin and Smyth, 2010). Diet components comprising part of the gut environment are also key risk factors associated with NE development (G). For example, feeds rich in water-soluble non-starch polysaccharides, such as a wheat-based diet, can increase digesta viscosity, prolong transit time, and promote pathogen retention (Annett et al., 2002; Shojadoost et al., 2012).

and NE disease (Stanley et al., 2014). Coccidiosis-induced epithelial extracellular matrix disruption, plasma protein leakage, and mucus production provide extra selective advantages for pathogenic *C. perfringens* which possess a stronger binding ability and mucolytic activity than nonpathogenic strains (Collier et al., 2008; Martin and Smyth, 2010). Diet components also constitute relevant key risk factors associated with NE development. Feeds rich in water-soluble non-starch polysaccharides, such as wheat-based diets, increase digesta viscosity, prolong transit time, and promote pathogen retention (Annett et al., 2002; Shojadoost et al., 2012). Increased NE occurrence is also associated with poor husbandry management, such as food deprivation, inadequate hygiene routines, and overcrowding (Hofacre et al., 2019).

Given the multifactorial nature of NE, the production of experimental infections similar to field conditions is known to be challenging. Typically, induced NE outbreaks should occur around week three post-hatch, reflecting the timing when animals in the field are most at risk (Williams, 2005; Moore, 2016). Additionally, the model should yield a high incidence of necrotic lesions without severe mortality in the flock (Dierick et al., 2021). This is relevant to the field situation where subclinical infections are more common and account for larger economic loss compared to the clinical form of NE (Wade & Keyburn, 2015). Efforts over the past decade suggest that concentrated live coccidial vaccines in combination with multiple dosages of *C. perfringens* culture result in NE infections that fulfill these criteria (Gholamiandehkordi et al., 2007; Dierick et al., 2021). Recent NE studies often adopt this dual-infection approach concurrently with the application of dietary and management risk factors (Dierick et al., 2019; Lee et al., 2013; M'Sadeq et al., 2015; Onrust et al., 2018; Wilson et al., 2018).

Induction of experimental NE using natural exposure to *C. perfringens* further mirrors conditions under which NE arises in commercial operations. This approach has gained prominence over the past decade (Abildgaard et al., 2010; Calik et al., 2019; Emami et al., 2019 and 2021; Fernando et al., 2011; Lovland et al., 2003; Palliyeguru et al., 2010). Compared to conventional models that drive infection *via* experimental application of *C. perfringens*, natural NE infection can A) simplify the disease challenge protocol, B) eliminate the variation between models caused by different bacterial culture conditions, challenge route, dosage, timing, and frequency, and C) develop subclinical infection most similar to the field condition. Importantly, *C. perfringens* undergoes a series of adaptations in response to fluctuation of the gut environment, which modify the disease-causing ability of this bacterium (**Figure 1**). Given the highly plastic phenotype that *C. perfringens* can display natural infection also overcomes deviations commonly associated with *in vitro* manipulation, including changes to colonization efficacy and toxin production (Parreira et al., 2016). Thus, the natural infection

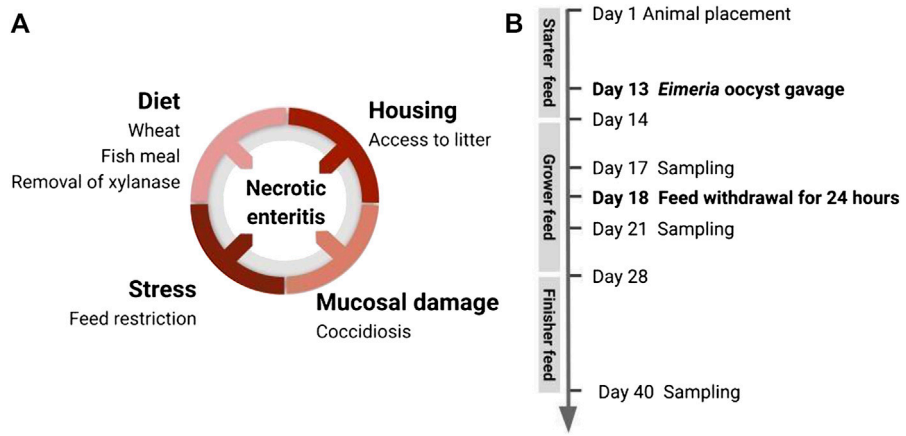

FIGURE 2 | Induction of subclinical NE in broiler chicken by application of disease predisposing factors**. (A)** To promote natural development of the already-existing virulent *C perfringens* in the gut, this disease model incorporated multiple predisposing factors: housing condition, diet components, coccidiosis-induced mucosal damage, and stressor leading to alteration of gut environment. **(B)** Timeline of animal handling and sampling schedule.

approach can better recapitulate the microbial loads and other relevant physiological factors that contribute to NE pathogenesis.

Our objective was to validate a natural, subclinical NE challenge model. Aiming to optimize the current natural NE model, we incorporated a novel stressor, a 24-h feed withdrawal at day 18 post-hatch, apart from other commonly used risk factors for inducing experimental NE. This nutrient alteration in the gut lumen aims to disrupt the intestinal microbial community and promote the development of pathogenic *C. perfringens*. To examine whether this infection protocol induces subclinical NE in different rearing conditions, we challenged three experimental flocks with different housing types, diet regimens, and two levels of coccidial challenge intensity. Our results suggested timely application of stress factors (**Figure 2**) resulted in a consistent NE infection similar to the field situation, characterized by a high incidence of gut lesions in the flock with a low mortality rate.

2 MATERIAL AND METHODS

2.1 Animals and Natural Necrotic Enteritis Challenge

One-day-old Ross 708 broiler chicks were obtained from a local hatchery (Sofina Foods) and housed in the Poultry Research Center at the University of Alberta, Edmonton, Canada. The natural NE infection model was developed stepwise using a total of 752 animals from three experimental flocks. Animals in flocks 1 and 3 were randomly assigned to two dietary treatments to evaluate the impact of antibiotic removal on NE development (flock 1: antibiotic treatment with 21 cages of 8 birds, and drug-free treatment with 22 cages of 8 birds; flock 3: antibiotic treatment with 8 pens of 18 birds, drug-free treatment with 8 pens of 18 birds). Animals in flock 2 were used for evaluating the immunomodulation effect of β-glucan and were randomly assigned to three injection treatments (each with 5 cages of 8 birds). Flocks 1 and 2 were reared in Specht pullet cages (21 × 23.5 × 17.5 inches, Specht Canada Inc.), and flock 3 was housed in the floor pens (0.9 m × 1.4 m). All three flocks were treated with a natural NE challenge procedure but with different levels of coccidiosis challenge intensity (**Table 3**).

Birds from all three flocks were fed a wheat-based diet formulated to meet or exceed the management guide recommendations for all nutrients. The experimental diets were administered as a starter diet, grower diet, and finisher diet (**Table 1**). The diet composition for flock 3 was adjusted as part of an adaptation of the model to include a more practical commercial-type diet. Feed and water were provided ad libitum. Temperature and lighting were monitored daily and adjusted according to the Ross 708 guidelines (Aviagen, 2019). On day 13, a 10× (flock 1) or 15× (flocks 2 and 3) dose of the Coccivac-B52 vaccine (Merck Animal Health) containing live, sporulated *Eimeria* oocysts (*E. acervulina*, *E. mivati*, *E. tenella*, and two strains of *E. maxima* at unspecified doses) was administered through oral gavage. Each bird received 1 ml of vaccine diluted in distilled water. On day 18, the feed was withdrawn for 24 h with animals being closely monitored for health over the subsequent 3 days. **Figure 2B** shows the predisposing factors application timeline in the natural NE challenge model.

2.2 Sampling and Lesion Scoring

For experimental flocks 1 and 2, animals were randomly selected and examined for NE disease status on days 17, 21, and 40 (flock 1: $n = 16$, flock 2: $n = 18$). Flock 3 was sampled on day 21 and day 40 ($n = 128$). The NE-specific lesions in the small intestine were scored as described by Shojadoost et al. (2012) with some modifications. Animals were scored from 0 to 3 based on the following criteria:

0: No gross lesion;

1: Thin or friable walls, or diffuse superficial fibrin;

2: Focal necrosis or ulceration, or non-removable fibrin deposit;

3: Multifocal necrosis or ulceration, or nonremovable fibrin deposit.

TABLE 1 | Ingredient and calculated nutrient composition of experimental diets for birds during starter, grower, and finisher stages.

	Flocks 1 and 2			Flock 3		
	Starter	**Grower**	**Finisher**	**Starter**	**Grower**	**Finisher**
Ingredients (%)						
Canola meal	5	7.5	10	7.5	10	12
Fish meal	4	4	4	-	-	-
Soybean meal	24.05	17.62	11.75	27.96	22.44	19.38
Wheat	62.25	65.44	67.18	59.18	61.46	61.40
Limestone	0.92	0.78	0.66	1.18	1.03	0.93
Monocalcium phosphate	0.43	0.20	-	1.00	0.75	0.57
NaCl	0.30	0.30	0.30	0.27	0.26	0.26
L-Lysine	0.06	0.06	0.92	0.10	0.07	0.02
DL-Methionine	0.26	0.22	0.2	0.30	0.25	0.23
L-Threonine	0.05	0.03	0.01	0.05	0.01	-
Hy-D® Premix[a]	0.05	0.05	0.05	0.05	0.05	0.05
Vitamin Mineral Premix[b]	0.5	0.5	0.5	0.5	0.5	0.5
Choline Chloride Premix[c]	0.05	0.05	0.05	0.05	0.05	0.05
Phytase[d]	0.01	0.01	0.01	0.01	0.01	0.01
Canola oil	1.62	2.79	3.92	1.86	3.12	4.63
Mycotoxin binder[e]	0.05	0.05	0.05	0.15	0.15	0.15
Xylanase[f]	—	—	—	0.05	0.05	0.05
Calculated nutrient composition						
Crude protein	25.5	23.62	22.65	25.2	23.58	22.73
ME, kcal/kg	3,000	3,100	3,200	3,000	3,100	3,200
Calcium	0.96	0.87	0.79	0.96	0.87	0.81
Available phosphorus	0.48	0.435	0.395	0.48	0.435	0.405

[a]*Provided 69 μg 25-hydroxycholecalciferol per kg diet.*
[b]*Provided per kilogram of diet: vitamin A (retinyl acetate), 10,000 IU; cholecalciferol, 4,000 IU; vitamin E (DL-α-tocopheryl acetate), 50 IU; vitamin K, 4.0 mg; thiamine mononitrate (B_1), 4.0 mg; riboflavin (B_2), 10 mg; pyridoxine HCL (B_6), 5.0 mg; vitamin B_{12} (cobalamin), 0.02 mg; D-pantothenic acid, 15 mg; folic acid, 0.2 mg; niacin, 65 mg; biotin, 1.65 mg; iodine (ethylenediamine dihydroiodide), 1.65 mg; Mn (MnSO4H2O), 120 mg; Cu, 20 mg; Zn, 100 mg, Se, 0.3 mg; Fe ($FeSO_4{\cdot}7H_2O$), 800 mg.*
[c]*Provided 100 mg choline per kg of diet.*
[d]*Provided 500 FTU phytase per kg of diet (Phyzyme XP, Danisco Animal Nutrition, Marlborough, United Kingdom).*
[e]*Biomin II (Biomin Canada Inc. Mont-St-Hilaire, Québec, Canada).*
[f]*Econase XT, 25 (AB, Vista, Marlborough, United Kingdom) provided 80,000 BXU, of endo-1, 4-beta-xylanase activity per kg diet.*

Lesions more severe than score 3 were not observed.

2.3 Bacterial Quantification

2.3.1 DNA Extraction and Purification

Cecal contents collected from birds in flocks 1 and 2 were kept at −20°C for *C. perfringens* quantification. 0.2 g of thawed cecal content was measured into a 2 ml tube with 0.3 g 0.1 mm diameter silica beads (Biospec). The cecal contents were washed with and resuspended in 1 ml of TN150 buffer (149 mM NaCl, 5.58 mM Tris-HCl, 4.38 mM Trometamol, and pH 8.0) followed by a 3 min bead-beating at 5,000 rpm (Mini BeadBeater, Fisher Scientific). After centrifugation at 14,600 g for 5 min, the supernatant was transferred to a new 2 ml microtube. The DNA was purified using the phenol and chloroform-isoamyl alcohol (24:1) method and precipitated with 100% ethanol at −20°C overnight. The DNA pellet was washed twice with 500 μL of 70% ethanol without disrupting the pellet and dissolved in 100 μL of Nuclease-free water. The concentration and quality of DNA were measured using an ND-1000 spectrophotometer (NanoDrop Technologies) at 260 and 280 nm.

2.3.2 Quantitative Real-Time PCR (qRT-PCR)

The total *C. perfringens* population was quantified by qRT-PCR targeting the 16s rRNA gene (**Table 2**). Commercial *C. perfringens* genomic DNA was serially diluted 7-fold (using 1.35×10^7 as a starting point) and included on each plate to generate a standard curve for the absolute quantification of the bacteria population.

The qRT-PCR experiment was performed in QuantStudio™ 6 Flex System (Applied Biosystems) and data were analyzed with a QuantStudio rt-PCR Software v.1.3 (Applied Biosystems). Reactions of each sample were triplicated on a 96-well plate containing a 20 μL reaction mixture in each well (1 μL 50 ng/μL DNA template, 1 μL 25 pmol/μL of forward and reverse primers, 10 μL Fast SYBR Green Master Mix, and 7 μL Nuclease-free water). The amplification process started with initial denaturation at 95°C for 20 s followed by 40 cycles of annealing including 95°C for 3 s and 62°C for 30 s. As an indicator of amplification specificity, the melting curve of PCR products was generated by fluorescence collection during slow heating from 60 to 95°C with a rate of 0.05°C/s. The copy number of the target gene was calculated by the following formula as described by Li et al. (2009) and expressed as copies/g digesta.

$$DNA\ (number\ of\ molecules) = \frac{[6.02 \times 10^{23}\ (\frac{molecules}{mol}) \times DNA\ amount\ \ (g)]}{[DNA\ length(bp) \times 660\ (\frac{\frac{g}{mol}}{bp})]}$$

To detect the presence of the netB gene in cecal digesta, the genomic DNA of a netB-positive strain (CA147, Arden Biotechnology Ltd., United Kingdom) was 7-fold serial diluted,

TABLE 2 | *Clostridium perfringens* qPCR targeted genes and primer sequences used in this study.

Target	Sense	Sequence	References
16S rRNA	Fw	GGGTTTCAACACCTCCGTG	AP017630.1
	Rv	GCAAGGGATGTCAAGTGTAGG	
netB	Fw	TGATACCGCTTCACATAAAGGTTGG	Yang et al. (2018)
	Rv	ATAAGTTTCAGGCCATTTCATTTTTCCG	

TABLE 3 | Mortality and intestinal lesion prevalence in three experimental flocks with different flock sizes, housing types, and coccidiosis challenge intensities. Coccidial pre-exposure was incorporated in the NE disease model through oral gavage of live Eimeria oocysts using the Coccivac-B52 vaccine (Merck Animal Health).

	Flock 1	Flock 2	Flock 3
Flock size	344	120	288
Housing type	cage	cage	floor
Eimeria dosage[a]	10×	15×	15×
Overall mortality (%)	1.15%	1.39%	2.86%
Mortality (%), day 18–35	0	0	1.51%
Overall lesion prevalence	10.42%	85.19%	80.08%

[a]*A concentrated Coccivac-B52 vaccine was applied at 10× (flock 1) or 15× (flocks 2 and 3) of the recommended dosage. Each bird received 1 ml of vaccine diluted in distilled water.*

as aforementioned, and included on each plate to generate a standard curve for the absolute quantification of the strains. Reactions of each sample containing a 20 μL reaction mixture were prepared as aforementioned. The amplification process started at 95°C for 20 s, followed by 40 cycles of annealing and elongation including 95°C for 3 s and 60°C for 30 s. The specific netB amplicon was differentiated from nonspecific products by the DNA melting curve. The copy number of the target genes (*C. perfringens* and netB-positive strains) were calculated according to Li et al. (2009) and expressed as copies/g digesta.

2.4 Intestine Histology

Following lesion scoring, a 3 cm intestinal section at the lesion site was collected and fixed in 10% formaldehyde for microscopic histology analysis. Fixed intestine tissue was dehydrated and embedded in paraffin wax, then sliced into 5 μm sections and stained with hematoxylin and eosin. The intestinal cross sections were examined under light microscopy. Images were collected by using a SeBaCam digital microscope camera with SeBaView software (Thermo Scientific).

2.5 Statistical Analysis

Statistical analyses were performed by the GraphPad Prism 8 software. The experimental unit was the individual bird. To understand the impact of the coccidial challenge on natural NE development, Fisher's exact test was conducted to compare the lesion score between low- and high-coccidial challenged animals. A nonparametric Mann-Whitney test was used to compare the rank of lesion score between two coccidial challenge levels of the same age. T-tests were conducted to compare bacteria abundance between the age of days 17 and 21. $p < 0.05$ was defined as being statistically significant.

3 RESULTS

3.1 Mortality and Clinical Signs

The two cage-reared flocks yielded similar mortality during the experiment period. The overall mortality (day 1–40) was 1.15% in flock 1 and 1.39% in flock 2 (**Table 3**). All mortalities occurred within the first week, prior to the coccidial vaccine dosing and feed withdrawal challenge. Birds did not show observable clinical signs, but bloody and mucous-containing feces were found after feed withdrawal, indicating the presence of diarrhea.

The floor-reared flock (flock 3) showed higher overall mortality at 3.82% compared to the cage-reared flocks (**Table 3**). Two mortality peaks were observed during the experiment period. The first peak occurred during week 1 and the second peak was found between weeks 3–5 after the 24-h feed withdrawal was applied. The second mortality peak was directly triggered by feed withdrawal on day 18, which also caused depression and decreased mobility in birds.

3.2 Detection and Quantification of *C. perfringens*

Cecal total *C. perfringens* was quantified by qRT-PCR targeting the 16 s gene. In flock 1, all sampled birds were found to be *C. perfringens* positive regardless of age (**Figure 3A**). The presence of netB, the hallmark of NE-causing strains, was detected in 75.0% of the samples on day 17, before the feed withdrawal challenge, and increased to 93.8% on day 21. Correspondingly, netB abundance on day 21 was significantly higher than day 17 ($p = 0.0242$) (**Figure 3B**). This is consistent with our expectation that the 24-h feed withdrawal on day 18 further contributed to the propagation of the virulent strains within the flock. The observed *C. perfringens* density was relatively high with 16s abundance ranging from 10^7 to 10^9 copies per gram of cecal content (**Figure 3B**). A relatively lower netB abundance was observed at around 10^6 copies per gram of digesta. Quantification of 16 s and netB copies showed no significant difference between day 17 and day 21 ($p > 0.05$), though day 21 tended to have a higher abundance of the examined genes. Flock 2 was reared in similar conditions as flock 1 but was challenged with a higher dosage of Eimeria oocysts on day 13. However, the higher coccidial challenge level did not increase bacteria detection rate or bacteria abundance (data not shown).

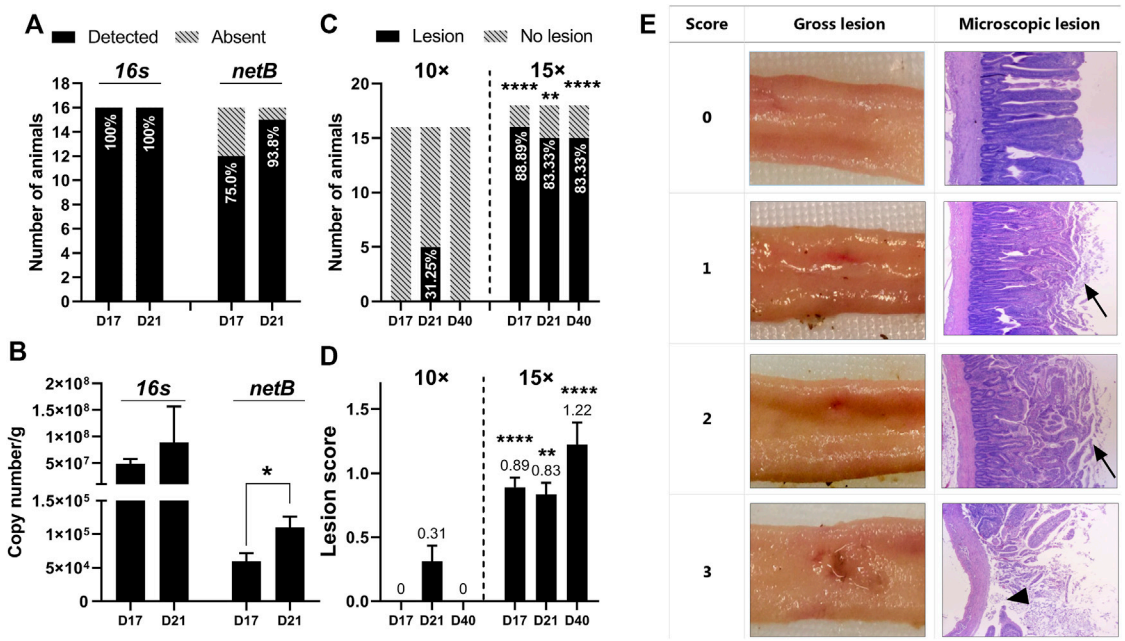

FIGURE 3 | Quantification of *C* perfringens and intestine lesion confirmed induction of subclinical necrotic enteritis using the natural infection model **(A)** Detection of *C perfringens* 16s and netB gene in cecal contents by qRT-PCR. The percentages of animals detected with 16s or netB are plotted within bars. Data were collected from flock 1 (n = 16) **(B)** Abundance of 16s and netB gene expressed as copy number/g of cecal content. Data were collected from flock 1. T-tests were conducted to compare gene abundance between the age of days 17 and 21. The netB abundance on day 21 was significantly higher than on day 17 (p = 0.0242) **(C)** Intestine gross lesion prevalence in flock 1 (challenged with 10× concentrated coccidial vaccine) and flock 2 (challenged with 15× concentrate/d coccidial vaccine). The percentages of animals detected with gross lesions are plotted within bars. Fisher's exact test was conducted to compare the difference between low and high coccidial challenged animals (D17: $p < 0.0001$, D21: $p = 0.0045$; D40: $p < 0.0001$) **(D)** Lesion scoring results from flock 1 and flock 2. A nonparametric Mann-Whitney test was used to compare the rank of lesion score between two coccidial challenge levels from the same age (D17: $p < 0.0001$, D21: $p = 0.0045$, D40: $p < 0.0001$) **(E)** Severity of NE-specific lesions was scored from 0 to 3 based on the intestine gross examination. The tissue at the lesion site was processed for the histology analysis. The original magnification of the images is ×25. The necrotic tissue was typically covered by a layer of mixed cellular debris (arrow). Sloughed mucosa leading to complete loss of villi (arrowhead) was observed in intestinal tissue with a score of 3.

3.3 Gross Examination of Intestine Lesion

In our study, mild lesions (scores 1 and 2) were predominantly observed with a few birds scored with 3 (**Figure 3E**). Severe lesions (score greater than 3) was not observed in any of the flocks. A total of 48 birds were sampled in experiment flock 1. Only 5 birds (10.42%) had NE-specific lesions under the 10× coccidiosis vaccine challenge (**Table 3**), and all the lesion-positive animals were observed on day 21. With increased intensity of coccidial challenge at (15× vaccine dose), flock 2 (n = 54) and flock 3 (n = 256) showed a higher prevalence of birds with necrotic lesions (85.19 and 80.08%, respectively). Lesion prevalence in flocks reared in the wire-floored cage environment (flock 1 and 2) showed that coccidial challenge levels have a profound impact on NE lesion development (**Figure 3C**). The 15× dosage of Eimeria vaccine gavage led to prevalent lesion development in the flock as early as day 17 and the lesions were also present on day 40. The high-coccidial challenge flock showed increased lesion score compared to the low-challenge flock on day 17 ($p < 0.0001$), day 21 ($p = 0.0045$), and day 40 ($p < 0.0001$) (**Figure 3D**). The floor-rear flock (flock 3) with 15× coccidial challenge yielded consistently higher lesion prevalence on both sampling days as expected. The observed lesion prevalence was 75% (96/128) on day 21 and 93.75% (120/128) on day 40. Animals in flock 3 were not sampled on day 17.

3.4 Microscopic Examination of Intestine Lesion

Representative histopathological images of the intestinal section are shown in **Figure 3E**. Intestine tissue scored at 0 with no gross lesion and generally showed intact villus structure. However, examination under higher magnification revealed pathological changes including the presence of Eimeria oocysts, mildly dilated capillaries, and capillary hemorrhage. Tissue lesions that scored 1 and 2 generally showed similar microscopic appearance though distinguished changes were observed in gross examination. Under microscopic examination, the necrotic region showed hyperemia, villus fusion, and separation of epithelium from the lamina propria. The necrotic tissue was usually covered by adherent fibrin and cellular debris. These pathological alterations were also observed in lesioned tissue with a score of 3. Noticeably, sloughed mucosa leading to complete loss of villi was observed in certain areas within the lesioned tissue (**Figure 3E**).

4 DISCUSSION

4.1 Confirmation of Necrotic Enteritis Development

Bacteria quantification together with the characteristic pathology of NE, such as clinical signs and gut lesions, is indicative of successful induction of NE disease (Williams et al., 2003; McReynolds et al., 2004; Palliyeguru et al., 2010). *C. perfringens* can be found in high populations in NE-affected animals ranging from 10^6 ~ 10^9 CFU (Williams, 2005; McDevitt et al., 2006; Abildgaard et al., 2010). The *C. perfringens* population observed in our trial is consistent with those typically found in NE-affected animals. Interestingly, the netB gene was more prevalent on day 21 compared to day 17 (**Figure 3A**). This may be associated with the observed diarrhea on days 18–19 following the feed withdrawal challenge, which could indicate that the experimental conditions promoted the spreading of the netB-carrying strains in the flock.

As noted in previous studies, there may be a poor correlation between the number of *C. perfringens* organisms in the digesta and the incidence or severity of necrotic enteritis, especially in the subclinical form of the disease (Fernando et al., 2011). Subclinical NE is usually mild with no clinical signs or sudden increase in mortality (Fernando et al., 2011). Thus, gut lesions are considered to be a sensitive disease indicator compared to clinical signs and mortality (Williams et al., 2003). In this study, gut lesions were found in all three experimental flocks and were confirmed with microscopic examination (**Figure 3E**). Together, the observed bacterial load, clinical signs, and pathological changes suggest NE occurrence in the flocks with disease severity peaked during weeks 3 to 4.

4.2 Prevalence of Gut Lesion

Many conventional NE disease models have shown lesion incidence peaks at a certain age and declines as the animal approaches slaughter (Lovland et al., 2003; Gholamiandehkordi et al., 2007). Natural NE infection induced by reused litter material from a previous flock (Palliyeguru et al., 2010), high stocking density, and housing of birds on litter (Lovland et al., 2003; Fernando et al., 2011) have resulted in lesion prevalence ranging from 6.9 to 68.6%. However, to our knowledge, most studies conducted only one lesion examination during the rearing period. In the field conditions, subclinical NE-affected animals can be detected at slaughter with *C. perfringens*-associated lesions in the liver and gut (Lovland and Kaldhusdal, 1999; Johansson et al., 2010). This suggests experimental NE models with persisting lesion occurrence may better reflect NE cases in the field, where birds suffering from the subclinical disease are kept without being treated. In this study, we thus involved multiple gut examinations throughout the rearing period. Results suggested coccidial challenge has a profound influence on the development of gut NE lesions. Coccidial challenge intensity affects the occurrence and duration of gross lesions present in the NE-affected flocks. As noted by Stanley et al. (2014), *Eimeria* spp. caused significant changes in gut microbiota diversity and enabled *C. perfringens* to persist post challenge. *C. perfringens* inoculated in the absence of this predisposing factor fail to establish and maintain themselves in the gut flora.

4.3 Mortality

Epidemiology studies suggest that NE occurrence is associated with specific housing conditions, including access to litter and floor-type housing (Kaldhusdal et al., 2016; Goossens et al., 2020). Our data shows NE-related mortality was not observed in our cage-reared flocks even with a 15× coccidial challenge dosage. A sudden but minor increase in mortality in the floor-reared flock was observed starting from day 18. In the E. maxima/*C. perfringens* dual infection model, NE lesions can be produced without mortality in animals reared in wire cages (Williams et al., 2003), while similar models in floor-reared broilers yielded NE-induced mortality ranging from 8 to 12% (Wu et al., 2010; Hofacre et al., 2019).

Natural NE infection induced by reused litter material from a previous flock resulted in mortality from days 15 to 30, ranging from 1.5 to 4.9% across dietary treatments (Palliyeguru et al., 2010). Fernando et al. (2011), by housing birds on wood-shaving litter and removing antibiotics, induced NE infection with mortality of 1.19–1.66% from days 20 to 36. These findings and our observation are consistent with the mortality range reported in subclinical NE affected flocks (Lovland and Kaldhusdal, 2001). Recent work by Calik et al. (2019) and Emami et al. (2019) described a new natural NE disease model by spraying the same coccidiosis vaccine, as used in our study, on litter and feed upon bird placement. An NE outbreak was observed on days 7–9 with overall mortality at around 12%. These findings showed higher mortality which peaked at an earlier age compared to our study but is consistent with our observation that the NE outbreak occurred 1–2 weeks after the coccidial challenge by concentrated Coccivac®-B52 vaccine.

4.4 Practical Aspects of the Natural Infection Model

Conventional clinical NE models usually involve repeated oral inoculations of coccidial oocysts and *C. perfringens* for consecutive days (Williams et al., 2003; McReynolds et al., 2004; Gholamiandehkordi et al., 2007; Park et al., 2008; Wu et al., 2010; Jayaraman et al., 2013). One of the advantages of the natural NE model is the simplicity of the challenge schedule. Coccidial inoculation typically takes 30 min for two experienced technicians to gavage 100 birds, while feed withdrawal can be done within an hour even in large flocks. Experience from our research facility showed consistent induction of NE across studies using this protocol. Reduced complexity in the challenge schedule can limit animal stress and treatment inconsistency between different personnel, thus contributing to persistent induction of NE disease.

This natural infection model also allows flexibility in designing dietary formulas. The wheat-based broiler chicken diets commonly include xylanase to break down arabinoxylans, decrease viscosity, and increase digestibility in the birds (Lee et al., 2017). Elimination of xylanase has been used to produce

natural NE infection (Abildgaard et al., 2010), by increasing feed transit time promoting *C. perfringens* persistence (Choct et al., 2006). Another commonly used dietary predisposing factor, fishmeal, supplies abundant glycine and methionine that enhance *C. perfringens* proliferation and toxin production (Wilkie et al., 2005; Dahiya et al., 2007; Shojadoost et al., 2012). However, the usage of fishmeal is limited in broiler feed due to its high cost and low availability (Frempong et al., 2019). We showed that birds fed with a fishmeal-free diet with xylanase inclusion (flock 3) showed prevalent NE lesions in the gut, suggesting fishmeal inclusion and xylanase elimination are not required for experimental NE induction.

The presence of pathogenic *C. perfringens* is required but insufficient to trigger NE infection. The virulence phenotype of *C. perfringens* is subject to the influence of host epithelium and complex lumen environment (**Figure 1**). This highlights the cooperative roles of a wide range of environmental factors that contribute to NE development. For research aimed at prophylaxis of NE, it is critical to conduct the evaluation under a condition accurately mimicking the disease development under practical production conditions. The infection model presented in this study was able to reproduce the commonly observed subclinical NE, regarding the severity of symptoms, timing of lesion development, and rate of mortality. By allowing the pathogen to develop *in vivo*, NE researchers will be able to evaluate a prophylactic strategy at an early stage of disease development, when the damage to the animal is most reversible and thus ideal to be targeted. However, more studies are needed to better understand this novel infection approach, such as alterations to mucus characterization, epithelial properties, and immunological function during natural NE induction.

Feed withdrawal introduced on day 18 in this natural NE challenge is important to trigger the timely development of the disease outbreak. At the same time, this approach is believed to cause limited stress to the animals and is considered humane when used properly. Pathogenic *C. perfringens* has a stronger ability in binding extracellular matrices and utilize nutrients released from host intestinal tissue, thus showing a selective survival advantage over nonpathogenic strains during feed withdrawal (Timbermont et al., 2009, 2014; Wade et al., 2015). Feed restriction is often used in conventional NE challenges, not as a designated stressor but as a measure to ensure uniformity of inoculation treatments (Shojadoost et al., 2012). In those situations, the *C. perfringens* inoculation is administered mixed with feed, and feed is usually withdrawn overnight so that the birds will eagerly eat the inoculated feed.

The NE-causing *C. perfringens* are of high diversity in terms of genomic content, with the varied ability to cause intestinal damage. The growing understanding of the differences between strains isolated from animals of different health statuses and geographical regions highlights the need to carefully select appropriate strains to use in experimental NE models. It was reported that 2 *C. perfringens* strains, both isolated from NE-affected chickens and carrying NetB, showed varied virulence and produced different levels of disease severity in experimentally-induced NE (Gharib-Naseri et al., 2019). Knowledge gained using this challenge model can likely be applied across wide geographic regions. The natural, subclinical infection NE challenge model would allow the propagation of pathogenic strains that are locally prevalent. This would be beneficial to the specific region in the understanding of pathogenesis and control strategies against the locally prevalent pathogenic strains.

5 CONCLUSION

The NE infection model presented in this study is based on the natural uptake of *C. perfringens* presented in the housing environment by the chicken. We incorporated multiple NE-associated risk factors to promote the natural development of pathogens, and successfully reproduce subclinical NE. This will contribute to future research aiming at understanding and preventing this disease, by mimicking the natural development of NE in commercial poultry production.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Alberta Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

WH, DK, and DB jointly conceived and designed the study. WH, EG, and DK performed the experiments. WH analyzed the data and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Novel strategies to improve chicken performance and welfare by unveiling host-microbiota interactions through hologenomics

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Fast optimisation of farming practices is essential to meet environmental sustainability challenges. Hologenomics, the joint study of the genomic features of animals and the microbial communities associated with them, opens new avenues to obtain in-depth knowledge on how host-microbiota interactions affect animal performance and welfare, and in doing so, improve the quality and sustainability of animal production. Here, we introduce the animal trials conducted with broiler chickens in the H2020 project HoloFood, and our strategy to implement hologenomic analyses in light of the initial results, which despite yielding negligible effects of tested feed additives, provide relevant information to understand how host genomic features, microbiota development dynamics and host-microbiota interactions shape animal welfare and performance. We report the most relevant results, propose hypotheses to explain the observed patterns, and outline how these questions will be addressed through the generation and analysis of animal-microbiota multi-omic data during the HoloFood project.

KEYWORDS

animal performance, genomics, metagenomics, multi-omics, sustainability

Introduction

With the ever-increasing human population on Earth, humanity is facing several major challenges to ensure long-term balance between natural resource use and environmental conservation (Reid et al., 2010). Many of the current agricultural practices are not sustainable due to excessive carbon emission, resource consumption, and waste production (Agovino et al., 2019). Hence, there is an urgent need to transition into a more resilient and sustainable agriculture model, in which the efficiency of production is improved, the use of antibiotics is reduced, and the welfare of animals is ensured (Eyhorn et al., 2019).

A fundamental step to optimise farming practices is to obtain in-depth understanding on the biological functioning of the animal production systems (Messerli et al., 2019). These systems often include biological elements beyond the actual animal that is being produced, among which host-associated microorganisms stand out due to their relevance for the optimal biological functioning of most animals (McFall-Ngai et al., 2013). Intestinal microorganisms not only modulate nutrient intake (Diaz Carrasco et al., 2019), but also shape intestinal immune and inflammatory processes (Zhou et al., 2020), intervene on host systemic growth parameters (Fraune and Bosch, 2010), and even influence host behaviour (Johnson and Foster, 2018). Microorganisms colonise the animal gut as soon as it is exposed to the environment (Sprockett et al., 2018), and develop communities with complex spatial and temporal dynamics (Debray et al., 2021), which continuously interact with the host animal (Khan et al., 2019; Ansari et al., 2020).

Animal-microbiota interactions have so far remained largely unexplored because of the limited capacity of scientists to properly characterise and analyse the key elements partaking in this interplay due to their excessive complexity (Alberdi et al., 2021). However, the development of high-throughput DNA sequencing and mass spectrometry technologies, linked to higher computing capacity and development of powerful bioinformatic tools, is changing this scenario (Graw et al., 2021). Today we are not only able to characterise the entire genetic information of animals and their associated microorganisms (namely the hologenome), but we can also quantify how genes are expressed, which proteins are synthesised and what metabolites result from enzymatic reactions happening in the gut (Nyholm et al., 2020). Such a holo-omic approach that considers multiple omic layers of both animals and associated microorganisms, is starting to unveil biological features and patterns that have remained hidden so far (Alberdi et al., 2021).

This technological revolution can contribute to many sources of variability that have been so far attributed to background noise, such as host microgenetic and microbiota variation among individuals, to be surfaced and included in the analyses (Alberdi et al., 2021). This requires an increased attention on the biological processes happening in each individual animal, rather than considering animals just as units that contribute to pen or tank statistic averages. The first attempts to implement individual-based multi-omic strategies in farm animals have provided detailed understanding of feed-microbiota-animal interactions (Andersen et al., 2021), by for example demonstrating which bacteria with which genes are able to degrade which carbohydrates (Michalak et al., 2020). We anticipate that such a mechanistic understanding of biological processes will contribute to generating refined hypotheses, predictive models and experimental treatments that will lead to a reduction of animals employed for research and an ultimate development of more optimal farming strategies.

HoloFood (HoloFood, 2019) is a multi-partner H2020 project that is pioneering such an approach, among other farming systems, on broiler chickens. The project aims at developing and implementing joint multi-omic analyses of animals and their associated microorganisms to generate in-depth knowledge of animal-microbiota interactions, and in doing so, improve the quantity, quality and safety of the produced food, the sustainability of the production process and the welfare of animals. To set the baseline to an upcoming series of publications that implement such multi-omic analyses, here we introduce the strategic vision, and experimental work conducted to generate biological samples and associated performance results of broiler chickens in the project HoloFood. We present the main performance results, discuss their relevance, and relate them to future multi-omic analyses that HoloFood partners will conduct to address the variety of biological questions raised from the initial screening of animal performance.

Material and methods

Animals and housing

The study consisted of three identical experiments (A, B, and C). In each trial a total of 960 days-of-hatch broiler chicks belonging to two fast growing genetic lines (Ross308® and Cobb500®) from two hatcheries (to increase genetic variability) were allocated at 24 pens upon arrival. The name of genetic lines has been blinded and each one is described along the manuscript under the letter X or Y. In order to avoid the possible influence of the parent stock, birds were distributed in such a way that each replicate received the same number of broilers from each hatchery tray. Each pen had a total surface of 2.25 m^2 with 40 birds per pen. Each pen was provided with one individual hopper feeder and two nipple drinkers. The barn is windowless and provided with automatic environment control with a gas heating system by screens and ventilation by depression. The room also has programmable lighting, provided by TL tubes evenly distributed. The temperature program was adjusted according to the standard program

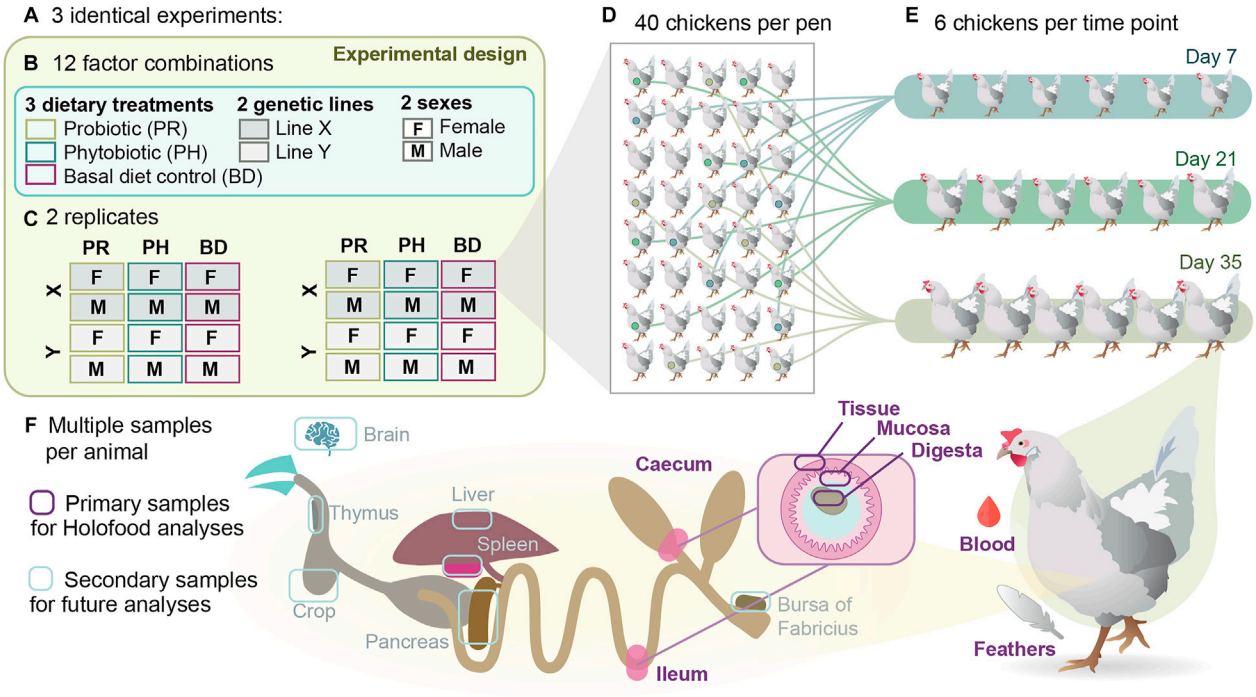

FIGURE 1
Experimental, sampling and analytical design of the HoloFood study focused on broiler chickens. **(A)** Three experiments with identical study design were conducted in 2019. **(B)** Each experiment contained 12 factor combinations of three dietary treatments, two genetic lines and two sexes. **(C)** Each combination was replicated twice per experiment, yielding 24 pens per experiment and 72 pens in total. **(D)** Each pen contained 40 chickens in the beginning of the experiment, 18 experimental animals that were randomly selected and tagged the first day of the experiment, and 22 more animals that provided commercial-like density conditions. **(E)** Six chickens were euthanised at day 7, six more at day 21 and six more at day 35 for collecting samples for performance, multi-omic and complementary analyses. **(F)** From each animal, 14 tissue and digesta samples were collected for a variety of analyses (see Table 1 for details), and complementary organ samples were also collected for future analyses.

used in the farm: from 0 to 2 days the temperature increased from 32 to 34°C; from 3 to 7 days the temperature was reduced to 29–31°C and continued decreasing for 3°C per week afterwards until reaching 21°C. The lighting program was 24 h of light the first 2 days, 18 h of light until 7 days, and 14 h of light per day afterwards. The litter was fresh wood shavings. All birds were vaccinated against Avian Infectious Bronchitis and Gumboro diseases according to the vaccination program usually practised at the hatchery. Moreover, a set of 240 Cobb500 animals from one of the hatcheries in experiment 1 were vaccinated against Marek disease.

The study had a randomized complete block design with a factorial 2 × 2 × 3 arrangement according to broiler line (X or Y), sex (male or female) and dietary treatment: 1) basal diet (BD), 2) BD plus a probiotic additive (PR), and 3) BD plus a phytobiotic additive (PH) (overview in Figure 1). The three trials were carried out in different seasons of the year: spring (trial A), summer (trial B) and autumn (trial C). Treatments were randomly assigned to one pen of each block (2 blocks per experiment), so that each treatment had 2 replicates (pens) per experiment (6 in total) with 40 animals per pen (20 animals from each hatchery). The experimental design deviates from conventional studies aimed at testing the effect of feed additives on performance, as it was designed seeking to maximise inter-individual genetic variability, and in doing so increase the probability to identify interactions between animal and microbial genomic features. The six animals to be slaughtered at days 7, 21, and 35 within each pen were randomly selected and marked at day 0 to avoid observer biases in subsequent samplings.

Diets, additives and feeding

Aimed at maximising the effects of the feed additives, the BD was designed as a pro-inflammatory diet, using wheat (a cereal rich in non-starch polysaccharides (NSP) with a more than 50% inclusion) and soybean meal as main ingredients without the addition of enzymes, antibiotics, or coccidiostats. Diets were formulated according to birds' requirements and commercial practices in three different periods: starter (0–9 days), grower (10–23 days) and finisher (24–37 days). Feeds were presented in crumble form for the starter period and in 3 mm pellets later on.

Composition of diets and estimated nutrient contents are presented in Supplementary Table S1. The PR treatment consisted of BD with a mixture of three strains, namely *Bacillus subtilis* DSM 32324, *B. subtilis* DSM 32325 and *B. amyloliquefaciens* DSM 25840 (level of inclusion at 0.75 g/kg feed, $>3.20 \times 10^9$ CFU/g), which has been recently authorised as a zootechnical product for poultry species (EFSA Panel on Additives and Products or Substances used in Animal Feed et al., 2020). The PH treatment consisted of BD with a phytobiotic additive obtained from white grapes and containing 78% of procyanidins and 22% of polyphenols as active ingredients (level of inclusion at 0.75 g/kg feed).

Batch feed samples were taken from each production for proximate analysis (Association of Official Analytical Chemists, 2000, moisture -dry matter-by oven drying–method 2-, nitrogen -crude protein-by combustion -Dumas method-, ether extract on a Soxtec system -method 3B- and ash after muffle furnace incineration -method 12) and to quantify concentrations of probiotic and phytobiotic additives. Data of analytical composition of diets is shown in Supplementary Table S1. In addition, water and litter samples were collected in each pen at days 0, 7, 21, and 35 for microbiological and litter quality analyses. Moreover, three samples of each feed per diet period were taken from three different bags for microbiological assessment.

Animal monitoring

Animals were counted and weighed by hatchery tray upon arrival and by replicate at days 7, 21, and 35. Growth performance was monitored to replicate the same days. Pen level (40 chickens/pen) analyses included average body weight (BW), daily gain (ADG), daily feed intake (ADFI), feed conversion ratio (FCR) and European production efficiency factor (EPEF). The incidence and severity of footpad dermatitis per pen was subjectively evaluated by trained personnel at days 7, 21 and 35 according the 5-point scale described by Butterworth (Butterworth and Welfare Quality Consortium and Normalisatie-instituut, 2009): 0) no evidence of footpad dermatitis; 1 and 2) minimal evidence of footpad dermatitis; 3 and 4) evidence of footpad dermatitis. Dead animals were weighted, and the most probable cause of death recorded. Animals (laggards) excluded from the trial during the first week were not considered.

Animal sampling

At days 7–8, 21–22 and 35–37 (multiple days were necessary due to workload), 6 animals per pen were randomly selected, individually weighed (iBW), evaluated for footpad dermatitis (data not shown), euthanised and sampled. Animals were euthanised according to RD 53/2013 (Spain), following the ethical requirements established. After the euthanasia, a total of 14 samples were collected from each animal to measure individual key performance indicators (KPIs - iBW, concentration of corticosterone (COR) in feathers, concentration of acute phase proteins (C-reactive protein (CRP) and chicken haptoglobin-like protein (PIT54)), and lipopolisaccharides (LPS) concentration in plasma, and pathogens detection in caecal contents (Salmonella spp., Campylobacter spp., and Clostridium spp.)) as well as to generate multi-omic and complementary analyses (Table 1). Measured KPIs aimed at quantifying quantity, quality and safety of the produced food, the sustainability of the production process and the welfare of the produced animals. Sections of ileum and cecum, intestinal content from ileum and cecum, feathers, and blood were obtained. Different aliquots were distributed and properly stored for downstream analyses. Liver, pancreas, thymus, bursa of Fabricius, brain, and spleen samples were also obtained for future analyses (Figure 1F).

TABLE 1 Overview of the biological samples collected from each individual animal within the project HoloFood and their corresponding multi-omic and complementary analyses with data included in this article bolded.

Sample	Analyses
Blood	**Lipopolysaccharide and acute phase proteins**
Ileum tissue	Mucus production
	Histology
	Targeted amplification of inflammatory markers
	Shotgun chicken transcriptomics
Ileum mucosa	Shotgun chicken transcriptomics
	16S rRNA amplicon sequencing
Ileum content	Shotgun metagenomics
	16S rRNA amplicon sequencing
	Shotgun metatranscriptomics
	Chicken genomics
	Metabolomics
Caecum tissue	Mucus production
	Histology
	Targeted amplification of inflammatory markers
	Shotgun chicken transcriptomics
Caecum mucosa	Shotgun chicken transcriptomics
	16S rRNA amplicon sequencing
Caecum content	**Pathogen detection through PCR**
	Shotgun metagenomics
	16S rRNA amplicon sequencing
	Shotgun metatranscriptomics
	Chicken genomics
	Metabolomics
Feathers	**Corticosterone measurement**

In addition, at day 37 (commercial slaughtering age), 6 extra animals per pen were randomly selected, euthanised and subjected to evaluation of meat quality traits (carcass, abdominal fat, breast, and leg yield). The oxidative stability of the thigh muscle was determined over a period of 7 days from randomly selected three animals used for meat quality assessment.

The data on each individual animal derived from the experiments were analysed at three different levels. The first level included the analyses of measurements obtained in the farm (e.g., iBW). The second level comprised analyses conducted *a posteriori* from samples obtained during the trials in order to control the health, welfare of animals (e.g., ELISA for CRP, PIT54, LPS and COR quantification, and PCR for pathogen detection), and meat quality traits (e.g., meat oxidative stability). The third level included multi-omic analyses, which characterises the animal-microbiota system at the highest level of breadth and resolution. These analyses will include whole-genome sequencing of chicken genomes, metagenomics of microbial communities (meta) transcriptomics and metabolomics. This third level of data will be carried out in the next steps of the project.

Analytical procedures

Pathogen detection

Opportunistic infections of different zoonotic pathogens (*Salmonella, Campylobacter and Clostridium* spp.) were controlled in caecal contents from sampled animals at days 7, 21 and 35 by conventional PCR (absence/*presence*). *Salmonella* spp. was detected using the primers 5′-GTG AAATTATCGCCACGTTCGGGCAA-3′ and 5′-TCATCG CACCGTCAAAGGAACC-3′, which are specific for the InvA gene of *Salmonella*, following the conditions described in Rahn et al. (1992). For the detection of *Clostridium perfringens*, the primers 5′-AAGATTTGTAAG GCGCTT-3′ and 5′-ATTTCCTGAAATCCACTC-3′ specific for alpha-toxin gene present in all strains of this bacteria species were used. The specific amplification program was as follows: 94°C/4'; (94°C/1′, 55°C/1′, 72°C/1′20″)x35; 72°C/15'; 4°C/end. The presence of *Campylobacter* spp. was detected using the primers 5′-TTGGAAACGACTGCTAATACTCTA-3′ and 5′-AGCCATTAGATTTCACAAGAGACT-3′, which amplify a specific segment of 16S ribosomal RNA gene specific of *Campylobacter* spp. The specific amplification program was as follows: 94°C/4' (94°C/1′, 48°C/2′, 72°C/1′) x35; 72°C/15'; 4°C/end.

Corticosterone in feathers

Corticosterone was determined in accordance with the method described by Bortolotti et al. (2008).

Acute phase proteins in plasma

Chicken haptoglobin-like protein (PIT54) was quantified using the haptoglobin ELISA Kit (Ref. ABIN1563052, antibodies-online.com) and C-reactive protein (CRP) was measured using the CRP ELISA Kit (Ref. ABIN4947413, antibodies-online.com) in accordance with manufacturer instructions.

Lipopolysaccharide in plasma

Lipopolysaccharide concentration in plasma was determined using Pierce LAL Chromogenic Endotoxin Quantification Kit (Ref. 88282; Thermofisher, United States) in accordance with manufacturer instructions.

pH of litter

Three samples of 50 g of litter per pen were collected for pH determination at days 7, 21 and 35 avoiding the areas near and below the feeders and drinkers. The three samples collected from the same pen and day were pooled and homogenised and the moisture was determined in a sub-sample of 100 g according to the AOAC method Association of Official Analytical Chemists, 2000 method 925.09). For pH analysis, a subsample of 10 g was placed in a beaker with 100 ml of distilled water, shaked with a glass rod and allowed to stand for 30 min. The pH value was obtained using a pH metre (Crison, L'Hospitalet de Llobregat, Spain).

Oxidative stability of meat

Samples (5 g of muscle from the thigh) were homogenised with an aqueous 7.5% trichloroacetic acid solution, filtered and brought to 20 ml. To proceed, 5 ml of the extraction solution and 5 ml of 0.02 M thiobarbituric acid were mixed and boiled for 15 min and then cooled in cold water. Absorbance of the peak was measured at 525 nm as malondialdehyde production in an ultraviolet-visible spectrophotometer (Shimadzu, Japan) using the third derivative of the spectrum between 425 and 650 to correct the baseline. The 1,1,3,3-tetraethoxypropane was used as standard (Botsoglou et al., 1994; Ruiz et al., 1999).

Statistical analyses

Data was explored to discard any possible outlier according to the Kolmogorov-Smirnov test (Massey, 1951). As no outliers were considered, the statistical analysis included all data. The GLIMMIX procedure of SAS software (SAS/STAT 14.1; SAS Institute Inc., Cary, NC, United States) was used to perform the analysis of the different variables. In the case of ELISA determinations, when the limit of detection was not reached, the missing values were replaced by the limit of detection (L)/$\sqrt{2}$ (Hornung and Reed, 1990). The statistical model used is shown below:

$$y_{ijklm} = \mu + T_i + B_k + S_l + E_m + \gamma_j + e_{ijklm}$$

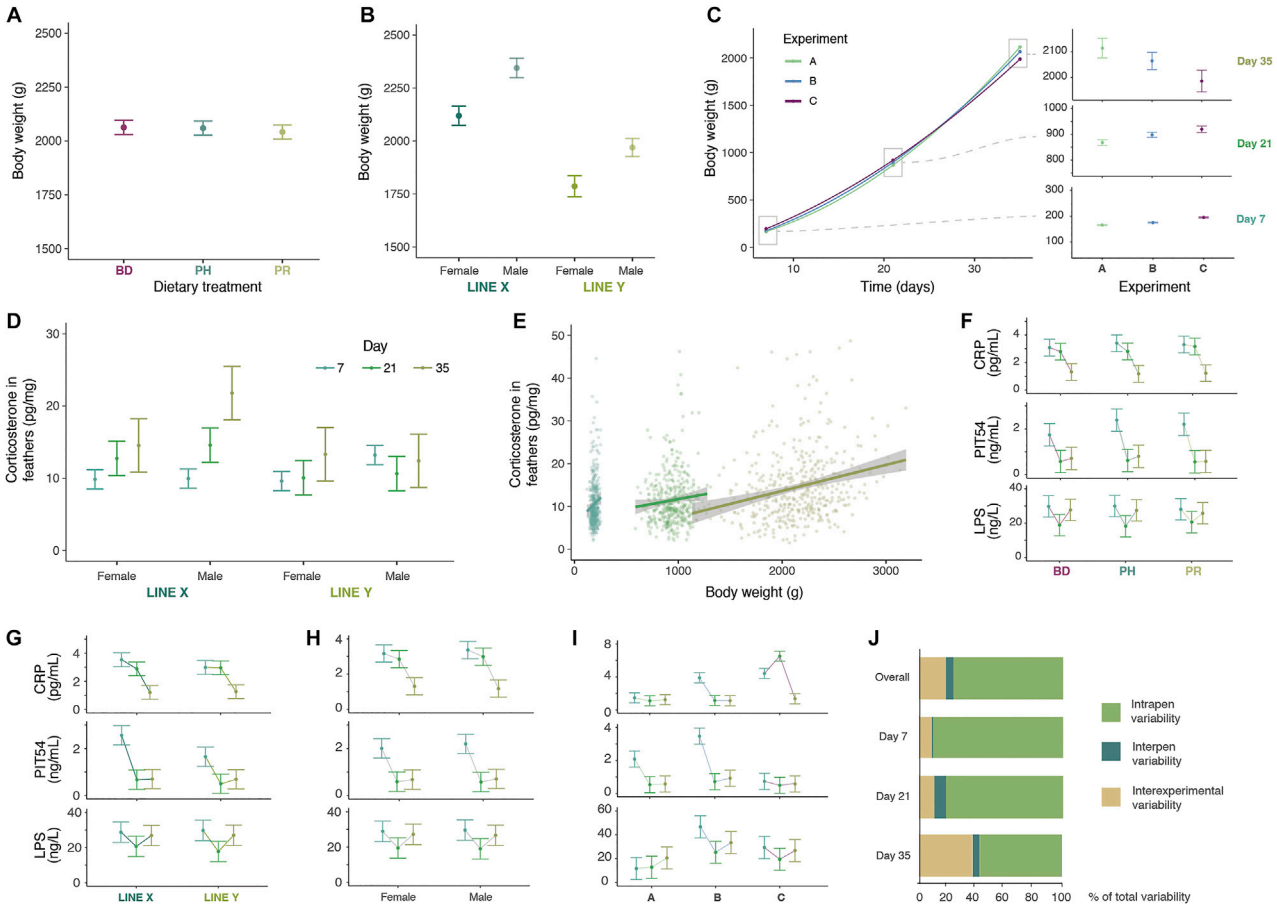

FIGURE 2
Overview of main results. **(A)** Body weight differences across dietary treatments, namely basal diet (BD), BD plus probiotic (PR) and BD plus phytobiotic (PH), at day 35. **(B)** Body weight differences across lines and sexes at day 35. **(C)** Body weight progression of the three experiments, with detailed overview of days 7, 21 and 35. **(D)** Corticosterone (COR) levels measured in feathers at different days, sexes, and genetic lines. **(E)** Linear correlation between COR levels and body weight at the three time points. **(F)** C-reactive protein (CRP), avian haptoglobin-like protein (PIT54) and lipopolysaccharide (LPS) levels in plasma across time points in different dietary treatments. **(G)** CRP, PIT54 and LPS levels in plasma across time points in different genetic lines. **(H)** CRP, PIT54 and LPS levels in plasma across time points in different sexes. **(I)** CRP, PIT54 and LPS levels in plasma across time points in different experiments. **(J)** Hierarchical decomposition of observed body weight variation within pens, among pens and among experiments.

Where y_{ijklm} is the response variable, T_i is the dietary treatment effect, B_k is the broiler line effect, S_l is the biological sex effect, E_m is the experiment effect, γ_j is the random block effect, and e_{ij} is the error of the experimental unit. The experimental unit for the statistics and the tables presented was the pen. Results in Supplementary Tables S2–S12 are expressed as least square means ± standard error. Differences were considered significant at $p < 0.05$, while those at $p < 0.1$ are reported as tendencies.

Hierarchical decomposition of the variance was carried out using ANOVA-estimation of variance components as implemented in the fitVCA function of the R package VCA (Schuetzenmeister and Dufey, 2017), using iBW as response variable, and pen and experiment as explanatory variables.

Results

In the following, we present the main results obtained from pen-level analyses, and the first two levels of the individual analyses sorted by topic. We explain their biological relevance based on current knowledge, and we project the potential of multi-omic analyses that will be conducted in the project HoloFood on top of these results to address the most relevant pending questions.

Effect of dietary treatments

No significant effect of administered dietary additives was observed in the performance of the animals (Figure 2A;

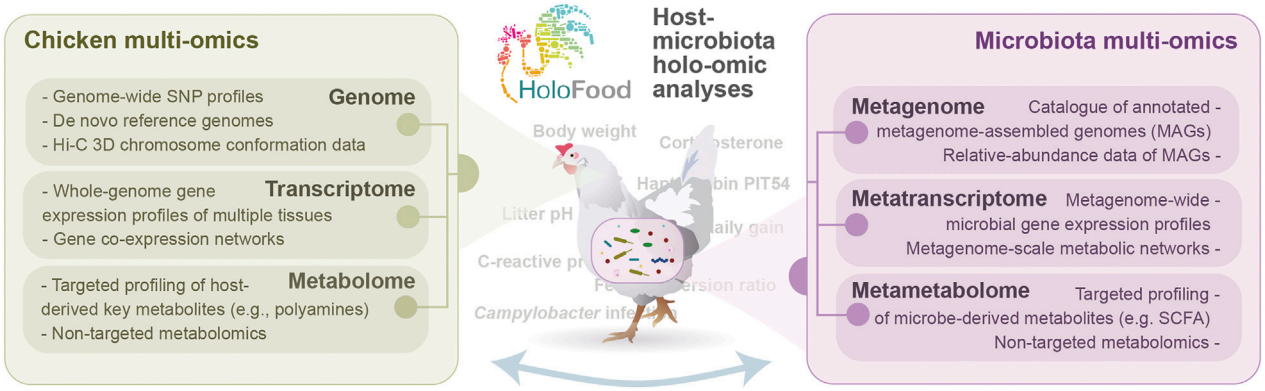

FIGURE 3
Overview of holo-omic analyses that will be conducted in the H2020 project HoloFood to deepen into the results outlined in this manuscript and address the questions raised and beyond.

Supplementary Tables S2-S12). Dietary treatments neither induced any significant change in the acute phase protein values measured in plasma (Figure 2F, Supplementary Table S7). The only parameter affected by dietary treatments was COR (Supplementary Table S6), which was increased with the PH diet at day 7, but these levels were not prolonged over time nor decreased compared to the BD.

Effect of broiler line

Broiler line X reached significantly higher BW, ADG, and ADFI and lower FCR than line Y at day 35 (2232 g and 1878 g for BW (standard error (SE) = 18.8), 62.6 and 52.4 g for ADG (SE = 0.54), 96.6 and 81.1 g for ADFI (SE = 0.80), and 1.497 and 1.542 for FCR (SE = 0.015), respectively; Figure 2B and Supplementary Table S2).

The accumulation of COR in feathers was significantly higher for line X (13.91 pg/mg) than for line Y (11.54 pg/mg) (SE = 0.914; $p < 0.001$) (Figure 2D, Supplementary Table S6) and individual COR levels were also higher in animals with higher iBW (Figure 2E). PIT54 levels in plasma were also higher in line X at days 7 ($p = 0.0687$) and 21 ($p = 0.001$) than in line Y (+35.5% and +25.4%, respectively) (Figure 2G, Supplementary Table S7B) and for the whole experiment ($p = 0.0354$; +27.5%).

Effect of biological sex

Male chickens exhibited larger average BW than females at all time points, reaching an 8.7% larger BW at day 35 (2157 and 1953 g, respectively (SE = 13.8); Figure 2B). The rest of performance traits were also improved in males compared to females (ADG, ADFI, FCR, and EPEF) (Supplementary Table S2).

Males showed higher COR levels than females in the period 0–35 days (13.8 vs. 11.7 pg/mg; $p < 0.001$; SE = 0.91) and also at day 7 and at day 35 ($p < 0.001$ and $p = 0.028$, respectively) (Figure 2D, Supplementary Table S6). In the last period of the experiment (at day 35), the levels of CRP in plasma were higher in females than males (1.31 vs. 1.18 ng/mg; SE = 0.08; $p = 0.045$) (Figure 2H, Supplementary Table S7B).

Effect of age and development

Corticosterone accumulation in feathers increased as animals grew (Supplementary Table S6; Figure 2E), while the levels of PIT54 and CRP in plasma peaked at day 7, and decreased through time (Supplementary Table S7B; Figures 2F,G).

Effect of zoonotic pathogens

Targeted detection of three common zoonotic pathogens, namely *Salmonella*, *Clostridium* and *Campylobacter* spp. was performed to detect whether natural colonisation of the chicken intestines occurred during the trials. Only one animal in trial A and another in trial B were positive for Clostridium, being all the animals negative for Salmonella and Campylobacter. However, in trial C, the analyses revealed that 13.7% of the sampled animals at day 7 were positive for *Salmonella*, but the colonisation vanished as the animals grew. Moreover, 99% of the birds sampled from day 21 onwards presented a *Campylobacter* colonisation, with no prevalence differences between dietary treatments. The detection of *Campylobacter* was correlated with a drop in BW from day 21 onwards compared to the two previous trials (Figure 2C). In addition, a peak of CRP values in plasma was detected in *Campylobacter* positive animals at day 21 (Figure 2I).

Hierarchical variance decomposition of chicken body weight

Due to our interest in generating systemic characterisation of individual chickens, we explored how the variance of iBW data for each combination of factors (i.e., treatment, biological sex and genetic line) was distributed across the three hierarchical levels of the study: 1) variation across the six animals sampled in each pen at each time point, 2) variation between the two pen replicates within each experiment, and 3) variation among the three experiments.

The average coefficient of variation for iBW of the six sampled animals within each pen was 11.08%, with maximum values reaching 22.5% (Supplementary Table S12). At day 35, the average BW difference between the largest and smallest animal sampled in each pen was over 31% of the mean value. The intrapen variability explained most (76.3%) of the variance observed within combinations of factors (Figure 2J). However, its weight with respect to experiment factor decreased with the age of the animal.

Discussion

Effect of dietary treatments

Bacillus spp. are commonly used as additives in broiler production (Irta, 2015), and the specific probiotic strains tested in our study have been previously shown to improve performance and physiological traits in broilers (Molnár et al., 2011; Goodarzi Boroojeni et al., 2018). However, contrasting observations that align with our results have also been reported (Li et al., 2018), which could be explained by varying experimental conditions, such as the specific *Bacillus* strain employed, the dose, or the basal diet. The supplementation of poultry diets with multi-strain *Bacillus* probiotic products is in general associated with competitive exclusion of common pathogens, improved nutrient digestion and absorption through the production of exogenous enzymes, enhanced intestinal morphology, and the modulation of relevant immune system pathways (Ramlucken et al., 2020; Tarradas et al., 2020). Regarding the phytobiotic, multiple modes of action have been attributed to additives containing polyphenols and procyanidins, including antioxidant and anti-inflammatory properties, promotion of beneficial bacteria in the gut, and enhancement of nutrient absorption through binding of dietary proteins and carbohydrates (Chamorro et al., 2019; Hasted et al., 2021).

As the beneficial effect of feed additives is usually not evident when animals grow under optimal conditions (Vilà et al., 2010), we deliberately induced a challenging condition through increasing the amount of dietary soluble NSP. These compounds are known to have deleterious effects on the bird's health and performance through increasing intestinal viscosity and hampering nutrient digestibility (Raza et al., 2019), and can thus maximise the beneficial effects of feed additives (Bortoluzzi et al., 2019; Whelan et al., 2019). Accordingly, the overall performance was 13.8% lower than the expected from reference performance tables (Cobb-Vantress, 2018; Aviagen, 2019), yet with no differences between treatments.

COR measured in feathers is used as a biomarker of accumulative stress (Bortolotti et al., 2008), as chronic levels of COR are associated with detrimental effects on growth and related biological traits (Scanes, 2016). The increase of COR with the PH diet at day 7, and the disappearance of this effect throughout the study contrasts with previous studies that reported a reduction of serum and feather COR in broilers fed with polyphenol extracts (Gong et al., 2018; Gopi et al., 2020).

To delve into the reasons under, among other questions, the lack of positive effect of the feed additives on commonly assessed nutritional parameters, HoloFood will generate deep genome-resolved metagenomic datasets (Almeida et al., 2019; Pasolli et al., 2019) from the ileal and caecal content. While most chicken-associated microbiota research is being conducted using targeted sequencing (Mohd Shaufi et al., 2015; Jurburg et al., 2019; Ocejo et al., 2019), the first shotgun-sequencing based studies have recently been published (Glendinning et al., 2020; Gilroy et al., 2021). The metagenome-assembled genome (MAG) catalogue of bacteria associated with broiler chickens generated in HoloFood, will not only complement such efforts for the high-resolution characterisation of chicken-associated microbiomes, but will also enable in-depth study of strain-level microbiota dynamics in the analysed production context through combining taxonomic and direct functional inferences. The reconstructed bacterial genomes will be functionally annotated, thus directly inferring the metabolic capacities (e.g., complex polysaccharide degradation, short-chain fatty acid (SCFA) production) of strains, and acknowledging the aggregated functional landscape of the entire community present in each animal (Shaffer et al., 2020). HoloFood will also generate whole genome sequences of the probiotic strains through hybrid short- and long-read DNA sequencing to ensure highest-quality genome reconstructions of the tested probiotics (Wick et al., 2017), and perform a pangenome analysis with other sequenced and annotated Bacillus strains to identify bacterial genes that could confer beneficial functional capabilities each of the strains. This will enable us to understand the specific means of action through which each strain can interact with the microbiota and various intestinal features of the host. All these analyses will enable ascertaining the relative abundances of *Bacillus* probiotics in different intestinal segments, and measuring whether the additives trigger broad-as well as fine-scale taxonomic and/or functional changes in the microbiota of broilers, that could contribute to explain the observed results.

Effect of broiler line

Although theoretically both lines tend to perform similarly in terms of growth and final BW (Cobb-Vantress, 2018; Aviagen,

2019; Livingston et al., 2020), line X reached significantly higher BW, ADG, and ADFI and lower FCR than line Y at day 35. The differences observed on performance between lines could partially be explained by the higher initial BW of line X (44.5 g) at day 0 than line Y (40.8 g) (SE = 0.31; Supplementary Table S2). A retrospective analysis of the breeders' features showed that the age of breeders was higher for line X (49.6 ± 10.1 weeks) than for line Y (44.1 ± 11.4 weeks), which probably caused the observed difference between the initial BW (Iqbal et al., 2017). However, the differences between lines at day 35 did not disappear even when BW at day 0 was included as a covariate in the statistical model, suggesting that other factors contributed to shape the differences observed between both lines. Therefore, performance results suggest that line X could exhibit a higher resistance to NSP or a more active feeding behaviour than line Y.

The higher accumulation of COR in feathers in the broiler line X or in the animals with a higher iBW could be explained by an increased growth rate promoting deposition of COR in feathers (Jimeno et al., 2018), in contrast to what would be expected from a stress indicator (Carbajal et al., 2014). On the other hand, PIT54 is an acute phase protein with an important inhibitory role in inflammation processes (Wicher and Fries, 2006), which is rapidly increased in the blood as a response to infectious agents or physiological stressors (O'reilly and Eckersall, 2014). PIT54 in the chicken plasma binds free haemoglobin to inhibit haemoglobin-mediated oxidation of lipid and protein (Ahn et al., 2019). Antinutritional effects of NSP are related to a reduction of BW and FCR, and can trigger a mild chronic inflammation in the gut (Cardoso Dal Pont et al., 2020). Host gut inflammatory response produces reactive oxygen species (ROS), which cause oxidative stress and have the potential to damage host tissue (Costantini and Møller, 2009). The increased antioxidant activity through the high levels of PIT54 in line X could explain the better performance compared with line Y through the amelioration of antinutritional effects of NSP.

The observed differences between broiler lines point to differences in systemic responses to the pro-inflammatory diet, which probably yielded the BW and associated KPI differences between the two genetic lines. The whole-genome analyses we will conduct in HoloFood will enable deepening into these observations through unveiling genome-wide differences between both lines. We will perform whole genome resequencing to generate single nucleotide polymorphism (SNP) profiles of all individuals (Li et al., 2009). This will enable testing whether both lines have genetic differences in key genes related to inflammatory responses induced by a high dietary concentration of NSP, as well as key metabolic pathways such as steroid hormone biosynthesis (Kanehisa et al., 2021). In addition, we will also generate *de-novo* reference genomes (Baker, 2012) and Hi-C maps (Lieberman-Aiden et al., 2009) of both lines, to explore the effects of structural genome variants (e.g., copy number variations (CNVs), translocations, inversions) in the performance differences observed. Such chicken genomic data will be coupled with the aforementioned microbial metagenomic information, which will allow exploring whether and how host-microbiota interactions orchestrate different physiological responses to nutritional stress in the two broiler lines, as previously reported in other taxa (Ma et al., 2019).

Effect of biological sex

The differences between biological sex among chickens observed in the current experiment are expected (Cobb-Vantress, 2018; Aviagen, 2019), as growth patterns of male chickens outperform that of females (Aggrey, 2002; Hausman et al., 2014; Livingston et al., 2020). However, unlike in previous studies (Carbajal et al., 2014), males showed higher COR levels than females, supporting again that COR cannot directly be negatively associated with welfare and performance of animals (Jimeno et al., 2018). On the other hand, females showed higher levels of CRP than in males at day 35. CRP is an acute phase protein used as a highly sensitive marker of inflammation and tissue damage (O'reilly and Eckersall, 2014). Some immune biomarkers including CRP are influenced by biological sex in other production animals such as pigs (Gutiérrez et al., 2018). However, the reasons behind biological sex differences for inflammatory markers are still unknown.

In HoloFood we will aim at further understanding these differences in growth between both sexes, as well as other questions mentioned above, by generating whole-genome transcriptomic data (RNAseq) to identify gene expression differences in the intestinal tissues. While intestinal expression of targeted genes is routinely measured in animal sciences (Slawinska et al., 2019; Farahat et al., 2021), how genome-wide gene expression varies across sexes and intestinal sections is still largely unknown. As intestinal gene expression is known to be modulated by the microbiota (Volf et al., 2017), and biological sex also contributes to shaping microbial communities (Lee et al., 2017), we will aim at detecting associations between host expression patterns and microbial communities to ascertain host-microbiota interactions related to sex differences. For instance, we will analyse expression of host genes involved in cholesterol (precursor of steroids, and thus related to COR) absorption, such as NPC1L1 and ABCG5/ABCG8, which have been shown to be modulated by the microbiota in rodents (Zhong et al., 2015). Sample collection in HoloFood was extended to organs beyond the gastrointestinal tract, including liver and brain, which are involved in appetite regulation and other processes related to the gut-brain axis (Cryan et al., 2019). The analysis of gene expression in such organs along with gut processes, will enable delving into the relationship between host genetics and microbiota factors with nutrient metabolism in shaping feeding behaviour and related performance differences in broiler chickens.

Effect of age and development

Animal development is linked to multiple changes in analysed metrics. For instance, COR in feathers increased as animals grew, mirroring previous observations (Nordquist et al., 2020). The pattern observed by the levels of PIT54 and CRP in plasma (peaked at day 7 and decreased through time), exhibit a trend that could be linked to vaccination as well as to microbiota development. On the one hand, vaccines are known to increase concentration of acute phase proteins and stress markers during the first days after administration (Kaab et al., 2018), and all chickens in the experiment were vaccinated against Avian Infectious Bronchitis and Gumboro diseases at hatch. On the other hand, early microbial colonisation of the intestine is also known to boost the development of the immune system (Broom and Kogut, 2018) through, for example, the production of the intestinal mucus layer (Duangnumsawang et al., 2021), which provides the first protective shield preventing a direct access of pathogenic bacteria to the epithelial surface.

HoloFood will also generate and analyse microbiota-wide gene expression in the chicken intestine, as well as metabolites that play essential roles in the host-microbiota interplay, such as SCFAs (van der Hee and Wells, 2021). Our study design, which entails euthanising animals at each sampling point, prioritises spatial resolution of intestinal sections over temporal development, which complicates tracking the temporal development of individual animals. However, sampling at three different time points will provide an overview of how much microbiota development varies across individuals (Sprockett et al., 2018; Debray et al., 2021; Ballou et al., 2016; Jurburg et al., 2019). Shotgun metatranscriptomic data will complement the metagenomic information, thus providing not only an overview of the relative abundances of different bacterial taxa in different time points, but also displaying the gene expression patterns of the bacteria. In addition, metabolomic data will enable validating whether the activated metabolic pathways are translated into different levels of SCFA concentrations. We will measure how the expression of microbial genes involved in carbohydrate metabolism and SCFA production vary across development, and how these changes are associated with animal growth and changes observed in acute phase protein levels.

Effect of zoonotic pathogens

Campylobacter and *Salmonella* are usually considered mere commensals in poultry, but they cause the highest numbers of foodborne diseases in humans globally (European Food Safety Authorityand European Centre for Disease Prevention and Control, 2021). Recent studies have nevertheless shown that *Campylobacter* colonisation in chickens can cause gut microbiota alterations and intestinal damage that occasionally facilitates bacterial colonisation of extraintestinal organs, which may eventually lead to a reduced animal performance and welfare (Awad et al., 2015, 2018). In accordance with these observations, the detection of *Campylobacter* was correlated with a drop in BW from day 21 onwards. In addition, the peak of CRP detected in *Campylobacter* positive animals at day 21, would most probably indicate systemic reaction to the colonisation (Liu et al., 2019; Zhang et al., 2020). Other opportunistic pathogens from the *Campylobacterales* order, such as *Helicobacter brantae,* which may be present along with *Campylobacter* but undetected with targeted approaches, might likewise be involved in performance drop (Kollarcikova et al., 2019).

The bacterial genome catalogue we will build in HoloFood will not only enable us to ascertain whether the natural *Campylobacter* colonisation was due to a single or multiple strains (Chaloner et al., 2014), but also to characterise the entire catalogues of genes of these strains and thus identify potential virulence factors that seem to be inducing an inflammatory response. We will be able to measure whether the *Campylobacter* colonisation correlated with any systemic change in the microbiota and in the intestinal response of the animals, through combining gene expression data of chickens and microorganisms as well as metabolomic information. *Campylobacter* induces the expression of various host pro-inflammatory cytokines through the activation of Toll-like receptor 4 (TLR4) and TLR21 signalling pathways (de Zoete et al., 2010). Our analyses will enable measuring changes in the expression levels of genes involved in these signalling pathways between Campylobacter positive and negative animals, to deepen into the effect of *Campylobacter* in chicken welfare and performance.

Hierarchical variance decomposition of chicken body weight

In the current study, the mentioned individual variability was higher than previously reported (Vasdal et al., 2019; Lundberg et al., 2021), although chicken BW variation can increase if animals are subjected to challenging diets (Gous, 2018). The reduction of the intrapen variability with the age of animals, would probably be due to augmenting environmental effects and potentially microbiota development factors. Although the three experiment replicates were identical, and abiotic conditions were controlled in the farm, these were conducted in spring, summer, and autumn, which could have entailed slight differences in the temperature and humidity of the barn.

The whole-genome analyses we will perform in HoloFood will enable ascertaining whether the interindividual genetic variability could be related to the observed dispersion of the data. Genotype-phenotype association studies in commercial chicken lines have identified several important genomic regions that explain a percentage of the BW variation (Tarsani et al., 2019; Wang et al., 2020; Dadousis et al., 2021). In addition, the variability in the intestinal microbiota can intensify differences in performance between individuals from the same population (Yan et al., 2017; Shah et al., 2019; Wen et al., 2021). Ultimately, we will aim at identifying chicken genetic variants associated with microbiota

changes with noteworthy impact on performance. These chicken genomic and microbial metagenomic analyses will enable ascertaining to which degree the observed intrapen and interexperiment variation can be attributed to differences in the genetic features of chickens and microbial communities.

Conclusion

To conclude, the range of analytical approaches outlined in this article will give us the opportunity to showcase the strength of implementing new multi-omic approaches to address relevant questions for farming practices. Many questions that would remain unanswered by employing traditional techniques can now be addressed using these new technologies, and most importantly, new questions that were not so far set out (e.g., the reasons for intra-pen variability) can be now proposed. We believe that the hologenomic approach being implemented in HoloFood will help us move from "Does factor X affect KPI Y?" to "How and why does factor X affect KPI Y?". That is to say, transitioning from a trial-and-error approach to a knowledge-based strategy in which understanding biological processes that underlie the administration of feeds, additives or drugs, as well as the observed interindividual variation, is prioritised. We will address all the questions outlined in this article and more through the collaboration of multiple academic and industrial partners, aimed at pioneering the large-scale implementation of hologenomics in animal farming (Figure 3). Although HoloFood will generate and analyse one of the largest multi-omic datasets in farm animals with characterisation of hundreds of specimens, we acknowledge the mathematical challenges of analysing such a complex and hyper-dimensional dataset. The dimensionality of the data will be reduced by leveraging the hierarchical structure of biology itself (e.g., enzymes embedded within metabolic pathways), as well as using the most advanced feature selection approaches to identify the most relevant molecular elements. Ongoing data analyses, which will be published in upcoming articles, will show us how far we can reach, what are the limitations of this novel approach, and how the field can best advance to make the most of the new technologies for a more secure, ethical and sustainable food production.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by This experiment followed the EU principles for animal care and experimentation and experimental procedures approved by Ethical Committee of Generalitat de Catalunya, Spain (Proceeding number 10226).

Author contributions

NT, EEG, DS, AA, and JT contributed to the conception and design of the study. NT, SM, FGB, APR, EEG, AA, and JT performed the material preparation, data collection, and analysis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.884925/full#supplementary-material

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