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Research Article

Evaluation of A Lactic Acid Based Probiotic on Leaky Gut and Microbiome Associated with *Salmonella Enteritidis* Infection and Feed Restriction in Broiler Chickens

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Abstract

The objective of the present study was to evaluate the effect of a lactic acid based probiotic on leaky gut and microbiome associated challenges with *Salmonella Enteritidis* infection and 24h feed restriction (FR) in broiler chickens. Chickens were orally gavaged with $2x10^4$cfu/chick of *S. Enteritidis* at 1 d of age and then were randomly assigned to one of four groups: 1) Control, 2) Probiotic control, 3) FR, and 4) Probiotic+FR. The probiotic was included in the drinking water for 16 days. Blood samples were collected for measuring leakage of FITC-d, and ceca content was also collected for microbiome evaluation. In the present study, the probiotic reduced FITC-d when compared with FR chickens without the probiotic. At Phylum level, both groups treated with probiotic had higher proportion of Firmicutes and Bacteroidetes. At the Class level, both control groups in this trial had an increase in Gammaproteobacteria. This study confirms that FR increases gut permeability in chickens, but these changes were prevented by the administration of a lactic acid based probiotic.

Keywords: Chicken; Feed restriction; Gut permeability; Microbiome; Stress

Abbreviations: FR: Feed Restriction; GIT: Gastrointestinal Tract; TJ: Tight Junction; LAB: Lactic Acid Bacteria; QIIME: Quantitative Insights into Microbial Ecology; OTUs: Operational Taxonomic Units; BWG: Body Weight Gain; GALT: Gut Associated Lymphoid Tissue; CRF: Corticotropin-Releasing Factor

Introduction

Due to intensive genetic selection, broiler chickens have become the most efficient meat-producing animals because of their fast growth, supported by a virtually unlimited voluntary feed intake. These characteristics cause many problems in the management of broiler breeder hens because of the negative correlation between muscle growth and reproduction ability. Hence, commercial restricted feeding programs in broiler breeders have been implemented, with negative effects on welfare and health, as birds are continuously hungry [1]. Previous research in poultry have showed that feed restriction (FR) increases the plasma levels of corticosterone, an accepted indicator of stress in birds, and it is associated with systemic and local inflammation in the gastrointestinal tract (GIT) as well as oxidative stress [2-4]. Oxidative stress is not only a causative factor of cellular injury but also a pivotal regulator of all crucial cellular metabolism pathways [5,6]. Directly or indirectly, oxidative stress contributes to the structural and functional derangement of the intestinal mucosa. Specifically, high levels of lipid peroxidation, protein oxidation, and glutathione redox state imbalance have been linked with disruption of gut barrier integrity through alterations of the tight junction (TJ) structural complex and enterocyte apoptosis, leading to increased intestinal permeability [7]. Similarly, recent studies conducted in our laboratory have demonstrated that the stress caused by 24h of FR [8,9] or 0.57ppm of dexamethasone in the diet of broiler chickens for six d [8] induce a significant increase in permeability of fluorescein isothiocyanate-dextran (FITC-d) in the blood circulation and is consistent with leakage from the lumen. This suggests the presence of a change in paracellular permeability rather than in transcellular transport. The purpose of the present study was to evaluate the effect of a lactic acid based probiotic on leaky gut and

microbiome changes associated with *S. Enteritidis* infection and feed restriction in broiler chickens.

Materials and Methods

Probiotic culture

FloraMax®-B11 is a defined probiotic culture derived from gastrointestinal poultry origin that contains proprietary strains of lactic acid bacteria (LAB), selected by their *in vitro* ability to inhibit enteropathogens [10]. Several published studies have shown that FloraMax®-B11 increased colonization resistance to *Salmonella* spp. Infections [11-14], reduced idiopathic diarrhea in commercial turkey brooding houses [15], as well as increased performance and reduced costs in poultry production [11,16,17].

Bacterial strains and culture conditions

The challenge organism used in this experiment was a poultry isolate of *Salmonella enterica* serovar *Enteritidis*, bacteriophage type 13A, obtained from the USDA National Veterinary Services Laboratory, Ames, IA, resistant to 25µg/mL of novobiocin (NO, catalog no.N-1628, Sigma) and selected for resistance to 20µg/mL of nalidixic acid (NA, catalog no.N-4382, Sigma) in our laboratory. For both trials, 100µL of *S. Enteritidis* from a frozen aliquot was added to 10mL of tryptic soy broth (Catalog no. 22092, Sigma) and incubated at 37 °C for 8h, and passed two times every 8h to ensure that all bacteria were in log phase. Post-incubation, bacterial cells were washed three times with sterile 0.9% saline by centrifugation at 1,864×g for 10min, reconstituted in saline, quantified by densitometry with a spectrophotometer (Spectronic 20D+, Spectronic Instruments Thermo Scientific), and diluted to an approximate concentration of 10^8cfu per milliliter. Concentrations of *S. Enteritidis* were further verified by serial dilution and plating on brilliant green agar (Catalog no. 70134, Sigma) with NO and NA for enumeration of actual cfu used to challenge the chickens.

Animal source

In this experiment day-of-hatch male broiler chickens were obtained from Cobb-Vantress (Siloam Springs, AR, USA) and were randomly housed in heated brooder batteries, in a controlled age-appropriate environment. Birds were provided *ad libitum* access to water and un medicated corn-soybean diet meeting the nutritional requirements of poultry recommended by National Research Council [18]. All animal handling procedures were approved by Institutional Animal Care and Use Committee at the University of Arkansas approval number 15006 entitled "Development of enteric inflammation models for investigation of antibiotic alternatives in poultry".

Serum determination of FITC-d leakage

Blood samples were collected from the femoral vein, kept at room temperature for 3h, and centrifuged (500xg for 15min) to separate the serum from the red blood cells. FITC-d levels of diluted serum samples (1:5 PBS) were measured at excitation wavelength of 485 nm and an emission wavelength of 528nm with a Synergy HT, Multi-mode microplate fluorescence reader (BioTek Instruments, Inc., Vermont, USA). Fluorescence measured was then compared to a standard curve with known FITC-d concentrations. Gut leakage for each bird was reported as ng of FITC-d per mL of serum.

Experimental design

Day-of-hatch chickens were randomly assigned to one of four groups (n=20/group), neck tagged, individually weighed and placed into battery cages. Experiment groups included: 1) Control no FR; 2) Probiotic in drinking water for 16 d no FR; 3) 24 h of FR; 4) Probiotic in drinking water for 16 d plus 24 h of FR. All chickens were orally gavaged with $2x10^4$ cfu/chick of S. Enteritidis at 1 d of age. Chickens were placed into battery cages in a controlled age-appropriate environment with unrestricted access to feed and water. Beginning at 15 d, chickens in the no FR groups, were allowed to continue with *ad libitum* access to feed, while chickens in FR groups, were subjected to 24h of feed withdrawal. At 16 d of age, chickens in all groups were weighed and given one dose of FITC-d (4.16mg/kg of body weight) by oral gavage. After 2.5h, they were humanely killed by CO_2 asphyxiation. Blood samples were collected for measuring leakage of FITC-d, and in this trial, cecal contents were also taken for DNA extraction as described below.

Ceca Microbial population assessment

Ceca contents (200mg) from each bird were collected for DNA isolation utilizing QIA amp DNA Stool Mini Kit (Qiagen, Valencia, CA). The concentration of extracted DNA was diluted to $10ng\mu L^{-1}$ for the preparation of a sequencing library targeting the V4 region of 16S rRNA gene. Isolated DNA samples were amplified via a PCR using dual-index primers and normalized the amplicons with a Sequal Prep™ Normalization kit (Life Technology, Carlsbad, CA) according to the manufacturers' recommendation. The library was constructed by combining 5µL of each normalized aliquot samples for further assessment. Library concentration and product size were confirmed using a KAPA Library Quantification Kit (Kapa Biosystems, Woburn, MA) via a quantitative PCR (qPCR, Eppendorf, Westbury, NY) and an Agilent 2100 Bioanalyzer system (Agilent, Santa Clara, CA), respectively. The 20nM of pooled library aliquot and the 20nM of PhiX control v3 were combined with 0.2N fresh NaOH and HT1 buffer and mixed a second time with 5% of the PhiX control v3. The 600µL of the mixture containing pooled library, PhiX control v3, NaOH and HT1 buffer was subsequently loaded onto a MiSeq v2 reagent cartridge to run sequencing.

Microbiome sequencing analysis by qiime pipeline

Raw sequencing read files were processed using quantitative insights into microbial ecology (QIIME) pipeline (version 1.9.0). Each of the operational taxonomic units (OTUs) was assigned to specific microorganisms to determine taxonomic levels and subjected to alpha and beta diversity analyses and tables were constructed by clustering sequences with 97% or higher identity based on Greengenes 16S rRNA gene database. In addition, OTUs that were not observed at least twice were excluded manually to eliminate possible erroneous reads from sequencing. Chimeras that were sequences generated by multiple templates or parent sequences were identified and filtered by ChimeraS layer script that utilizes BLAST. Also, the OTU table was sub sampled or rarefied

using a minimal observed OTU value to discard any samples that have unusually fewer sequences. Subsequently, OTUs tables were converted to taxonomic tables for further analysis. Weighted and unweighted version of UniFrac graphs and rarefaction plots were generated for beta and alpha diversity test respectively.

Data and statistical analysis

Body weight gain (BWG), serum FITC-d concentration and proportion of bacterial composition were subjected to analysis of variance as a completely randomized design, using the General Linear Models procedure of SAS [19]. Significant differences among the means were determined by Duncan's multiple-range test at $P<0.05$.

Results

Table 1 shows the results of the effect of a lactic acid bacteria probiotic on serum FITC-d associated with 24h feed restriction and *S. Enteritidis* infection in broiler chickens. In the present study, control and probiotic chickens with no FR showed no leakage of FITC-d. However, a significant reduction in serum FITC-d concentration was observed in chickens that received the probiotic and were exposed to 24h of FR at d 16 when compared with FR control non-treated chickens. Interestingly, a significant increase in BWG was observed in chickens that received the probiotic when compared with control chickens without the probiotic. Chickens that received the probiotic and FR for 24 h showed a numerical increase in BWG when compared with FR control chickens (Table 1).

Table 1: Effect of a lactic acid bacteria probiotic on serum FITC-d associated with *Salmonella enteritidis* infection and 24 hours feed restriction in broiler chickens.

	Serum FITC-d (ng/mL), day 16	Body weight gain (g), day 16
Control	0.00 ± 0.00^{b}	408.45 ± 16.06^{b}
Probiotic control	0.00 ± 0.00^{b}	453.35 ± 7.93^{a}
Feed restriction(24h)	9.70 ± 2.84^{a}	349.55 ± 11.73^{bc}
Probiotic+ Feed restriction (24h)	1.02 ± 0.72^{b}	371.60 ± 8.74^{b}

On day 1, chickens were orally gavaged with 2 $x10^{4}$ *Salmonella Enteritidis*

Data expressed as mean±standard error.

a-cMeans within a column with different superscripts differ ($P<0.05$), n=20 chickens/group

Table 2: Effect of a lactic acid bacteria probiotic on Phylum distribution (cumulative percentage lowest common ancestor) and class direct assignment in percentage for all ceca samples of broiler chickens associated with Salmonella Enteritidis infection and 24 hours feed restriction.

	Control	Probiotic Control	Feed Restriction (24h)	Probiotic+ Feed Restriction (24h)
Phylum (%)				
Firmicutes	54.79 ± 3.89^{b}	86.32 ± 2.74^{a}	24.76 ± 4.48^{c}	74.73 ± 4.75^{a}
Bacteroidetes	7.59 ± 1.76^{c}	27.60 ± 4.02^{b}	68.88 ± 3.51^{a}	30.46 ± 5.80^{b}
Proteobacteria	13.73 ± 2.41^{a}	4.74 ± 0.93^{b}	3.92 ± 1.71^{b}	4.96 ± 2.57^{b}
Actinobacteria	0.005 ± 0.02^{a}	0.029 ± 0.01^{a}	0.003 ± 0.003^{a}	0.029 ± 0.02^{a}
Class				
Gammaproteo bacteria	13.07 ± 2.58^{a}	4.16 ± 0.083^{b}	19.95 ± 2.76^{a}	4.35 ± 2.55^{b}
Clostridia	5.61 ± 2.22^{a}	3.25 ± 1.30^{a}	1.36 ± 0.29^{a}	4.00 ± 2.04^{a}
Betaproteo bacteria	0.005 ± 0.001^{a}	0.021 ± 0.01^{a}	0.001 ± 0.006^{a}	0.003 ± 0.001^{a}

$^{a-b}$Superscripts within rows indicate significant difference at $P<0.05$, n = 6.

The results of the effect of a lactic acid bacteria probiotic on Phylum distribution (cumulative % lowest common ancestor) and Class direct assignment in % for all ceca samples of broiler chickens associated with *S. Enteritidis* infection and 24h feed restriction are summarized in Table 2. At the Phylum level microbiome analysis, both groups of chickens treated with the probiotic had the higher proportion of Firmicutes, followed by control chickens with no FR and chickens that received FR had the lowest number of Firmicutes. Control chickens with no FR and chickens that received FR also showed the higher numbers of Bacteroidetes, followed by both groups that received the probiotic. Control chickens with no FR had the lowest numbers of Bacteroidetes but also had the higer proportion of Proteobacteria. Proportion of Actinobacteria was very low in all groups with no significant differences among them. At the class level, it was interesting to observe that both the control and 24h feed restriction group in this trial had an increase in Gammaproteobacteria, when compared with both groups that received the probiotic, but Clostridia and Betaproteobacteria proportions were similar in all groups (Table 2).

Discussion

Chronic FR represents a permanent stress for any organism, particularly for poultry with relatively high metabolic requirements, where increased plasma corticosterone concentrations are often associated with chronic stress observed in FR programs [20,21]. Stress can induce a variety of changes in normal gastrointestinal function, including changes in gut motility and permeability, as well as alterations in ion, fluid, and mucus secretion and absorption [22-25]. Animal models of acute and chronic stress demonstrate that stress induces changes in intestinal barrier function increasing transcellular and paracellular intestinal permeability associated with a temporary redistribution of TJ proteins [26-30]. These

changes have been linked to Mast cells who are important effectors of the brain-gut axis that translate the stress signals into the release of a wide range of neurotransmitters and proinflammatory cytokines, with dramatic effects on gastrointestinal physiology [31,32]. Since the mucosal barrier of the GIT represents the largest body surface in contact with the external environment, this fragile barrier plays a crucial role in the biology of metazoans [6,33,34]. The single line of intestinal epithelial cells (IECs), are responsible of maintaining its selective barrier function through the formation of complex protein networks: desmosomes, adherent junctions, and TJ [35-37]. Tight juntions are molecules that prevent paracellular permeability [38,39]. Intestinal epithelial cells play important roles in mechanisms of innate immunity as part of the gut associated lymphoid tissue (GALT), displaying a wide array of immune functions such as pathogen recognition, release of anti-microbial compounds, and secretion of several hormones, neurotransmitters, enzymes, cytokines and chemokines [40-42]. Furthermore, IECs such as goblet cells secrete several mucins that reinforce the overall intestinal barrier [43,44]. Therefore, any injury to IECs could lead to dramatic changes in gut permeability that result in disruption of the GIT homeostasis, followed by intestinal and systemic inflammation [45]. Published studies have shown the mechanisms linked with the disruption of TJ by inflammatory mediators, among them: hormones, oxygen free radical species, enzymes as well as multiple proinflammatory cytokines released by pathogens, diet ingredients, or stress [39,46].

More recently, we have demonstrated that a rye-soybean ration, 24 h of feed restriction, or dietary administration of dexamethasone induce leaky gut, bacterial translocation, and dysbacteriosis in broiler chickens [8,47,48]. In the present study, the commercial probiotic was able to reduce the intestinal permeability of FITC-d into the serum in models previously published of FR (Table 1). FITC-d is a large molecule (3-5kDa) which does not usually leak through the intact gastrointestinal tract barrier. Nevertheless, when epithelial TJ are disrupted, serum FITC-d leaks to the blood stream as demonstrated by an increase in trans-mucosal permeability associated with the stress caused by 24 h of FR [49]. As previously reported, the fact that gut permeability was significantly higher in FR chickens suggests that this stress practice has a strong impact on the epithelial barrier, altering gut permeability in broiler chickens [8,50]. On the other hand, it has been studied in chickens that elevated serum concentrations of corticosteroid are associated with environmental stress [51-54]. The stress-induced intestinal disturbances caused by corticosteroids is mediated by corticotropin-releasing factor (CRF), which increases intestinal paracellular permeability via mast cell dependent release of TNF-α and proteases causing systemic and local inflammation in the GIT by oxidative stress [1-3,37,55-58]. Oxidative stress is not only a causative factor of cellular injury but also a pivotal regulator of all crucial cellular metabolic processes [5,6]. Directly or indirectly, oxidative stress contributes to the structural and functional derangement of the intestinal mucosa. Specifically, high levels of lipid peroxidation, protein oxidation, and glutathione redox state imbalance have been linked with disruption of gut barrier integrity through alterations of the TJ structural complex and enterocyte apoptosis leading to increased intestinal permeability [7,59,60]. In the present study, in addition to the stress caused by FR, all chickens were also challenged with *S. Enteritidis* at day-of-hatch. Similar to a previous study conducted by our laboratory using S. Heidelberg [61], in the present study *S. Enteritidis* challenge did not increase the leakage of FITC-d (Table 1) by itself. Metagenomic analysis of cecal content using the MEGAN software can be used to interactively analyze and compare metagenomic and metatranscriptomic data, thereby providing a percent identity filter that can be used to enforce the following levels of percentage sequence identities for an assignment at a given taxonomic level [62].

Chickens treated with the probiotic had the higher proportion of Firmicutes, followed by control chickens with no FR and chickens that received FR had the lowest numbers. Control chickens with no feed restriction and chickens that only received FR also showed the higher numbers of Bacteroidetes, followed by both groups that recived the probiotic. Control chickens with no FR had the lowest numbers of Bacteroidetes, however, these chickens also had the higher proportion of Proteobacteria. Control chickens with no FR had the lowest numbers of Bacteroidetes but had the higher proportion of Proteobacteria. Proportion of Actinobacteria was very low in all groups with no significant differences among them. At the Class level, it was interesting to observe that both control groups in this trial had an increase in Gammaproteobacteria, when compared with both groups that received the probiotic, but Clostridia and Bacilli proportions were similar in all groups. Changes in the proportion of Phylum and Class were associated with the challenge of S. Enteritidis which belongs to phylum Proteobacteria, class Gamma proteobacteria (Table 2). In contrast, chickens that received the probiotic had the highest proportion of Firmicutes and Bacteroidetes, but the lowest amount of Proteobacteria. Probiotic treated chickens also showed significant reduction in Gamma proteobacteria, but similar to the control group, a higher proportion in Clostridia and Bacilli. The shift in these bacterial populations, had a similar trend as previously reported with S. Heidelberg in previous research [63].

The results of the present study confirm that 24h of FR to broiler chickens increased gut permeability as was indicated by the detection of FITC-d in the serum, but these changes were prevented by the administration of a lactic acid based probiotic. In addition to their well-recognized immune modulator properties [64-68] several investigators have reported that both lactic acid based probiotics as well as *Bacillus sp*. based probiotics maintain intestinal homoeostasis and improve the integrity of the intestinal epithelial cells through their anti-inflammatory and anti-oxidative properties [69-71]. Studies to evaluate anti-inflammatory and anti-oxidant properties of previously selected probiotic in chickens under different stress conditions are currently being evaluated [72-76].

Conflict of Interest Statement

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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