UNIVERSIDADE FEDERAL DO PARANÁ

RICARDO MITSUO HAYASHI

PHENOTYPIC AND GENOTYPIC FEATURES OF THE *Salmonella enterica* serovar Heidelberg STRAIN UFPR1 AND INSIGHTS ABOUT INTESTINAL MICROBIOTA IN FERAL AND BROILER CHICKENS

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Orientação: Prof.ª Dr.ª Elizabeth Santin

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS VETERINÁRIAS da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **RICARDO MITSUO HAYASHI** intitulada: **Phenotypic and genotypic features of the *Salmonella enterica* serovar Heidelberg strain UFPR1 and insights about intestinal microbiota in feral and broiler chickens**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua Aprovado no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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Avaliador Externo

ANDERSON FERREIRA DA CUNHA
Avaliador Externo

RODERICK MACKIE
Avaliador Externo

RICARDO RAUBER
Avaliador Externo

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“Somewhere, something incredible is waiting to be known”

(Carl Sagan, 1977)

“The states of health or disease are the expressions of the success and failure experienced by the organism in its efforts to respond adaptively to environmental challenges”

(Rene Dubos, 1965)

RESUMO

Salmonelas paratíficas são patógenos relevantes sob uma perspectiva clínica e de saúde pública. A *Salmonella enterica* sorovar Heidelberg (SH) frequentemente causa doenças transmitidas por alimentos em humanos e é frequentemente encontrada em avicultura em vários países, causando perdas econômicas e preocupações relacionadas à saúde pública. Neste cenário, gerou-se uma riqueza de informações sobre uma cepa emergente brasileira de SH, UFPR1, a fim de compreender sua variação genética, patogenicidade, resistência e medidas alternativas de controle. No Capítulo 1, o primeiro genoma completo da cepa SH UFPR1 é relatado, revelando 11 fragmentos genômicos ausentes em comparação a uma cepa SH resistente a múltiplos fármacos, o que explica a alta suscetibilidade a antibióticos e resistência a ácidos orgânicos de cadeia curta. No Capítulo 2, um probiótico composto por três cepas de *Bacillus subtilis* melhorou o desempenho animal quando alimentado a 250 g/ton e reduziu a colonização de SH UFPR1 no trato gastrintestinal. O probiótico mobilizou as células imunes e promoveu importantes alterações histológicas, relacionada à ativação da resposta de defesa e absorção intestinal. Além disso, a suplementação de probiótico aumentou a diversidade da microbiota cecal e alterou alguns grupos bacterianos comensais no íleo. Estudos sobre o microbioma intestinal e a imunidade inata em frangos geneticamente diferentes e inseridos em ambientes mais naturais são raros e podem nos fornecer ideias sobre a evolução e a ecologia dos patógenos, comensais e hospedeiro. No Capítulo 3, estudamos duas populações de aves feralizadas em Bermudas e no Havaí, comparando a composição da microbiota intestinal e a resposta imune inata a um grupo de frangos de corte comerciais. O sequenciamento de alto rendimento revelou a presença de uma microbiota central e algumas comunidades exclusivas em aves feralizadas e comerciais. A perda progressiva da diversidade microbiana no grupo de frangos de corte pode estar correlacionada a uma resposta imune inata vulnerável e interessantemente mediada por toll-like receptors (TLRs). Mais estudos serão necessários para distinguir características genotípicas e fenotípicas de diferentes cepas e variantes de *Salmonella*, a fim de melhorar e identificar medidas específicas de controle. Além disso, mais pesquisas envolvendo a feralização são úteis para esclarecer e explorar possíveis elos relacionados

a mudanças evolutivas concomitantes, visando melhorar a produtividade e produzir produtos avícolas mais seguros.

Palavras-chave: feralização, high throughput sequencing, microbioma, *Salmonella* Heidelberg, saúde intestinal, whole genome sequencing, probiótico.

ABSTRACT

Non-typhoidal *Salmonella* are relevant pathogens under a clinical and public health perspective. *Salmonella enterica* serovar Heidelberg (SH) frequently causes food-borne illness in humans and are frequently found in broiler operations in several countries, causing economic losses and health-related concerns. This research effort has generated a wealth of information on an emergent Brazilian SH, UFPR1 strain, in order to understand its genetic variation, pathogenicity, resistance and alternative measures of control. In the Chapter 1, the first complete genome of SH UFPR1 strain is reported, revealing 11 missing genomic fragments in comparison with a multidrug resistant SH strain, which explains the high susceptibility to antibiotics and short-chain organic acids resistance. In the Chapter 2, a probiotic composed by three strains of *Bacillus subtilis* improved animal performance when fed at 250 g/ton and reduced SH UFPR1 colonization in the gastrointestinal tract. The probiotic mobilized immune cells and promoted important histologic alteration, related to activation of defense response and gut absorption. In addition, the supplementation of probiotic increased the diversity of cecal microbiota and increased some commensal bacterial groups in ileum. Studies about gut microbiome and innate immunity in chickens genetically different and inserted in more natural environments are rare and can provide us insights about evolution and ecology of the pathogens, commensals and the host. In the Chapter 3, we studied two populations of feral chickens in Bermuda and Hawaii, comparing their gut microbiota composition and innate immune response to a group of modern broiler chickens. High throughput sequencing revealed the presence of a core microbiota and some exclusive taxa in feral and broiler chickens. The progressive loss of the microbial diversity in the broilers group may be correlated to a vulnerable and interesting innate immune response mediated by TLRs. Further work will be needed to distinguish genotypic and phenotypic features of different *Salmonella* serovars and strains in order to enhance and identify specific measures of control. Also, more research involving feralization is useful to clarify and explore possible links related to concomitant evolutionary changes, aiming to improve productivity and produce safer poultry products.

Key-words: feralization, gut health, high throughput sequencing, microbiome, probiotic, *Salmonella* Heidelberg, whole-genome sequencing.

SUMMARY

INTRODUCTION

Salmonellosis remains one of the most frequent food-borne zoonosis, constituting a worldwide major public health concern. Currently, at a global level, the main sources of infection for humans include the consumption of contaminated poultry products, despite the deployment of several control strategies such as integrated surveillance, multi-sectorial investigations, biosecurity and vaccination. Non-typhoid *Salmonella enterica* serovars are among the most common causative agents of food-borne diseases, being responsible for causing around 93.8 million illnesses and 155.000 deaths each year worldwide (CHEN *et al*., 2013). The Centers for Disease Control and Prevention (CDC), the USA Food and Drug Administration (FDA), the European Food Safety Authority (EFSA), the Public Health Agency of Canada (PHAC), and the Brazilian Health Regulatory Agency (ANVISA) each frequently report outbreaks caused by *Salmonella*-contaminated poultry products. According to the CDC, in 2013, 47% of food outbreaks were caused by bacteria, and of these outbreaks, 26% were caused by *Salmonella* spp., with *S*. Enteritidis, *S*. Typhimurium, *S*.Heidelberg, *S*. Newport, and *S*. Javiana as the main serovars (CDC, 2013). In Brazil, between 2007 and 2017, 95.9% of the outbreaks were caused by bacteria, and *Salmonella* spp. was the second most frequent causative agent (ANVISA, 2017).

In recent years, a shift in *Salmonella* serovars related to poultry and poultry production has been reported in diverse geographical regions, being particularly associated with the spread of certain well-adapted strains. Moreover, antimicrobial resistance in non-typhoidal *Salmonella* is considered one of the major public health threats related with food-animal production, including the poultry production chain and poultry meat (DHANANI *et al*., 2015)

The control of some foodborne pathogens such as *Salmonella* is complicated once their complex ability to survive during animal production, food processing, storage and improper cooking. Therefore, it is important to understand the ecology of *Salmonella* and the genetic variation of different serovars found in broiler chickens in order to design specific management practices to reduce risks associated with this pathogen. For example, understanding the genetic variation in different or similar *Salmonella* serovars and their relation with the host (colonization, immunity and microbiota) would provide

insights into their survival mechanisms in the chicken gut, and may lead to the development of innovative tools to prevent or limit their spread.

One of the most prevalent serovar is *Salmonella enterica* serovar Heidelberg, which is commonly isolated from patients with salmonellosis in North America, where its prevalence is greater than in other continents (CDC, 2014). SH provokes more invasive human infections (*e.g.*, myocarditis and bacteremia) than others non-typhoidal *Salmonella* (AFRC, 1989). Since 1962, SH has been isolated and reported from poultry and their products worldwide (LJUNGH; WADSTRÖM, 2006) and in Brazil (LA RAGIONE; WOODWARD, 2003). VOSS-RECH *et al.* (2015) reported 20 different *Salmonella* serovars in samples from broilers, and SH was prevalent in 7.31% of those.

SH UFPR1 strain was isolated from commercial broiler carcasses in South of Brazil, region that accounts almost 60% of the Brazilian broiler production. As an emergent pathogen, the UFPR1 strain has been used in our researches in order to understand its pathogenicity and potential measures of control. The LABMOR/CERIA (Laboratory of Microbiology and Ornithopatology / Center of Avian Immune Response) at UFPR had tried extensively to find effective solutions to control this strain in broiler chickens. Vaccines and feed additives such as organic acids, prebiotics and some strains of probiotics could not decrease the colonization of the UFPR1 strain in different organs at variated ages.

In chapter 1, we performed the whole-genome sequencing to investigate genomic features of the SH UFPR1 strain and compared against a multidrug resistant strain. Also, we observed clinical parameters of broilers challenged with UFPR1 strain and its resistance and susceptibility to antibiotics and organic acids.

In order to obtain an efficient feed additive to control SH UFPR1, the chapter 2 describes the ability of a probiotic composed of three different *Bacillus subtilis* strains to reduce the invasiveness and gut colonization of the SH UFPR1 strain, its effects on performance, immune response and diversity of gut microbiota by the high-throughput sequencing of the 16S rRNA gene. Also, we applied an innovative system to evaluate intestinal health by histology.

After this project, the gut microbiota has gained our interest and we sought to understand how the environment and the host impact the structure of the gut microbiome, specifically by correlations with immune response and gut microbiota composition.

Different environments and host genetic profile have been linked to differences in microbiome composition, thus suggesting that both factors can shape the gut microbiome of the host. However, cause-consequence mechanisms behind these links are still unclear.

In chapter 3, a partnership involving UFPR, University of Illinois and Michigan State University allowed us to make the first step to comprehend and characterized the gut microbiota and innate immune response of two populations of feral chickens in Bermuda and Hawaii, in comparison to a commercial broiler chickens group. Feralization, considered as the reverse of domestication, occurs when a domestic population recolonizes the wild, escaping its previous restricted environment. This project offers insights on how the modern poultry industry has selected the animals on growth characteristics that could adversely affect the gut microbes, leaving chickens, or not, more susceptible to diseases. Understanding the feral chicken's gut microbiota and its relation with immunity may provide insights to enhance performance and the microbiological safety of poultry products.

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CHAPTER 1

PHENOTYPIC AND GENOTYPIC FEATURES OF A *Salmonella* Heidelberg STRAIN ISOLATED IN BROILERS IN BRAZIL AS RELATED TO ANTIBIOTICS AND SHORT CHAIN ORGANIC ACID RESISTANCE AND SUSCEPTIBILITY

ABSTRACT

Salmonella enterica serovar Heidelberg (SH) frequently causes food-borne illness in humans and frequently found in broiler operations in several countries, causing economic losses and health-related concerns. Although the importance of SH, few data have been reported on its resistance and susceptibility to feed additives, generally used in the poultry industry such as: (*i*) antimicrobials, (*ii*) organic acids, (*iii*) probiotics etc. There is also little information about genetic diversity of different strains of SH. In Brazil, isolations of this serovar have been increasing since 2011 in broiler farms, indicating tolerance to traditional control measures. It was herein evaluated: (*i*) clinical parameters of broilers challenged with Brazilian SH (UFPR1 strain), (*ii*) its susceptibility to antibiotics and (*iii*) to organic acids, contextualizing with comparative genomics. UFPR1 strain did not affect broiler zootechnical performance parameters, there was no difference in clinical signs and few changes in organ histology and immune response of challenged broilers compared to non-challenged group. The use of organic acids in broiler feed did not significantly decrease SH isolation in cecum and liver of broilers compared to non-supplemented and challenged group. UFPR1 strain was susceptible *in vitro* to amikacin, amoxicillin+clavulanate, ceftiofur, cephalexin, doxycycline and oxytetracycline and presented intermediary resistance to ampicillin+sulbactam, cephalothin, ciprofloxacin, enrofloxacin, gentamycin and penicillin. The first complete genome of UFPR1 strain is reported herein. It consists of a circular chromosome, spanning 4760321 base pairs with 52.18% of GC-content and encodes 84 tRNA, 22rRNA and 4427 protein-coding genes. Moreover, the comparison between the genomes from UFPR1 strain and the multidrug resistant SL476 strain, revealed 11 missing genomic fragments. Crucially, the genes in these regions code proteins associated with: (*i*) cell cycle regulation, (*ii*) virulence, (*iii*) drug resistance, (*iv*) cellular adhesion, (*v*) salt efflux and (*vi*) several genes related to transposases and integrases could be involved in these deletions. Therefore, these

findings of missing genes in UFPR1 strain genome were in line with the phenotypic results of low-pathogenicity and antibiotic susceptibility of this strain.

Key words: antibiotic, comparative genomics, organic acids, resistance, UFPR1 strain.

INTRODUCTION

Despite recent advances in the treatment of infectious diseases, pathogenic microorganisms, including *Salmonella,* remain an important threat to human and animal health worldwide (EL GHANY *et al*., 2016). Non-typhoid *Salmonella* are known pathogens but they also silently infect animals, particularly poultry, as transient members of the intestinal microbial population without causing disease (BARROW *et al*., 1987; MUNIZ *et al*., 2015). Often, colonization of several serovars of *Salmonella enterica* have no effect in poultry zootechnical performance (MUNIZ *et al*., 2015). Kogut *et al*. (2016) described that *Salmonella* Enteritidis-infected chickens induces an immunological tolerogenic response beginning around three to four days post-primary infection which decreases the host responsiveness resulting in the establishment of persistent colonization. These asymptomatic infections could increase the probability of transmission to humans via contaminated food (CARTER *et al*., 2009).

Therefore, reducing *Salmonella* colonization and fecal shedding in live chickens and subsequently in chicken meat contamination can reduce the burden of salmonellosis in humans. Short chain organic acid (SCOA) have been added to feed, drinking water, and other matrices, in order to prevent *Salmonella* colonization in animal tissue and transmission through the food chain with many positives results, however the mode of action has not been totally understood (VAN IMMERSEEL *et al*., 2006).

One of the most prevalent serovar is *Salmonella enterica* serovar Heidelberg (SH), which is commonly isolated from patients with salmonellosis in North America, where its prevalence is greater than in other continents (CDC, 2013). SH provokes more invasive human infections (*e.g*., myocarditis and bacteremia) than others non-typhoidal *Salmonella* (HOFFMANN *et al*., 2014). Since 1962, SH has been isolated and reported from poultry and their products worldwide (FDA, 2013) and in Brazil (HOFER *et al*., 1997). Voss-Rech

et al. (2015) reported 20 different *Salmonella* serovars in samples from broilers, and SH was prevalent in 7.31% of those. There is no study comparing the genome of Brazilian SH (UFPR1 strain) to the SH described worldwide.

Nowadays, whole-genome sequencing (WGS) is an important molecular tool to investigate genomic features of any organism. Using this technique, several genomes from *Salmonella* strains were elucidated in order to better comprehend some aspects of their evolutionary biology, distinguish outbreak-related strains of sporadic infections (ALLARD *et al*., 2012) and comparing genomes of strains with different clinical history and resistance profile (EL GHANY *et al*., 2016; OGUNREMI *et al*., 2017; REIMSCHUESSEL *et al*., 2017). Moreover, WGS technique was recently used to comprehend the differences among serotypes of SH (DHANANI *et al*., 2015; LABBÉ *et al*., 2016) and resistance to different antibiotics (DONADO-GODOY *et al*., 2015; EDIRMANASINGHE *et al*., 2017).

The objective of this study was to evaluate clinical parameters of broilers challenged with UFPR1 strain and its resistance and susceptibility to antibiotics and organic acids, contextualizing with comparative genomic findings between UFPR1 and a multidrug resistant SH strain SL476 (GenBank Accession number NC_011083.1) (FRICKE *et al*., 2011).

MATERIAL AND METHODS

IN VIVO EXPERIMENT

UFPR1 strain was isolated from commercial broiler carcasses in South of Brazil according with procedures described in BRASIL MAPA (2003). The strain was serologically identified by Fiocruz Institute (protocol number 6830/2012).

Two experiments were conducted at CERIA (Center of Immune Response in Poultry) at Federal University of Parana, Curitiba, Brazil to evaluate the clinical effect of UFPR1 strain on broilers and the use of SCOA to control this infection. In Trial 1, a treatment with 0.05% of an organic acid blend (30% of formic acid and 18% of propionic acid) offered in drinking water was evaluated. It was given from 1 to 7 days and from 15 to 21 days of age, associated to a treatment with 3,0 Kg/ton of a product with minimum

92% of fumaric and benzoic acids in feed, from 1 to 21 days of age. In Trial 2, a product constituted with coated 89% of calcium butyrate at 2 Kg/ton in feed from 1 to 21 days was evaluated. The experiments were approved by the Ethical Committee of Agricultural Sector of Federal University of Parana under approval number: 037/2016 and 014/2016, respectively. All other procedures were the same for both trial as follow described.

HOUSE, CHICKEN AND SAMPLE COLLECTION

Eight isolated rooms previously disinfected with negative pressure were used. Each room contained four battery cages (replications) stacked vertically with sterilized litter, nipple drinkers and automatic temperature and lighting control. One-day-old male chicks (Cobb® 500; n=192) from one to 21 days of age were distributed in a completely randomized design with four treatments (n=48 birds per treatment, with four replicates per treatment, 12 birds per replication [i.e., pen]). T1: Non-challenged plus control diet, T2: Non-challenged plus SCOA treatment according to Trial, T3: Challenged with SH plus control diet, T4: Challenged with SH plus SCOA according to Trial.

At day 1 and 21, birds and feed were weighed to evaluate feed intake (FI), body weight gain (BWG) and feed conversion (FC).

At day 1, 10 chicks and swabs from each wall rooms and cages were collected to confirm the negativity in both *in vivo* experiments by qualitative analysis.

At day 7 and 21 in Trial 1 and day 14 and 21 in Trial 2, 12 birds from each treatment were euthanized by cervical dislocation, necropsied and liver and cecum were collected for *Salmonella* sp. counting procedure. In Trial 2 at day 14, liver and cecum of five birds per treatment were collected for histology and the liver was also collected in RNAlater for evaluation of mRNA expression of IL10 and IL-12.

DIETS

The nutritional density of experimental diets resembled those fed commercially in Brazil (ROSTAGNO *et al*., 2005) . Diets were based on corn and soybean meal and they were offered in mash form, fed *ad libitum* and formulated without coccidiostatic or antibiotics. Diets were designed for a unique feeding phase (Starter) from 1 to 21 days of age for all treatments.

All basic feed (with all ingredients except amino acids, vitamin and mineral premix) was sterilized by autoclave at 120ºC/15 minutes. After this process, the organic acid treatment, amino acids, vitamin and mineral premix were added according to each treatment and diet formulation and mixed for 10 minutes in a 50 Kg mixer. Batches were mixed in such an order to avoid interference among treatments.

SH CHALLENGE AND QUANTIFICATION

At 3 days of age in Trial 1 and at 7 days in Trial 2, chicks from T3 and T4 were challenged by gavage with 10^7 CFU/chick of SH.

In order to quantify typical colonies of *Salmonella* sp. (quantitative analysis), samples of liver and cecum were processed using the modified methodology by (PICKLER *et al*., 2012). Briefly, the organs were weight, mashed and homogenized in 2% buffered peptone water (1:9). Further dilution was conducted by successively placing 1 mL of the solution in a test tube with 9 mL 0.1% peptone water until a 10^{-3} dilution was achieved. Then 100 μL aliquots of each dilution were transferred to duplicate plates in Brilliant Green Agar (BGA) medium and uniformly spread with a sterile Drigalsky loop. The plates were incubated at 35ºC for 24h, after which the typical colonies were counted. For all samples, preenrichment was performed in 2% buffered peptone water at 35ºC for 24h. The samples that did not show typical *Salmonella* colonies in directly BGA counting were enriched into 10 mL Rappaport-Vassiliadis broth and incubated at 42ºC for 24h. Thereafter, a drop of the enrichment broth was placed on BGA medium. The samples that were negative after direct BGA plate counting, but positive after enrichment were assumed to have 10^1 CFU/g. The samples that were negative after enrichment were assumed to

have 0 CFU/g. For confirmation of the *Salmonella* serotype, the samples isolated were sent to the Sector of Enterobacteria of the Oswaldo Cruz Institute for serotyping.

HISTOLOGY EVALUATION OF CECUM AND LIVER (TRIAL 2)

Samples of cecum and liver were processed according with Kraieski *et al*. (2016). Briefly, samples were embedded in paraffin and 5 *μ*m sections were cut and stained with hematoxylin and eosin and for cecum was also added Alcian Blue. Liver samples were evaluated in 5 fields per bird in 10X objective and 100X of magnification, and it was observed congestion, hydropic degeneration, cell vacuolation, bile-duct proliferation, immune cells infiltration, pericholangitis and lymphocytic aggregate. The “I See Inside” - ISI methodology applied is in process of patent (SANTIN *et al*., 2015) and was developed based on a numeric score of alteration. In this methodology, an impact factor (IF) is defined for each alteration in microscopic analysis according to the reduction of organ functional capacity, based on previous knowledge of literature and background research. The IF ranges from 1 to 3, where 3 is the most impactful for the organ function, e.g. necrosis has the highest IF because the functional capacity of affected cells is totally lost. In addition, the extent of each lesion (intensity or observed frequency compared to non-affected organs) is evaluated in each organ/tissue per animal and the score ranges from 0 to 3: score 0 (absence of lesion or frequency), score 1 (alteration up to 25% of the area or observed frequency), score 2 (alteration ranges from 25 to 50% of the area or observed frequency), and score 3 (alteration extent more than 50% of the area or observed frequency). To reach the final value of the ISI index, the IF of each alteration is multiplied by the respective score number, and the results of all alterations are summed. The scales range from 0 to 39 for the liver.

For cecum samples, it was evaluated 5 fields per bird in 40X objective and 400X of magnification and was measured villus height, villus thickness, presence of erythrocytes and infiltration of immune cell on lamina propria.

CYTOKINES MRNA EXPRESSION IN LIVER (TRIAL 2)

Six birds per treatment were euthanized had their liver sampled in RNAlater and immediately stored at -20°C until further analysis. Briefly, total RNA from the tissues was isolated using Trizol reagent (15596-018, Invitrogen, Carlsbad, CA) and the procedure was followed according to the manufacturer's instructions. Turbo-DNAse kit (AM1907, Applied Biosystems, Foster City, CA) was used to treat the samples. RNA concentrations were quantified by NanoDrop Spectrophotometer (ND1000, Thermo Scientific, Bonn, Germany) and integrity was determined by Experion Automated Electrophoresis System (700-7000, Bio-Rad, Hercules, CA). RNA samples were reverse transcribed and RT-qPCR analyses were performed with a MyiQ System (170-9740, Bio-Rad). One microgram of RNA was converted to cDNA in a 20 µL reaction volume using the iScript™ Reverse Trasncription Supermix kit (170-8841, Bio-rad) at 25°C for one hour, 42°C for 30 minutes, and then 85°C for 5 minutes.

The genes analyzed by RT-qPCR were: IL-10 (5'-cgggagctgagggtgaa-3' and 5'-gtgaagaagcggtgacagc-3'), IL-12 (5'-agactccaatgggcaaatga-3' and 5'-ctcttcggcaaatggacagt-3') and GAPDH (5'-ggtggtgctaagcgtgttat-3' and 5'-acctctgtcatctctccaca-3'). The final 20 µL PCR reaction contained 2 µL reverse transcription product, 2 µL of the forward and reverse gene, and 10 µL of iTAq® Universal SYBR Green Supermix (172-5122, Bio-Rad). PCR cycle conditions of all primer pairs used an initial 60s denaturation step at 95°C followed by 40 cycles of denaturation (15s at 95°C), annealing and extension (30s at 60°C). The melting profile of each sample was analyzed after every PCR run to confirm PCR product specificity and was determined by heating samples at 65°C for 30s and then increasing the temperature at a linear rate of 20°C/s to 95°C while continuously monitoring fluorescence. Sample PCR amplification efficiencies were determined in the log-linear phase with the LinRegPCR program (RAMAKERS *et al*., 2003). Additionally, the delta–delta equation subtracts sample and reference Ct values from an endogenous control; however, the endogenous control (GAPDH) Ct was affected by treatments in this study ($P<0.05$), and therefore was removed from the equation. All data were normalized to the mRNA level of the control group (group non-challenged and without SCOA) and reported as the fold-change from the reference, which was calculated

as $E_S^{(40\text{-Ct Sample})}/E_R^{(40\text{-Ct Reference})}$, where E_S and E_R are the sample and reference PCR amplification efficiencies, respectively (HUMPHREY, 2004).

STATISTICAL ANALYSIS OF *IN VIVO* STUDIES

Data were analyzed using the statistical software Statistix 9. For microbiological analysis, data were evaluated by the Shapiro-Wilk normality test. Parametric data were subjected to analysis of variance (ANOVA) and Tukey's test ($P<0.05$). Nonparametric data (quantitative microbiological data and histology data) were submitted to the Kruskall-Wallis test ($P<0.05$). The chi-square test was used in microbiological results of presence/absence (qualitative) of *Salmonella* in liver of Trial 2. For results of zootechnical performance, and immunohistochemistry analysis, data were submitted to analysis of variance (ANOVA) as factorial design 2x2.

IN VITRO EXPERIMENT: ANTIMICROBIAL SUSCEPTIBILITY TEST

UFPR1 strain was assayed for susceptibility to a panel of 12 antimicrobials commonly used in human and veterinary clinic in Brazil. Antimicrobial minimum inhibitory concentration of SH was determined by dilution antimicrobial method using Mueller-Hinton agar after incubation at 37ºC for 18-24h. MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) results were interpreted in accordance to the interpretative criteria provided by Clinical and Laboratory Standards Institute (CLSI, 2004). The 12 antimicrobials tested included amikacin (250 mg/mL), amoxicillin+clavulanate (14 g + 3.5 g/100 mL), ampicillin+sulbactam (1 g **+** 0.5 g/10 mL), ceftiofur (50 mg/mL), cephalexin (250 mg/5 mL), cephalothin (1 g/10 mL), ciprofloxacin (2 mg/mL), doxycycline (4.6 g/100 mL), enrofloxacin (10 g/100mL), gentamycin (40 mg/mL), penicillin (6000000 UI/15 mL) and tetracycline (20 g/100 mL). The *Escherichia coli* ATCC 25 922 was used as reference strain. The MIC breakpoints were set based on CLSI (CLSI, 2004) and FDA (FDA, 2013).

GENOMIC ANALYSIS AND COMPARATIVE ANALYSIS

Isolated UFPR1 strain was cultured overnight in liquid LB medium and genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) and quantified using the NanoVue spectrophotomer (GE Healthcare). A total of 70 µg of DNA was sent to the High-Throughput Sequencing Facility at University of North Carolina. The library was prepared using the PacBio's 20 Kb template prep protocol (PN_100-286-000-06) and the library was size-selected on using a range setting of 8000 bp to 50,000 bp. De Novo assembly was performed using PacBio native pipeline (BERLIN *et al*., 2015). Comparative genomic analysis was independently performed with MAUVE v.20150225 (DARLING; MAU; PERNA, 2010) and Mummer v.3.23 (KURTZ *et al*., 2004) programs, using the annotated genome of SH strain SL476 as reference (GenBank assembly accession: GCA_000020705.1) (FRICKE *et al*., 2011). The shared genomic fragments between UFPR1 and SL476 were identified with Mummer and the regions without match in the other genome were identified with a Perl script (available in https://github.com/CaioFreire/Scripts). PROKKA v.1.12 software was used for genome annotation (SEEMANN, 2014) and the circular map was drawn using DNAPlotter v.10.2 (CARVER *et al*., 2009). The genome was deposited at the NCBI genome database under the number CP020101. In addition, missing fragments found for each genome with Megablast (https://blast.ncbi.nlm.nih.gov) were compared to the deleted genome, checking if the gene sequences in these missing fragments were present in other parts of the genome.

RESULTS

In the *in vivo* experiment, SCOA were given to the birds in the early days of life to provide adaptation to the organic acid treatment in the gut before challenge with SH. Findings regarding production parameters showed no significant effect ($P>0.05$) of SH challenge, SCOA treatment or interaction on zootechnical performance in any age period. Table 1 presents the results of SH challenged factor in both trials showing no effect on animal development.

TABLE 1 - MEAN ± STANDARD DEVIATION OF FEED INTAKE (FI), BODY WEIGHT GAIN (BWG) AND FEED CONVERSION (FC) DURING THE PERIODS OF 1 TO 21 DAYS OF AGE OF BROILERS ON TRIALS 1 AND 2 IN NON-CHALLENGED AND CHALLENGED BIRDS.

	Period	**Non-challenged**	**Challenged**	***P value**
Feed intake	1-21d (Trial 1)	1078.2±19.03	1088.3±14.74	0.680
	1-21d (Trial 2)	1214.1±33.20	1197.4±17.51	0.727
Body weight gain	1-21d (Trial 1)	884.98±19.89	914.46±25.32	0.367
	1-21d (Trial 2)	836.58±23.01	804.50±24.08	0.341
Feed conversion	1-21d (Trial 1)	1.221±0.01	1.198±0.02	0.468
	1-21d (Trial 2)	1.452±0.04	1.496±0.03	0.499

*Tukey test.

In the microbiologic results, as expected, non-challenged groups were negative for *Salmonella* and data were statistically evaluated only in challenged groups in a completely randomized design. In Trial 1 it was possible to count SH in liver and cecum as presented in figure 1 and the treatment with SCOA on feed and water did not significantly decrease ($P>0.05$) percentage of SH-positive in both organs. In Trial 2, SH counting was possible only in cecum and is presented in figure 2 showing no difference ($P>0.05$) between treatments. Microbiological results of liver in Trial 2 was only qualitative and showed 100 and 42% of samples positive for SH and SH+SCOA, respectively, at 14 days of age, and 25 and 58% positive for SH and SH+SCOA, respectively, at 21 days, which was significantly different ($P<0.05$) between treatments in chi-square test. It seems that the challenge in older birds (7 days in Trial 2 versus 3 days in Trial 1), reduced the recovery of SH from liver.

FIGURE 1. TRIAL 1 – *SALMONELLA* SP. QUANTIFICATION (LOG CFU/G) IN LIVER AND CECUM AT 7 AND 21 DAYS OF AGE IN TREATMENTS CHALLENGED WITH SALMONELLA HEIDELBERG (SH) OR *SALMONELLA* HEIDELBERG CHALLENGED + SHORT CHAIN ORGANIC ACID (SH + SCOA).

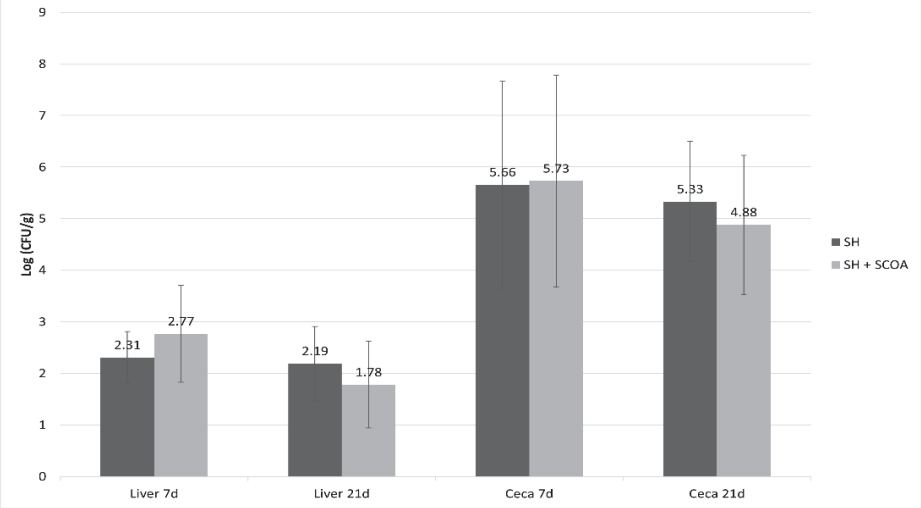
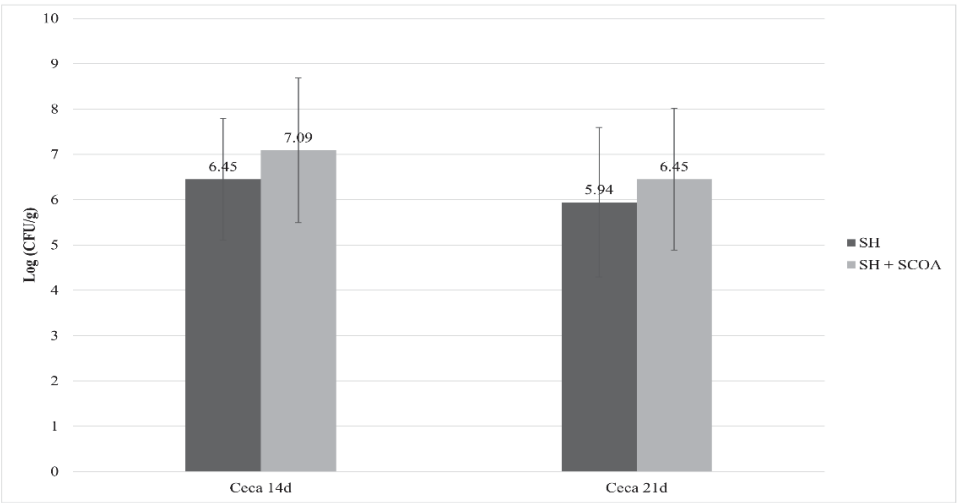
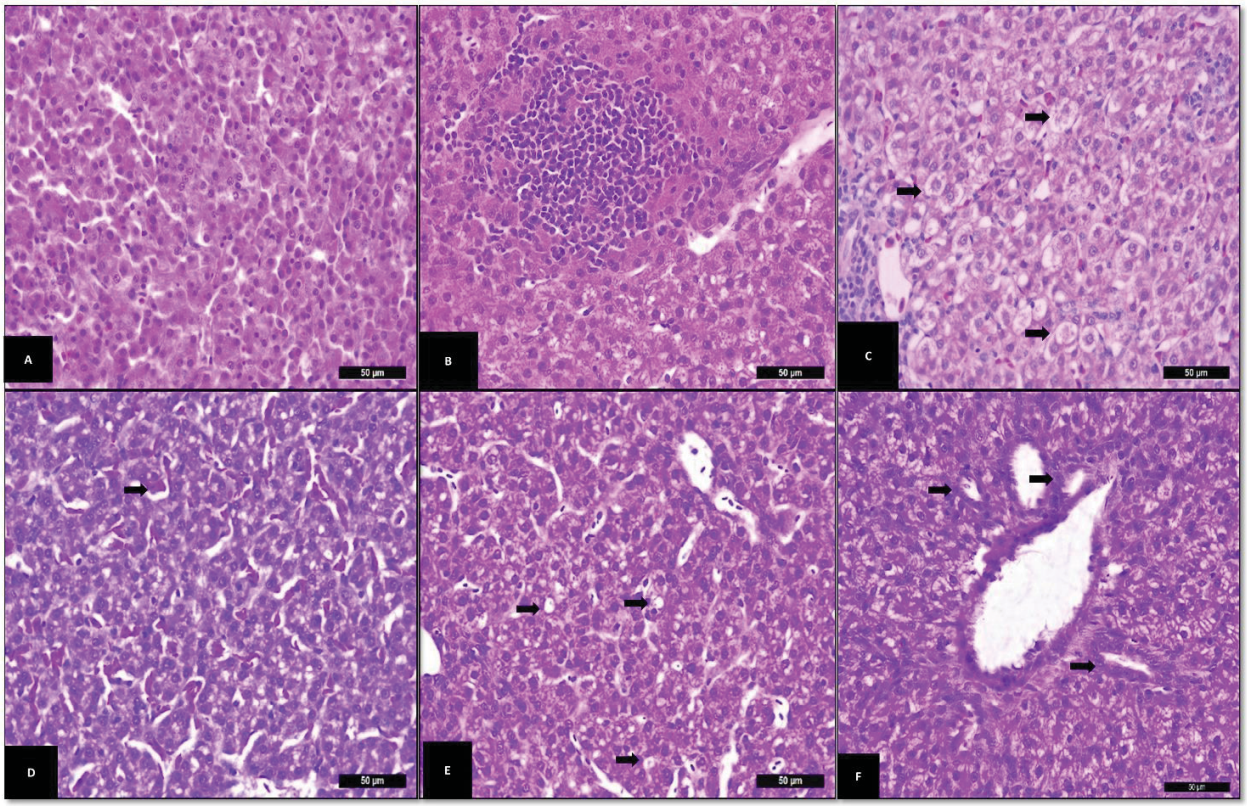


FIGURE 2 - TRIAL 2 – *SALMONELLA* SP. QUANTIFICATION (LOG CFU/G) IN CECUM AT 14 AND 21 DAYS OF AGE IN TREATMENTS CHALLENGED WITH *SALMONELLA* HEIDELBERG (SH) OR *SALMONELLA* HEIDELBERG CHALLENGED + SHORT CHAIN ORGANIC ACID (SH + SCOA).



Liver histology of SH-challenged birds showed higher ISI score at 14 days compared to non-challenged birds. The main alteration was congestion, vacuolation and immune cell infiltration as presented in figure 3. The SCOA treatment did not affect the liver histology.

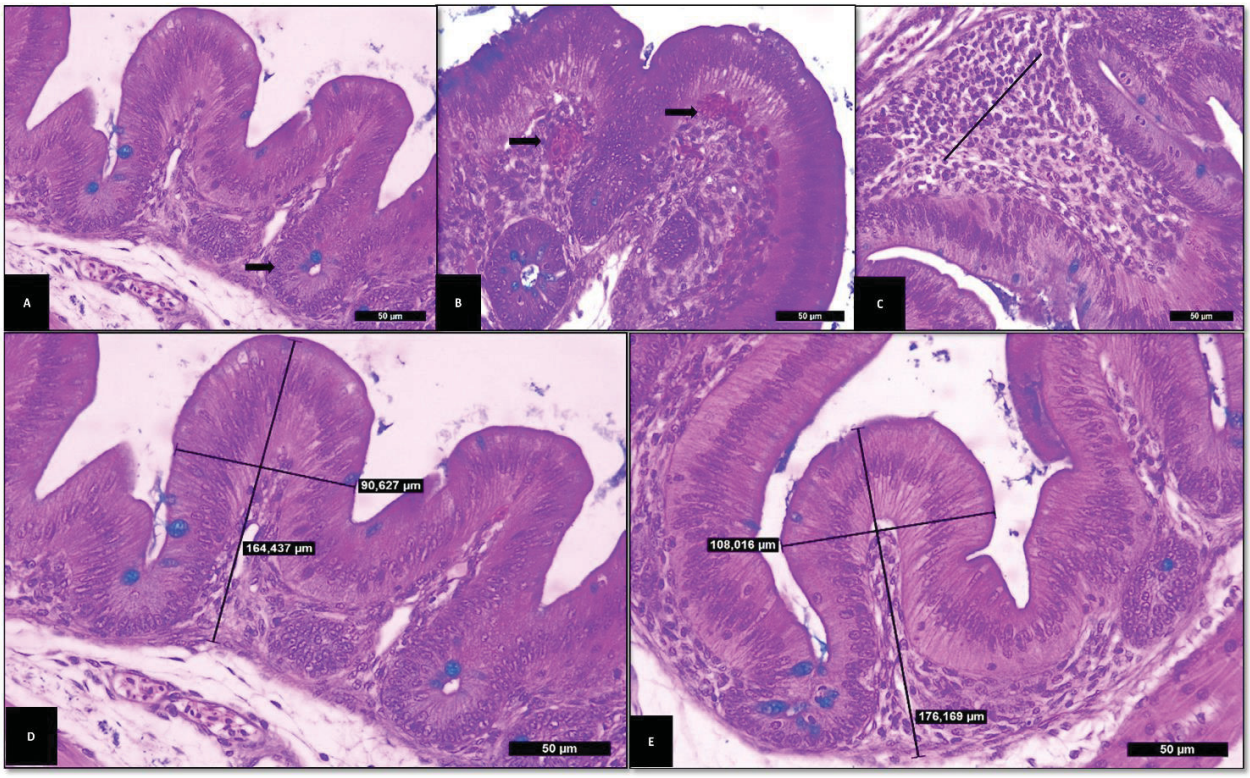
FIGURE 3 - TRIAL 2 – LIVER OF BROILERS (14 DAYS). A) Non-challenged group - normal tissue (parenchyma), ISI score 23. B) SH-Challenged group - ISI score 25, cell infiltrate in parenchyma grade II. C) SH-Challenged group - Hydropic degeneration grade III. D) SH-Challenged group - Congestion grade II. E) SH-Challenged group - Vacuolization grade II. F) SH-Challenged group - Bile duct proliferation grade II. Hematoxilin and Eosin, 400X.



At 14 days, data from cecum histology evaluation showed that the SH-challenged group increased villi height, thickness and area compared to non-challenged group

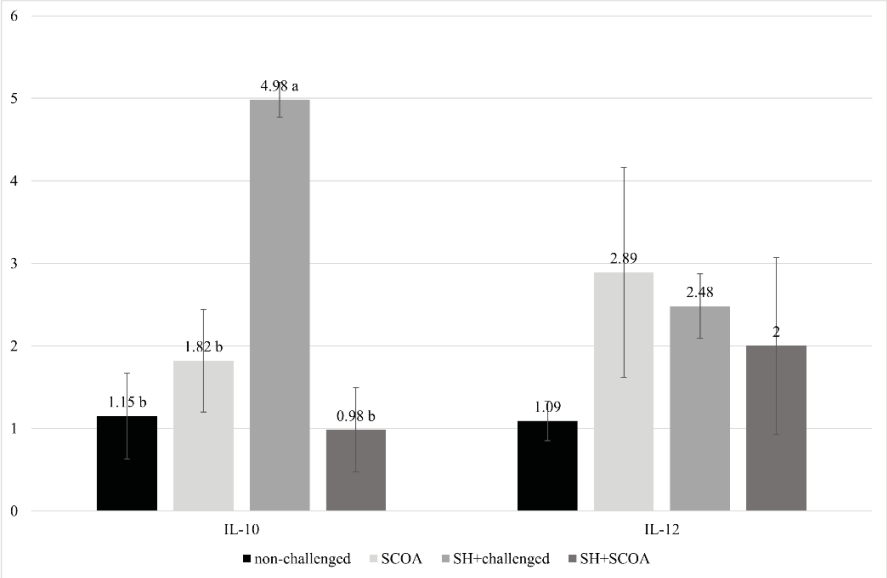
(FIGURE 4). The SCOA treatment did not affect the histology of cecum parameters and there was no interaction between challenged and SCOA.

FIGURE 4 - TRIAL 2 – CECUM OF BROILERS (14 DAYS). A) Non-challenged group - Normal villi and cripts of Lieberkhün. B) SH-Challenged group - Congestion grade III. C) SH-challenged group - Cell infiltrate in lamina propria grade II. D) Non-challenged group - Villus Height and thickness axes of measurement. E) SH-challenged group - Villus Height and thickness axes of measurement. Hematoxilin and Eosin plus Alcian Blue, 400X.



The results of mRNA expression of cytokines on liver at 14 days (FIGURE 5) showed higher IL-10 ($P<0.05$) cytokines in SH-challenged group compared to other treatments. There was no statistical difference between groups for IL-12 mRNA expression.

FIGURE 5 - TRIAL 2 – MRNA EXPRESSION OF IL-10 AND IL-12 AT 14 DAYS OF AGE IN NON-CHALLENGED GROUP, SH-CHALLENGED GROUP, SHORT CHAIN ORGANIC ACID (SCOA) AND SCOA+SH-CHALLENGED GROUP. [a,b] DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES AT P<0.05 AT KRUSKALL-WALLIS TEST.



In this study, UFPR1 strain was susceptible *in vitro* to amikacin, amoxicillin+clavulanate, ceftiofur, cephalexin, doxycycline and oxytetracycline and presented intermediary resistance to ampicillin+sulbactam, cephalothin, ciprofloxacin, enrofloxacin, gentamycin and penicillin, as presented in Table 2.

To investigate the genotypic particularities of UFPR1 strain, the whole-genome of this strain was sequenced and compared to the genomic sequence from the multidrug resistant SH SL476 strain. As shown in figure 6, the assembled genomic sequence from UFPR1 strain was 128 kb smaller than SH SL476 sequence, with important deletions of 11 chromosomal fragments in the Brazilian strain. Three of them were greater than 30, 40 and 50 kb, encompassing several important genes. Genomic regions without similar sequences in the compared genome can be observed by red dashes in Figure 6A and 6B. Nevertheless, the comparison between the genomes of these strains revealed high

similarity with few translocation events and conserved synteny (FIGURE 7). Moreover, no plasmid-sequences were found in the assembled sequences from reads of UFPR1 strain (BioProject NCBI number PRJNA378710), using Canu software v1.3 (KOREN *et al*., 2016) to correct all input data.

TABLE 2. MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM BACTERICIDAL CONCENTRATION (MBC) OF BRAZILIAN Salmonella enterica serovar Heidelberg (UFPR1 STRAIN).

Antibiotic	**MIC (µg/mL)**	**MBC (µg/mL)**	**Breakpoint**
Amikacin	1.90	61	Susceptible
Amoxicillin + clavulanate	≤0,06 + 0,15	875000+218750	Susceptible
Ampicillin + Sulbactam	24.41 + 3.05	6250	Intermediate
Ceftiofur	1.52	25000	Susceptible
Cephalexin	0.7625	-	Susceptible
Cephalothin	24.41	50000	Intermediate
Ciprofloxacin	0.24	-	Intermediate
Doxycycline	1.40	718.75	Susceptible
Enrofloxacin	0.76	3125	Intermediate
Gentamycin	1.22	9	Intermediate
Penicillin	0.61 UI/mL	6250	Intermediate
Oxytetracycline	1.64	13500	Susceptible

Inoculum SH UFPR1: 2 x 10^8 CFU/mL

Figure 6 - CHROMOSOME FEATURES OF A BRAZILIAN UFPR1 STRAIN (B) COMPARED TO SL476 STRAIN (A) ISOLATE. The circular map was drawn using DNA Plotter. Different features are shown in different colored bars. The coding sequences are shown in light blue (forward and reverse). The complete genome is shown in grey, the red dashes represents unique chromosome regions that have no homologous sequence in the genome of the other strain, green and purple in the major circle represent the GC content, while in the central circle show the GC skew [(G − C)/G + C]. Regions with GC-content below the average are shown in purple and those with content above the average are shown in green.

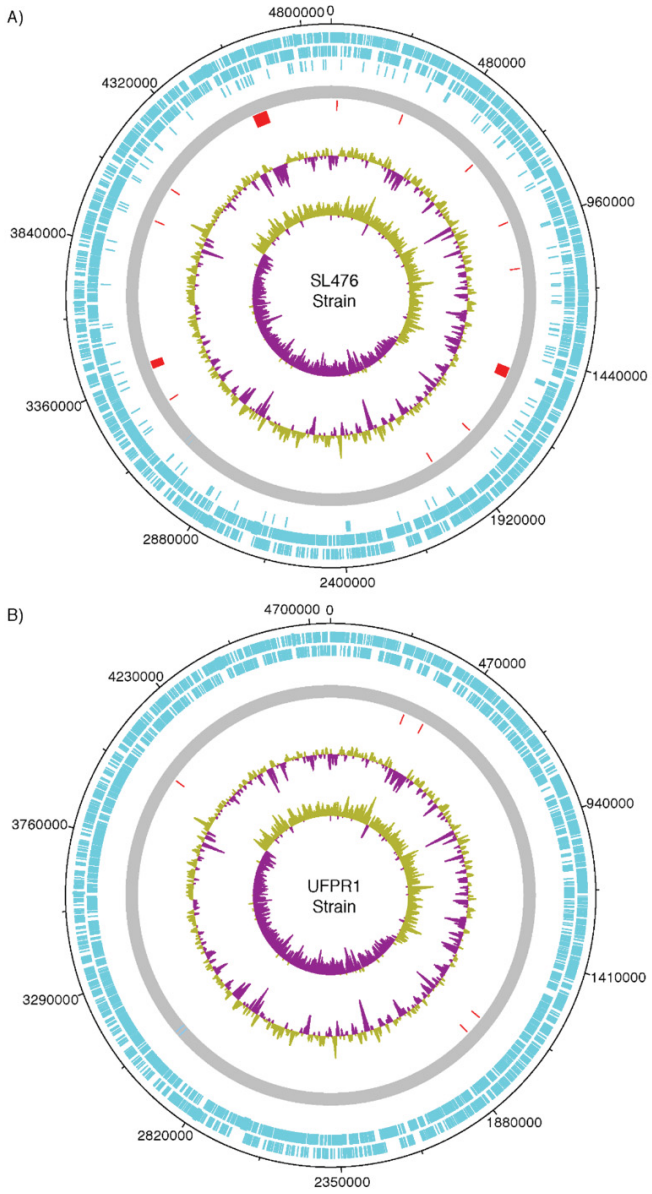
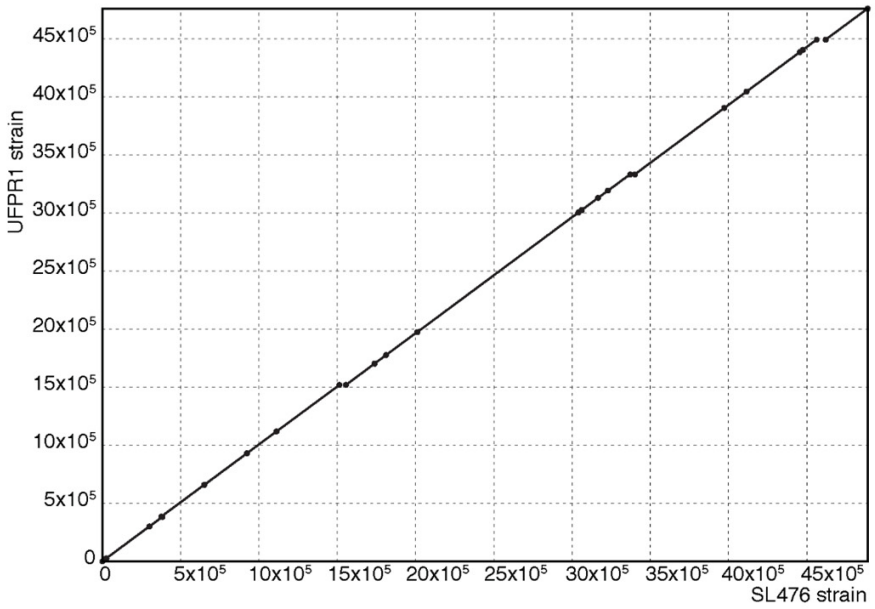


Figure 7 - ALIGNMENT BETWEEN GENOMIC SEQUENCES FROM SL476 AND UFPR1 STRAINS. This dot-plot was generated with Mummer Software. The exact matches between genomic sequences are represented on the diagonal, showing the high conservation between the genomes with few missing fragments, as shown in Figure 6.



This genomic investigation revealed that in the genomic regions deleted, 171 genes were present in SH SL476 strain and absent in UFPR1 strain (Datasheet S1 in Supplementary Material). Among them, 16% encoded proteins related to the DNA recombination process (transposases and invertases genes), 10% encoded for virus proteins (conjugal transfer, integrase, capsid and tail proteins), 46% were related to the codification for hypothetical proteins and 28% were mRNA used to produce proteins with known functions involved in cell cycle regulation, DNA replication, virulence, drug resistance, and salt efflux. However, some of these genes have more than one copy or are located in another position in UFPR1 strain genome. In the other hand, some of them are completely absent in UFPR1 strain. Among them it was found the *aph3* and *aph*6 genes that codify two isoforms of aminoglycoside O-phosphotransferase proteins, the *tem-1* gene that codify a protein associated to an antibiotic resistance mechanism in

bacteria, the *qacEΔ1* gene involved in resistance to a large spectrum of quaternary ammonium compound (QAC), the *sul1* gene involved in the resistance of sulfonamide, the *tetB* gene involved in the tetracycline efflux and the *lysR* gene that codifies the transcriptional activator of *lysA* gene, which encodes the diaminopimelate decarboxylase involved in pathway of lysine production. The *lysR* belong to the LYSR-type family transcriptional regulator, which regulates a varied set of genes involved in virulence, metabolism, quorum sensing and motility (MADDOCKS, OYSTON, 2008). In the alignment of the genomes it was observed five insertions in the UFPR1 strain coding some genes like *bgt*, *bgr* and *rpoS*. These genes are also present in the SH SL476 strain in other genomic regions and are correlated with important phenotypes found in UFPR1 strain, such as virulence and organic acids resistance. Only five chromosomal fragments were found only in UFPR1 strain compared to SL476 (Datasheet S2 in Supplementary Material). However, these fragments were identified in several other strains demonstrating that they were not exclusively of UFPR1 strain.

DISCUSSION

The results from *in vivo* studies, showed that SH UFPR1 strain did not affect zootechnical performance of broilers and no clinical signs were observed. Many other studies have described that non-typhoid *Salmonella* infection in chickens did not result in morbidity or severe clinical signs, although produce colonization of intestine and spread to liver and spleen infection (BARROW *et al*., 1987; BERNDT *et al*., 2007; MUNIZ *et al*., 2015). UFPR1 strain infection produced a mild histological alteration in the liver and cecum compared to non-challenged birds, mainly associated to inflammatory process. Interestingly, it was not observed any difference of IL-12 mRNA expression on liver of SH-challenged and non-challenged group.

In the present study, increased IL-10 mRNA expression on liver of birds from SH-challenged group compared to other groups was observed. Shanmugasundarm *et al*. (2015) presented that the amount of CD4+CD25+ (Treg) increases in the cecum of chickens infected with *Salmonella* Enteritidis. These CD4+CD25+ cells collected from cecal tonsils of *S.* Enteritidis-infected birds and re-stimulated *in vitro* with *Salmonella*

antigen had higher (P<0.05) IL-10 mRNA content compared to those in the control group. This cell was associated to suppress the immune response and maintain the infection in the host (KOGUT *et al*., 2016). The Treg cell was not marked in this study, but the absence of clinical signs or effect on zootechnical parameters with the increase of IL-10 mRNA suggested that it could happen with UFPR1 strain, however other studies should be done to corroborate it.

Whole-chromosome alignments made in the present genomic studies showed that besides phenotypic differences, the UFPR1 strain has the genome very similar to the multidrug resistance SH SL476 strain (FIGURE 6). However, several chromosomal fragments were lost in the UFPR1 strain that harboring several important genes (Datasheet S1 in Supplementary Material). Among them, the *aph3* and *aph6* genes encoding two isoforms of aminoglycoside O-phosphotransferase that participate in the primary mechanism of resistance to aminoglycosides like kanamycin, gentamicin, streptomycin and neomycin, which are frequently found together with transposable elements (WRIGHT, 1999). The *tem-1* gene, that codifies the β-lactamase protein in bacteria, is associated to β-lactam antibiotic resistance and was also deleted on UFPR1 strain. The protein produced by the translation of this mRNA is able to hydrolyze penicillin and the first-generation cephalosporin (SHAIKH *et al*., 2015). Fragments of chromosome in which genes related to the production of proteins involved in DNA replication, as DNA polymerase, DNA helicase, DNA resolvase, and DNA topoisomerase were also found deleted in UFPR1 strain. However, copies of these genes were found in another genomic DNA regions, indicating that the deletion of these regions did not affect the replication of this strain, as well it could be observed in its normal growth when cultured under laboratory conditions. Another important result found in this study was the deletion of *qacEΔ1, sul1* and *tetB* genes. The first one is involved in resistance to a large spectrum of cationic compounds such as intercalating dyes, diamidines and biguanides (JAGLIC, CERVINKOVA, 2012), while *sul1* is involved in the resistance of sulfonamide (BYRNE-BAILEY *et al*., 2009) and *TetB* that is involved in the tetracycline efflux (LI *et al*., 2004). Although other genes involved in the resistance to tetracycline were found in UFPR1 strain, such as *tetA* class B and *tetA* class C genes, the deletion of *tetB* gene could explain the intermediary resistance observed herein. Deletion of these genes could increase the

susceptibility of UFPR1 strain to the antimicrobial drugs as observed in the present phenotypic experiments. These results could be reinforced by the deletion of *LysR* gene that codify a transcriptional regulator, and it has been proposed that *Brucella abortus* strain mutant for *lysR* gene was significantly attenuated in mice macrophages, therefore this gene is required for virulence (MADDOCKS; OYSTON, 2008; SHEEHAN *et al*., 2015).

Gene deletion is used as an evolutionary process in bacteria in which small genomes have evolved from large genomes, with the natural selection acting as a significant driver of gene loss and reductive genome evolution (KOSKINIEMI *et al*., 2012). However, bacteria genome could be increased by the acquisition of genetic fragments transferred horizontally (DAVISON, 1999). Interestingly, no sequence from plasmids among the assembled sequences was observed. Transposons are threatening genome stability, which are able to create repetitive sequence islands that can initiate ectopic recombination (JR, 2004). The present results showed that the UFPR1 strain have several transposases gene deletion suggesting that DNA transposition could be decreased in this strain. On the other hand, UFPR1 strain genome had the presence of five different fragments that are absent in SL476. However, when these fragments were compared to other *Salmonella* genomes, a high similarity with several strains was observed, evidencing that they are not exclusively of UFPR1 strain. In these five fragments important genes were found, which are related to phenotypic characteristic found in the UFPR1 strain. Among them, the *Bgt* and *Bgr* genes that are related to serotype transformation and the *rpoS* gene involved with sensitivity for lower temperatures. The proteins produced by the expression of *Bgt* and *Bgr* genes are involved in the glucosylation of the O-antigen repeat units of lipopolysaccharide (LPS) and are correlated to serotype conversion in *Shigella flexneri* and also in *Salmonella* (MAVRIS, MANNING, MORONA, 1997). The presence of this gene was also correlated to the increase of virulence and resistance of oxidative stress (SZEMES *et al*., 2012).

Activation of *rpoS* gene is involved in cold sensitivity of *Salmonella enterica* serovar Typhimurium (KNUDSEN *et al*., 2014). The *rpoS* gene codifies an alternative sigma factor that regulates many cellular responses to environmental stress conditions, like heat, alkaline, and acid, and mutations in this gene have been detected in pathogenic bacteria (BHAGWAT *et al*., 2006). Bacteria are submitted to acid stress situations, such as the

extreme low pH in the stomach and the organic acids in the intestine, where they are produced in large quantities, including acetic, propionic and butyric acids. In both situations, the mechanism of acid tolerance response (ATR) must be activated to minimize the lethal effects of the acid stress, and *rpoS* is a key regulator of the ATR. *Salmonella rpoS* mutant fail to provide the same level of protection when compared to a wild-type strain, therefore *rpoS* mutant is ineffective to sustain the ATR, resulting in rapid cell death upon pH 3.0 (FOSTER, 1993; LEE *et al*., 1995). It was proposed that the product of the *rpoS* gene regulates virulence gene expression in *Salmonella* Thyphimurium in response to conditions encountered in the host tissue. Mutations in the *rpoS* gene are unable to develop a complete ATR and significantly reduce the potential of virulence in *Salmonella* Typhimurium strains (LEE *et al*., 1995). The presence of *rpoS* gene in the UFPR1 strain should be involved to the resistance of the organic acid tolerance found for this strain. Other study (BAIK *et al*., 2016) have also observed that the alternative sigma factor clearly plays an important role in protecting *Salmonella enterica* serovar Typhimurium against the lethal effects of weak acids.

In a recent study, Dhanani *et al*. (2015) demonstrated in an *in vivo* experiment that the resistant genes found in SL476 strain may explain its pathogenicity, colonization ability and persistence in chicken. The absence of several genes involved in antibiotics resistance and in salt efflux in the present genomic analysis could explain the SH UFPR1 strain high susceptibility to antibiotics and SCOA resistance (*rpoS* gene presence). The knowledge of this genotypic and phenotypic difference between SH strains could help to develop effective strategies to control this agent and prevent the food-borne illness in humans.

CONCLUSION

The infection of *Salmonella enterica* serovar Heildelberg UFPR1 in broilers did not affect zootechnical performance and promote mild inflammatory reaction on cecum and liver.

The use of different SCOA in drinking water or feed did not protect against *Salmonella enterica* serovar Heildelberg UFPR1.

The absence of several genes involved in antibiotics resistance and in salt efflux, and the presence of *rpoS* gene, could explain the SH UFPR1 strain high susceptibility to antibiotics and SCOA resistance.

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CHAPTER 2

EFFECT OF FEEDING *Bacillus subtilis* SPORES TO BROILERS CHALLENGED WITH *Salmonella enterica* serovar Heidelberg BRAZILIAN STRAIN UFPR1 ON PERFORMANCE, IMMUNE RESPONSE AND GUT HEALTH

ABSTRACT

Salmonellosis is a poultry industry and public health concern worldwide. Recently, *Salmonella enterica* serovar Heidelberg (SH) has been reported in broilers in Brazil. The effect of feeding a blend of three strains of *Bacillus subtilis* (PRO) was studied in broilers orally challenged (10^7 CFU/chick) or not with a SH isolated in south of Brazil (UFPR1 strain). Twelve male Cobb 500 broilers per pen were randomly assigned to 6 treatments in a 3x2 factorial experiment where PRO was added at 0, 250 or 500g/ton of broiler feed and fed to either SH-challenged (SH Control, SH+PRO 250 and SH+PRO 500) or non-challenged birds (Control, PRO 250 and PRO 500). Broiler performance, histologic alterations in intestinal morphology, *Salmonella* quantification and immune cells counts in liver (macrophages, T CD4+ and T CD8+) were analyzed. Changes in the intestinal microbiota of broilers were also studied by metagenomics for Control, SH Control, SH+PRO 250 and SH+PRO 500 only. Feeding PRO at 250 or 500g/ton reduced SH counts and incidence in liver and cecum at 21 days of age. It was observed that PRO groups increased the macrophage mobilization to the liver in SH-challenged birds ($P<0.05$) but reduced these cells in the liver of non-challenged birds, showing an interesting immune cell dynamics effect. PRO at 250g/ton did not affect gut histology, but improved animal performance ($P<0.05$) while PRO at 500/ton did not affect animal performance but increased histologic alteration related to activation of the defense response in the ileum in SH challenged birds compared to control birds ($P<0.05$). SH+PRO 500 group presented a more diverse cecal microbiota (Shannon-Wiener index; $P<0.05$) compared to Control and SH Control groups; while SH+PRO 250 had greater ileal richness (JackkNife index) compared to Control ($P<0.05$). PRO was effective in reducing *Salmonella* colonization in liver and cecum when fed at 250 or 500g/ton to broilers inoculated with SH strain UFPR1. PRO promotes positive alterations in

performance (at 250g/ton), immune modulatory effect in the gastrointestinal tract, SH reduction and intestinal microbiota modulation.

Key words: 16S, gut health, gut microbiome, immunity, poultry, probiotic, salmonellosis.

INTRODUCTION

Despite advances in the treatment of infectious diseases, pathogenic microorganisms such as *Salmonella* are an important threat to both human and animal health worldwide (KIM *et al*., 2007). *Salmonella* is a pathogen but it also has the ability to live in animals and poultry as a transient member of the intestinal microbial population without causing disease. Colonization of most types of *Salmonella enterica* does not often affect poultry performance and consequently asymptomatic infections may increase the likelihood of zoonotic transmission to humans through the food chain (CARTER *et al*., 2009). *Salmonella* enterica serovar Heidelberg (SH) ranks among the top 3 serovars isolated from patients with salmonellosis in North America, higher than in other regions of the world (CDC, 2014), provoking more invasive infections (e.g. myocarditis and bacteremia) than others non-typhoidal *Salmonella* (HOFFMANN *et al*., 2014). The Brazilian SH strain used in this trial (UFPR1) had its complete genome described recently, showing high resistance to short-chain organic acids and intermediate resistance to some antibiotics (SANTIN *et al*., 2017). Oral administration of probiotics may reduce the intestinal colonization of *Salmonella* (HIGGINS *et al*., 2007; PASCUAL *et al*., 1999), along with the inflammation caused by this bacterium, in broiler chickens (CHEN *et al.,* 2012). Probiotics are live microorganisms that offer an advantage to their hosts by enhancing the hosts' beneficial microbiota (AFRC, 1989; LJUNGH E WADSTROM, 2006). Studies have demonstrated that *Bacillus* spp. and *Bacillus subtilis* spores may be successful competitive exclusion agents (LA RAGIONE, WOODWARD, 2003). *Bacillus subtilis* modulates the intestinal microbiota and favors the growth of lactic acid bacteria with recognized health-conferring properties (KNARREBORG *et al*., 2008). A spore monoculture has the advantage of being readily produced, having a long shelf life, and, in the case of *Bacillus subtilis*, being avirulent (LA RAGIONE, WOODWARD, 2003). *Bacillus*

subtilis has been studied and used as a feed additive to improve broiler performance (HARRINGTON *et al*., 2015; REN *et al*., 2013), modulate immune response (LEE *et al*., 2015; SADEGHI *et al*., 2015) and act as a prophylactic agent against bacterial diseases, by balancing gut microbiota (JAYARAMAN *et al*., 2013; KNARREBORG *et al*., 2008).

Some probiotics may be able to decrease the invasiveness of pathogens, which use inflammation to enhance their own colonization, by decreasing innate inflammatory responses, including macrophage activation phenotypes. Probiotics are also well documented to increase modulation of adaptive immunity (HARDY *et al*., 2013). These findings suggest a specific immune interaction of each probiotic strain used, and its abilities to improve protection against certain pathogens, maintaining health and homeostasis through intestinal and systemic immunomodulation, in order to enhance animal performance and health.

The objective of this trial was to evaluate the ability of a probiotic composed of three different *Bacillus subtilis* strains to reduce the invasiveness and gut colonization of the Brazilian SH UFPR1 strain, and its effects on performance, intestinal mucosa morphology, immune cells dynamics (macrophages, CD4+ and CD8+ cells) in liver, and gut microbiota in broiler chickens.

MATERIAL AND METHODS

ANIMALS AND EXPERIMENTAL DESIGN

The experiment was conducted at CERIA (Center of Immune Response in Poultry) at Federal University of Parana, Curitiba, Brazil, and was approved by the Ethical Committee of Agricultural Sector of Federal University of Parana under approval number: 037/2016.

Six, previously disinfected, BSL-2 rearing rooms were used. Each room contained four battery cages (replications) stacked vertically with sterilized litter, nipple drinkers, automatic temperature and lighting controls, all under a negative pressured air system.
A total of 288 one-day-old male Cobb 500® broilers were distributed in a completely randomized block design (each block is a room) with six treatments of four replicates and

12 birds each where PRO was fed at 0, 250 or 500g/ton of feed in either SH-inoculated or non-inoculated birds, as shown in table 1. At the initiation of the trial, birds were allocated at in such a way that equal average initial body weight per cage was obtained. The trial was carried out from one to 21 days of age.

TABLE 1 – TREATMENTS DESCRIPTION.

Treatments	***Salmonella* Heidelberg Challenge**	**Probiotic[1] added (g/ton of feed)**
Control	No	0
PRO 250	No	250
PRO 500	No	500
SH Control	Yes	0
SH+PRO 250	Yes	250
SH+PRO 500	Yes	500

[1]Live spores of *Bacillus subtilis* (PRO) strains (NP122, B2 and AM0904; Sporulin®, Novus International Inc.).

Aiming at minimizing the possibility of unexpected *Salmonella* contamination, the chickens used in this trial corresponded to the male line of a grandparent stock farm not vaccinated against any type of *Salmonella*.

PRODUCT AND DOSAGE

The probiotic (PRO) used in this trial is a feed additive manufactured with three isolated live spores of *Bacillus subtilis* strains (NP122, B2 and AM0904; Sporulin®, Novus International Inc.). PRO was provided at three different levels: 0 g/ton (Control and SH Control groups); 250 g/ton (PRO 250 and SH+PRO 250); or 500 g/ton (PRO 500 and SH+PRO 500; TABLE 1). The recommended dosage by the manufacturer is 250g/ton, which provides 10^6 spores per g of feed, while 500g/ton will be evaluated against pathogens such as SH strain UFPR1.

FEED FORMULATION AND MIX

A balanced basal diet was offered in mash form and was formulated to provide nutrients at or above requirement levels (ROSTAGNO *et al*., 2005). Corn and soybean meal were used as main ingredients and no antibiotics or growth promoters were added. The diet was designed for a unique feeding phase (Starter) and it was offered to broilers *ad libitum* from one to 21 days of age for all treatments.

The basal diet was sterilized by autoclave at 120ºC for 15 minutes. After this process, PRO, amino acids, vitamin and mineral premix were added according to each treatment and mixed for 10 minutes using a 50 Kg “V” mixer. Batches were mixed in such an order to avoid interference among treatments. PRO diets containing live spores were mixed at last. The mixer was cleaned after each batch.

Salmonella enterica serovar Heidelberg

Salmonella enterica serovar Heidelberg (SH), strain UFPR1 sequences were submitted to the database NCBI/biosample identified as SAMN06560104, GenBank: CP020101. This pathogen was isolated from commercial broiler carcasses obtained from a broiler farm located in the south of Brazil. Samples from 20 livers and ceca were collected randomly from one-day-old chicks and tested negative for *Salmonella*. At 3 days of age, chicks from SH Control, SH+PRO 250 and SH+PRO 500 were orally challenged with 10^7 CFU of SH per chick. At 7 and 21 days of age, 12 birds from SH Control, SH+PRO 250 and SH+PRO 500 were subjected to necropsy, while *Salmonella* sp. counts were quantified in liver and cecum samples. A pool of four ceca and four livers per treatment (Control, PRO 250 and PRO 500) were also collected to evaluate the presence or absence of *Salmonella* sp. (qualitative analysis). In order to quantify typical colonies of *Salmonella* sp. (quantitative analysis), samples were processed using the modified methodology of (COX *et al*., 2010). The abundance of *Salmonella* in ileum and cecum was also measured using metagenomic analysis.

PERFORMANCE

Birds and feed were weighed weekly to evaluate feed intake (FI), body weight (BW), body weight gain (BWG) and feed conversion ratio (FC). All birds used for tissue sampling were weighed individually to estimate FC corrected for mortality. Mortality due to other causes rather than sampling procedures was not observed in this trial.

MACROPHAGES, CD4+ AND CD8+ CELLS QUANTIFICATION BY IMMUNOHISTOCHEMISTRY

At 7 and 21 days of age, 12 birds per treatment (3 birds per replicate) were euthanized and the accessory lobe of their livers were collected. Immunohistochemistry was performed to obtain macrophage, CD4+ and CD8+ lymphocyte counts according to (LOURENÇO *et al*., 2015)using the rabbit macrophage clone RAM-11 Dako. The labeled cells were counted in an optical microscope (Nikon Eclipse E200, Sao Paulo-SP- Brazil) with a 100X magnification objective. Five fields per bird, totalizing 25 microscopic fields per treatment of liver, were measured using only the hepatic parenchyma aiming at avoiding lymphoid aggregates.

EVALUATION OF INTESTINAL HEALTH – HISTOLOGY BY ISI (I SEE INSIDE METHODOLOGY)

At 7 and 21 days of age, 12 birds per treatment (3 birds per replicate) were euthanized, liver and ileum samples collected and further subjected to microscopic evaluation using the ISI Methodology (“I See Inside”; Pat. INPI-BR1020150036019) (43) as published by (KRAIESKI *et al*., 2016)Shortly, this methodology was developed based on a numeric score of histological alterations. For each alteration observed during microscopic analysis, an impact factor (IF) is defined according to its importance in affecting organ functional capacity based on previous knowledge of literature and background research (e.g. necrosis has the highest IF because the functional capacity of affected cells is totally lost). The IF ranges from 1 to 3, where 3 represents an IF of the greatest significance in terms of the organ function. In addition, the extent of each

alteration (intensity or observed frequency compared to non-affected tissue) is evaluated per field (liver) or per villi (intestine) and scored ranging from 0 to 3. To reach the final ISI value, the IF of each alteration is multiplied by the respective score number, and the results of all alterations are summed.

GENOMIC DNA PURIFICATION OF LUMINAL GUT MICROBIOTA AND DNA SEQUENCING

The ileal (distal) and cecal luminal contents from 12 birds (3 birds per replicate) of treatments Control, SH Control, SH+PRO 250 and SH+PRO 500 were collected, frozen in liquid nitrogen and stored at -80ºC until further analysis. Genomic DNA from each sample was purified using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer, and then DNA quantification and quality were evaluated using the NanoVue Plus spectrophotometer (GE Healthcare, Marlborough, USA). Samples were diluted at 50 ng/μL and pooled using the same volume for each one (three samples were used to form one pool, resulting in 4 replicates per treatment). The pooled samples from ileum and cecum were used to amplify approximately 460 bp of the 16S ribosomal RNA by PCR using specific primers V3 and V4. PCR products were used to build the metagenomics library for sequencing using MiSeq Reagent kit v3 (600 cycle) (Illumina Inc.). The sequencing of partial 16S ribosomal RNA was performed by next-generation sequencing method using Illumina MiSeq platform that produced thousands of 300bp paired-end reads (2x300bp) for each library. The full-length primer sequences to follow the protocol targeting this region are: 16S Amplicon PCR Forward Primer = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and 16S Amplicon PCR Reverse Primer = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC.

PROCESSING OF THE READS AND PHYLOGENETIC ANALYSIS

The sequencing data were analyzed in the Bioinformatics Lab of the UNICAMP (www.lge.ibi.unicamp.br). The paired-end reads from each treatment were submitted to quality filtering and adapter trimming using Trim Galore software

(http://www.bioinformatics.babraham.ac.uk/projects/trim_galore). The trimmed paired-end reads were merged into single reads using PEAR software (ZHANG *et al*., 2014). The single reads were then submitted to phylogenetic analysis and taxonomic assignments of the V3-V4 portion of the 16S rRNA gene using QIIME package (CAPORASO *et al*., 2010) configured for constructing Operational Taxonomic Units (OTUs) with 97% of identity and assign taxonomy based on the Greengenes reference database (currently version 13_8). The full data sequence has been registered at NCBI BioProject and the information should be available at the following link: http://www.ncbi.nlm.nih.gov/bioproject/413291. The rarefaction curves were conducted to evaluate the coverage of OTUs (Figure 3).

DIVERSITY ANALYSIS AND COMPARISON AMONG TREATMENTS

Only taxonomic groups with abundance higher than 1% at the deepest level identified were submitted to cluster analysis. The clustering of different treatments was done using the Multiple Experiment Viewer (MeV) software (SAEED *et al*., 2003). Ecological indexes, such as diversity $H' = -\sum_{i=1}^{S} p_i \, ln \, p_i$; where p_i is the proportion of characters belonging to the i*th* type of letter in the string of interest (BERGER; PARKER, 1970), richness and equitability $J = \frac{H'}{H_{max'}}$; where $H_{max'} = \log_b S$, were calculated using the program R. For all ecologic indexes, all OTUs obtained were used except those that appeared only once.

STATISTICAL ANALYSIS

Data were analyzed using the statistical software Statistix 9®. The microbiological data were evaluated by the Shapiro-Wilk normality test. The parametric data were subjected to analysis of variance (ANOVA) and Tukey's test to establish differences among treatment means. The nonparametric data were submitted to the Kruskal-Wallis test at a 5% probability value. When presence or absence of *Salmonella* was assayed, the chi-square test was used to establish statistical differences. For performance, immunohistochemistry and histology analysis, data were submitted to analysis of variance (ANOVA) using a 2 x 3 factorial design, once no difference for block were observed.

Changes in the populations of individual bacteria were analyzed by ANOVA and Tukey's test accordingly. For heat maps, only bacteria with abundance higher than 1% were used. A complete list of microorganisms identified are showed in table S1 for ileum and table S2 for cecum.

RESULTS

There was no interaction between SH and PRO for live performance and SH did not affect these parameters at any age period. The addition of PRO at 250 g/ton increased ($P<0.05$) FI and BWG from 1-21 days compared to Control (TABLE 2).

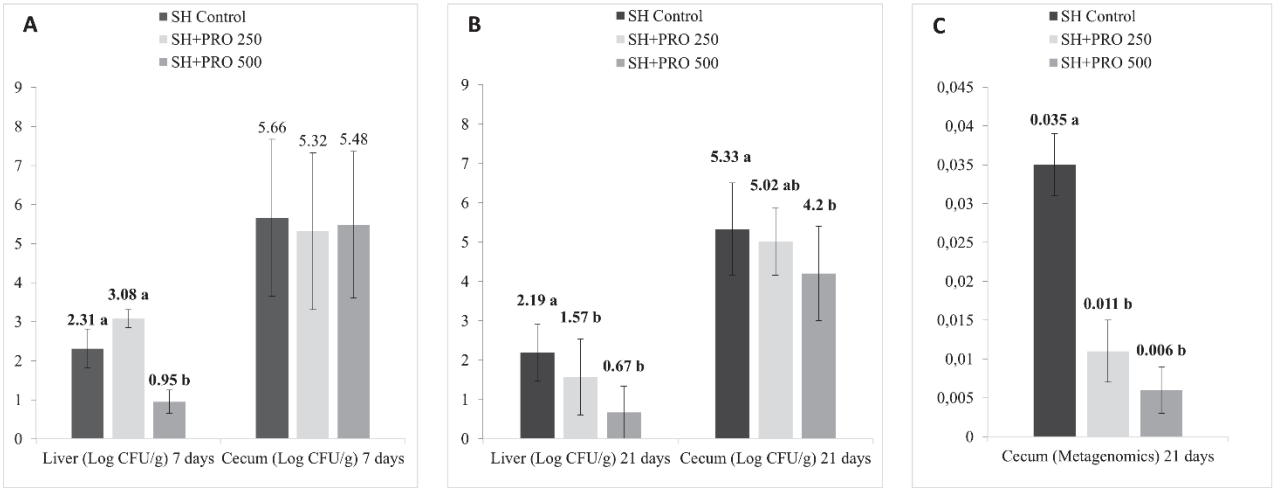
As expected, the non-challenged groups (Control, PRO 250 and PRO 500) tested negative for *Salmonella* therefore data were analyzed using the SH challenged treatments only as a completely randomized design. In liver, SH+PRO 500 had reduced SH counts ($P<0.01$) compared to SH Control at 7 days (FIGURE 1A), whereas both SH+PRO 250 and 500 birds had reduced SH counts at 21 days ($P<0.01$) compared to the SH Control group (FIGURE 1B). In ceca, only the SH+PRO 500 group had reduced ($P<0.05$) *Salmonella* counts (FIGURE 1B) using the bacteriological quantification (COX *et al*., 2010). However, PRO when fed at either dose significantly reduced *Salmonella* frequencies in cecum according to the more refined metagenomic analysis (FIGURE 1C) at 21 days of age.

TABLE 2 – MEAN ± STANDARD DEVIATION OF FEED INTAKE (FI) (G), BODY WEIGHT GAIN (BWG) (G) AND FEED CONVERSION (FC) DURING THE PERIODS 1 TO 7, 1 TO 14 AND 1 TO 21 DAYS OF AGE.

	FI 1-7 (g)	**FI 1-14 (g)**	**FI 1-21 (g)**	**BWG 1-7 (g)**	**BWG 1-14 (g)**	**BWG 1-21 (g)**	**FC 1-7**	**FC 1-14**	**FC 1-21**
Challenge					**Main effects**				
Control	122.99±8.54	468.06±35.08	1079.7±83.76	110.53±2.49	390.83±12.09	886.97±27.03	1.112±0.01	1.202±0.02	1.220±0.01
SH	116.99±13.03	463.20±35.56	1093.0±62.82	107.20±2.98	408.63±14.76	916.90±32.57	1.091±0.01	1.142±0.02	1.202±0.03
Probiotic									
Control	114.79±14.63	447,58±49.59	**1035.2±83.95 b**	103.25±3.86	374.65±19.95	**839.11±38.50 b**	1.110±0.02	1.204±0.03	1.243±0.03
250	127.45±5.88	482.56±18.51	**1131.3±48.07 a**	114.24±1.85	427.26±13.61	**965.98±31.46 a**	1.116±0.01	1.137±0.03	1.176±0.02
500	116.98±7.53	466.56±17.51	**1094.5±43.77ab**	108.89±3.13	398.22±8.29	**902.68±23.25 ab**	1.075±0.02	1.173±0.01	1.214±0.01
					Probabilities				
Challenge (P_1)	0.204	0.763	0.659	0.374	0.350	0.483	0.496	0.098	0.655
Probiotic (P_2)	0.058	0.163	**0.031**	0.071	0.062	**0.040**	0.411	0.229	0.278
Interaction (P_1*P_2)	0.639	0.755	0.865	0.743	0.259	0.410	0.787	0.061	0.336

[a,b] Different letters in the same column indicate significant differences at P<0.05 at Tukey test.

FIGURE 1 – *SALMONELLA* SP. QUANTIFICATION. A: *Salmonella* sp. counts (Log CFU/g) in liver and cecum at 7 days of age (4 days after inoculation) in treatments SH Control, SH+PRO 250 and SH+PRO 500 according to adapted methodology by Cox *et al*., (2010). B: Salmonella sp. counts (Log CFU/g) in liver and cecum at 21 days of age in treatments SH Control, SH+PRO 250 and SH+PRO 500according to adapted methodology by Cox *et al*., (2010). C: relative abundance using metagenomics analysis in ceca at 21 days of age in treatments SH Control, SH+PRO 250 and SH+PRO 500. Non-challenged groups (Control, PRO 250 and PRO 500) were negative for *Salmonella* in both methodologies. [a,b] Different letters indicate significant differences at P<0.05 at Kruskal-Wallis.



Liver histologic alterations by ISI and immunohistochemistry analysis are summarized in tables 3 and 4, respectively. No differences in ISI scores in liver were found among treatments in non-challenged birds at 7 days still, immunohistochemistry analysis revealed that PRO fed at 500 g/ton reduced macrophages and CD4+ cells recruitment in the liver of those chickens compared to Controls (P<0.05).

The challenged birds fed PRO had livers with lower histological alteration scores compared to the SH Control group (P<0.01) at 7 days of age. A reduction on hydropic degeneration and necrosis of liver parenchyma were associated to those observations. In addition, higher macrophage counts in liver were found in both SH+PRO 250 and 500 groups compared to the SH Control (TABLE 4). This could be related to SH reduction in this organ (at least for PRO fed at 500 g/ton). The opposite was observed in non-challenged birds when the PRO 500 exhibited reduced (P<0.01) macrophages and CD4+ cells in liver parenchyma.

TABLE 3 – MEAN ± STANDARD DEVIATION OF HISTOLOGICAL ALTERATIONS (ISI) IN LIVER (SCORE PER FIELD) AND ILEUM (SCORE PER VILLI) AT 7 AND 21 DAYS OF AGE.

	Liver		**Ileum**	
	7 days	**21 days**	**7 days**	**21 days**
Challenge				
Control	**23.49±6.53 a**	**12.26±5.78 b**	5.29±4.39	9.99±4.55
SH	**20.25±7.13 b**	**20.63±6.61 a**	4.56±4.54	10.42±3.54
Probiotic				
Control	**24.09±5.49 a**	**16.84±6.74 b**	**4.36±4.32 b**	**9.11±4.16 b**
250	**20.82±7.63 b**	**17.48±7.41 b**	**4.42±4.24 b**	**11.52±3.95 a**
500	**19.08±7.09 b**	**19.19±7.06 a**	**5.71±4.84 a**	**10.21±3.20 b**
Interaction				
Control	**23.70±5.82 a**	**9.93±5.25 c**	5.01±0.57	**10.50±5.22 ab**
PRO 250	**23.10±7.27 a**	**11.40±5.28 c**	4.11±0.57	**10.47±4.94 ab**
PRO 500	**23.67±6.52 a**	**15.45±5.42 b**	6.75±0.57	**9.00±3.13 bc**
SH Control	**24.28±5.34 a**	**20.31±6.39 a**	4.03±0.40	**8.42±3.33 c**
SH+PRO 250	**19.67±7.58 b**	**20.52±6.38 a**	4.57±0.40	**12.04±3.24 a**
SH+PRO 500	**16.79±6.23 c**	**21.06±7.06 a**	5.13±0.42	**10.79±3.08 a**
	Probabilities			
Challenge (P_1)	**<0.001**	**<0.001**	0.081	0.204
Probiotic (P_2)	**<0.001**	**<0.001**	**0.002**	**<0.001**
Interaction (P_1*P_2)	**<0.001**	**<0.001**	0.106	**<0.001**

[a,b,c] Different letters in the same column indicate significant differences at $P<0.05$ at Kruskal-Wallis test.

At 21 days of age, PRO 500 birds had increased ISI liver scores compared to the Control and PRO 250 groups in non-challenged birds (TABLE 3). No differences were found in SH-challenged broilers on this parameter. Still, increased CD4+ cells counts were observed in both SH+PRO 250 and SH+PRO 500 groups compared to SH Control (TABLE 4). The macrophage counts were higher in liver at 21 days of age regardless of SH challenge.

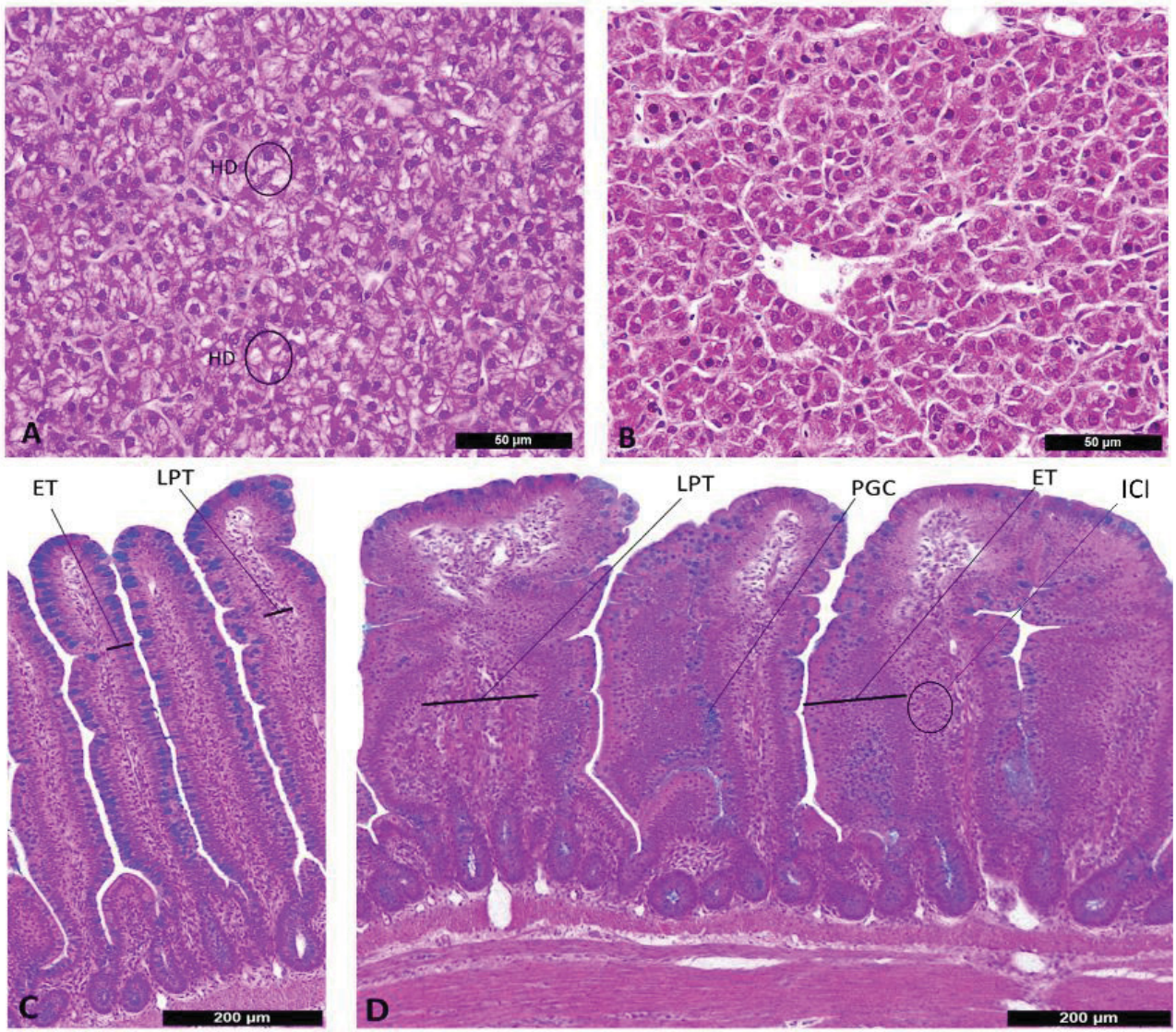
TABLE 4 – MEAN ± STANDARD ERROR OF MACROPHAGES, CD4+ AND CD8+ CELLS QUANTIFICATION BY IMMUNOHISTOCHEMISTRY IN LIVER (CELLS PER FIELD AT 100X OF MAGNIFICATION) AT 7 AND 21 DAYS OF AGE.

	Macrophages		**CD4+**		**CD8+**	
Challenge	**7 days**	**21 days**	**7 days**	**21 days**	**7 days**	**21 days**
Control	19.25±1.09	10.88±0.66	3.48±0.26	4.35±0.28	**4.63±0.31 b**	4.50±0.29
SH	23.96±0.33	11.78±0.61	3.53±0.10	4.12±0.25	**5.30±0.13 a**	4.78±0.25
Probiotic						
Control	**20.97±0.95 b**	**8.32±0.65 b**	**4.00±0.22 a**	3.50±0.28	**5.53±0.33 a**	3.92±0.28
250	**25.35±0.47 a**	**13.21±0.79 a**	**3.63±0.16 a**	4.85±0.35	**5.05±0.15 ab**	5.76±0.34
500	**20.87±1.01 c**	**12.91±0.80 a**	**2.92±0.17 b**	4.25±0.35	**4.67±0.20 b**	4.38±0.33
Interaction			**Interactions**			
Control	**20.15±1.35 b**	7.70±1.30	**4.40±0.57 a**	**5.15±0.56 a**	**4.65±0.79 ab**	4.1±0.56
PRO 250	**26.80±1.11 a**	11.4±1.3	**4.15±0.24 a**	**4.25±0.43 ab**	**5.30±0.30 ab**	5.50±0.56
PRO 500	**10.80±1.13 c**	13.55±1.3	**1.90±0.22 b**	**3.65±0.41 ab**	**3.95±0.40 b**	3.90±0.56
SH Control	**21.37±0.72 b**	8.62±0.92	**3.80±0.16 a**	**2.67±0.22 b**	**5.97±0.28 a**	3.82±0.39
SH+PRO 250	**24.62±0.41 a**	14.12±0.92	**3.37±0.20 a**	**5.15±0.47 a**	**4.92±0.17 ab**	5.90±0.39
SH+PRO 500	**25.90±0.26 a**	12.6±0.92	**3.42±0.18 a**	**4.55±0.49 a**	**5.02±0.21 ab**	4.65±0.39
			Probabilities			
Challenge (P_1)	**<0.001**	0.332	0.817	0.567	**0.020**	0.475
Probiotic (P_2)	**<0.001**	**<0.001**	**<0.001**	0.233	**0.050**	**0.001**
Interaction (P_1*P_2)	**<0.001**	0.271	**<0.001**	**0.001**	**0.035**	0.576

[a,b,c] Different letters in the same column indicate significant differences at P<0.05 at Kruskal-Wallis test.

Birds fed PRO at 500 g/ton had higher ISI scores in ileum at 7 days of age (TABLE 3). The main alterations observed in challenged birds were an increase in lamina propria thickness, epithelial thickness and proliferation of goblet cells (P<0.05). At 21 days of age, a significant interaction for ileal ISI scores was found, where both SH+PRO 250 and SH+PRO 500 groups presented higher ISI scores than the SH Control, while no significant differences were observed in non-challenged birds (TABLE 3). The main histologic alterations found in the PRO 500 g/ton group at that age were also observed at 7 days (FIGURE 2C, 2D).

FIGURE 2 – HISTOLOGICAL ALTERATIONS IN LIVER (A, B) AND ILEUM (C, D) ACCORDING TO ISI (I SEE INSIDE) SCORING METHODOLOGY (100X). A) Liver from SH Control, presenting score 3 of hydropic degeneration (HD) at 7 days of age. B) Liver from SH+PRO 500, normal hepatocytes at 7 days of age. C) Ileum from SH Control, villi with scores zero for epithelial thickness (ET) and lamina propria thickness (LPT) at 21 days of age. D) Ileum from SH+PRO 250 with score 2 for epithelial thickness (ET), score 2 for proliferation of goblet cells (PGC) and score 2 for lamina propria thickness (LPT) with inflammatory cells infiltration (ICI) at 21 days of age.



The metagenomic analysis of gut microbiota revealed an average of 411.360 and 157.658 reads per sample of cecum and ileum, respectively. Based on 97% species similarity, an average of 9.330 and 1.942 operational taxonomic units (OTUs) were obtained in cecum and ileum, respectively. The rarefaction curves suggested that in all treatments enough sequence reads per sample were collected, showing that sampling has been exhaustively sequenced and was enough to uncover major OTUs (FIGURE 3). The diversity index by Shannon-Wiener revealed that cecal microbial composition of the SH+PRO 500 group was significantly more diverse compared to Control and SH Control groups. The SH+PRO 250 birds had significant ($P<0.05$) higher richness (JackkNife test) in ileal microbiota compared to the Control group, while evenness test (Hill) revealed that SH+PRO 500 birds have lower species evenness in the cecum compared to the SH Control group (FIGURE 4).

FIGURE 3 – RAREFACTION PLOT FROM ILEAL (A) AND CECAL (B) MICROBIOTA OF GROUPS CONTROL, SH CONTROL, SH+PRO 250 AND SH+PRO 500. *$P<0.05$. **$P=0.08$. Rarefaction analysis suggested that the number of sequences from all experimental samples were enough to uncover major OTUs.

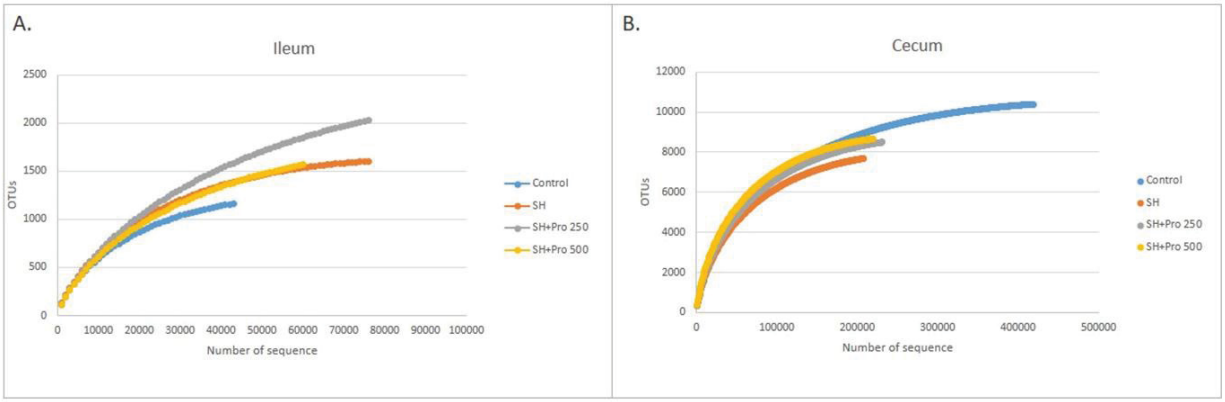
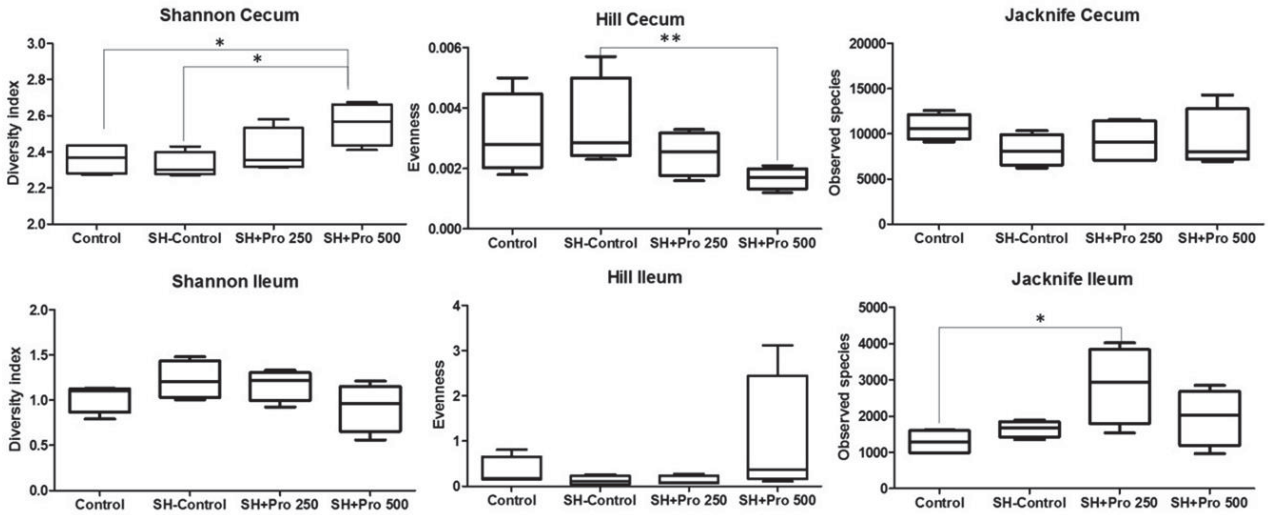


FIGURE 4 – ILEAL AND CECAL DIVERSITY (SHANNON-WIENER), EVENNESS (HILL) AND RICHNESS (JACKKNIFE) INDEX OF GROUPS CONTROL, SH CONTROL, SH+PRO 250 AND SH+PRO 500.



The family profiles of the corresponding ileal microbial populations are shown in figure 5A. As expected, data on microbiota presented high coefficients of variation addressing the difficulties in establishing statistical differences. The Clostridiaceae family (mostly represented by *Clostridium perfringens*) presented numerically lower abundance in SH+PRO 500. *Clostridium perfringens* were detected in high quantity in ileum because the samples were collected in the distal section. The unidentified members of Clostridiales order (group 1) revealed higher numerical abundance in the SH+PRO 500 broilers as opposed to other groups. The unidentified members of *Enterococcus* genus (phylum Firmicutes) and members of Peptostreptococcaceae family (group 1; class Clostridia) were significantly higher ($P<0.05$) in SH+PRO 250 compared to the Control group (FIGURE 6A). Another significant difference in ileum ($P<0.05$) is related to unidentified members of Streptophyta order, within the Cyanobacteria phylum. This bacterium was more abundant in SH+PRO 250 group compared to the Control and the SH+PRO 500 treatments (FIGURE 6A).

In cecal microbiota, the majority of Clostridiales detected fall primarily into Ruminococcaceae, Lachnospiraceae and Clostridiaceae families (FIGURE 5B).

An unidentified member of RF39 order (phylum Tenericutes, class Mollicutes) presented a statistical difference (P=0.041) between Control and SH Control (FIGURE 6B). The abundance of *Salmonella sp* in ceca was lower than 1% (i.e., up to 0.035%) been significantly lower in broilers fed PRO at both dosages comparing to Control and SH Control birds (FIGURE 1C; P<0.05).

FIGURE 5 - RELATIVE ABUNDANCE OF BACTERIA POPULATION IN ILEAL MICROBIOTA (A) AND CECAL MICROBIOTA (B) OF GROUPS CONTROL, SH CONTROL, SH+PRO 250 AND SH+PRO 500 AT 21 DAYS OF AGE, ANALYZED BY SEQUENCING USING ILLUMINA MISEQ SYSTEM.

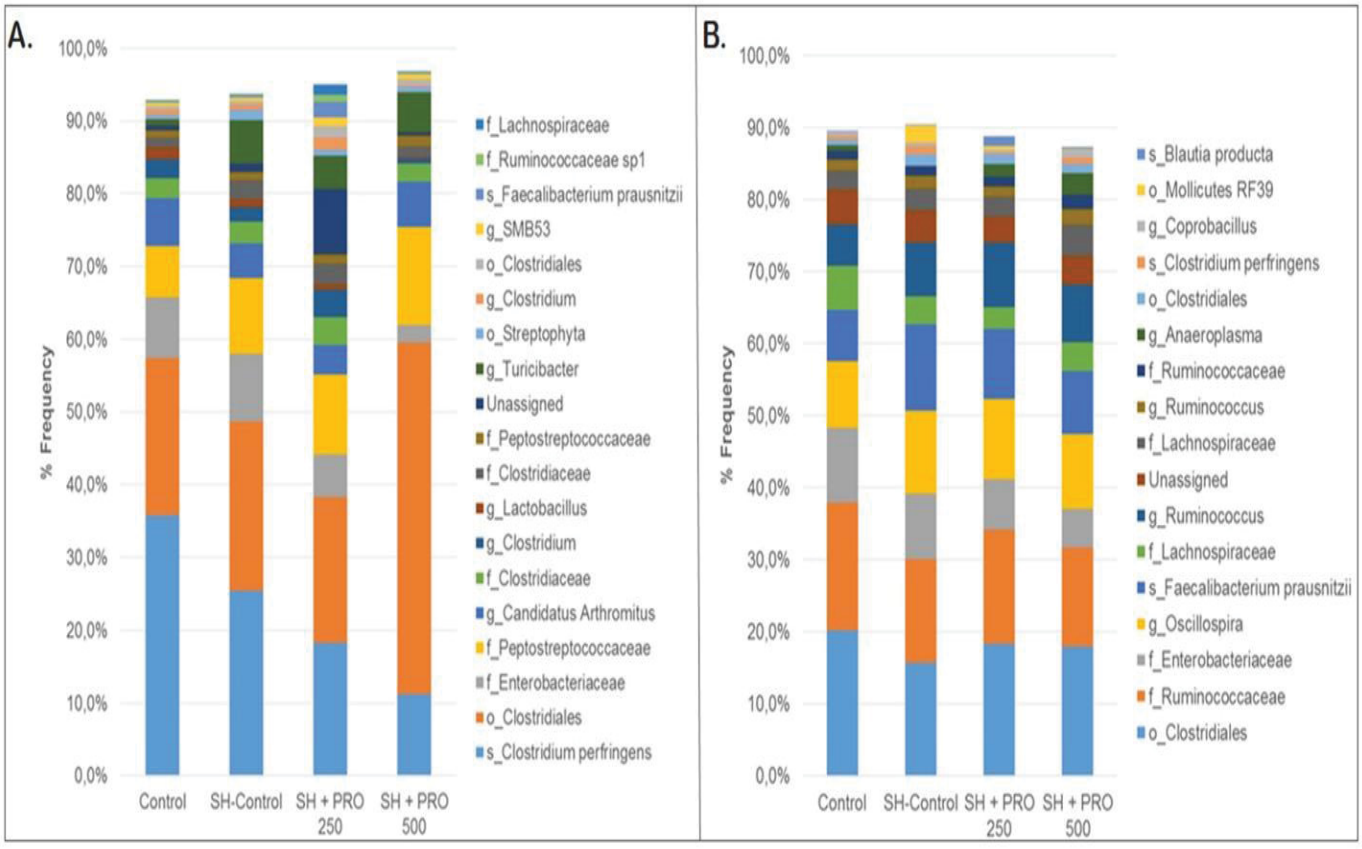
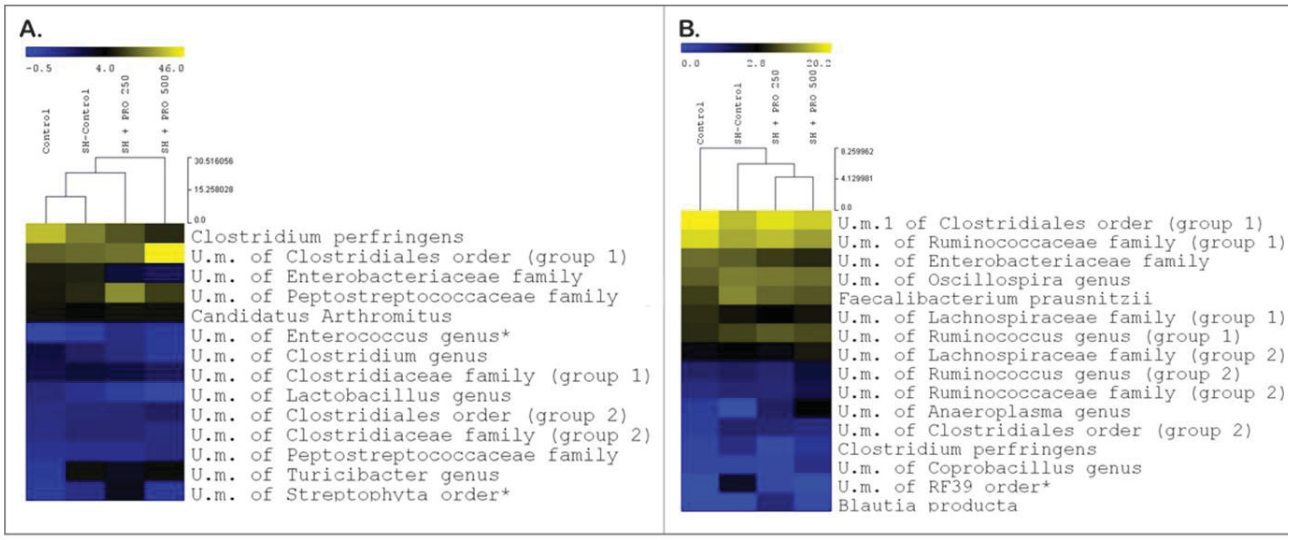


FIGURE 6 – RELATIVE ABUNDANCE OF DISTINCT GROUPS AT THE DEEPEST LEVEL IDENTIFIED IN ILEUM (A) AND CECUM (B) OF GROUPS CONTROL, SH CONTROL, SH+PRO 250 AND SH+PRO 500. A yellower color depicts a greater bacterial abundance to up to 46.2% in ileum (A) abundance and up to 20.2% in cecum (B) abundance. Groups with abundance less than 1% were not considered. U.m. = unidentified members. * Indicate significant differences at P<0.05.



DISCUSSION

No loss in performance resulted from challenging birds with SH at any time. This agrees with previous studies in our laboratory which showed that not all *Salmonella* influence the performance of broilers (MUNIZ *et al*., 2015). As the current trial was not primarily designed to test performance, the experimental layout had low statistical power to detect differences in parameters such as intake and weight gain. Still, a significant improvement in performance resulted from feeding PRO at 250 g/ton. This has also been observed by other workers when feeding some *Bacillus subtilis* strains to broilers (HARRINGTON, SIMS, KEHLET, 2015; REN *et al*., 2013; SHIVARAMAIAH *et al*., 2011).

It is worth noticing that the resulting abundance of *Salmonella* in cecum was relatively low (up to 0.035% for the Control group) compared to other bacterial groups (FIGURES 5 and 6); and that it was not detected in the ileum of chickens even in those orally challenged with SH, confirming the low affinity of *Salmonella* for that organ. Still, Feeding PRO at 500g/ton reduced *Salmonella* counts in both liver and cecum by the end of the trial. In the latter organ, metagenomics showed that both dosages were equally effective in reducing *Salmonella* abundance.

Other studies (KNAP *et al*., 2011) have also shown that adding *Bacillus subtilis* spores in the diet could reduce SH colonization at 42 days of age by up to 58%. The most commented mechanism been competitive exclusion by which *Bacillus subtilis* bacteria occupy adhesion locations of the membranes of enterocytes, goblet and enteroendocrine cells regularly used by *Salmonella*, therefore preventing it from establishing itself in the gut (SALMINEN, ISOLAURI, 1996). An agonist effect caused by the secretion of substances by *Bacillus subtilis*, such as bacteriocins, organic acids and hydrogen peroxide, can also inhibit the growth and development of pathogenic bacteria. Likewise, some strains of *Bacillus subtilis* are known to favor the growth of lactic acid-producing bacteria (KNARREBORG *et al*., 2008) with a subsequent acidification of the intestinal environment (VAN IMMERSEEL *et al*., 2006). These effects could modulate the host´s microbial populations and the intestinal immune response potentially reducing the frequency of *Salmonella* in the gut and its capacity to migrate from

the intestinal lumen into other organs. These is in agreement with the observations in the current trial.

PRO may help to reduce some deleterious alterations in liver parenchyma caused by SH. Hydropic degeneration is an intracytoplasmic fluid accumulation, secondary to disturbance of cell membrane integrity causing vacuolation of hepatocytes (FIGURE 2A and 2B). One of the causes is bacterial infections with differing lobular localization and may be a precursor to hepatocyte necrosis (GKRETSI *et al*., 2007). Also, the interesting transport of immune cells of PRO in liver was reported by other study (ZHANG *et al*., 2008) where probiotic bacteria reduced monocyte and macrophage recruitment to the intestines and spleen compared to control animals. Probiotics may ameliorate pro-inflammatory immune cell recruitment to systemic lymphoid tissues such as liver and other organs. This could save metabolic energy and have positive effect on performance, which in the present trial was observed in broilers fed PRO at 250 g/ton of feed. This performance improvement was not observed when feeding PRO at the highest dose, however this group of birds showed a significant reduction in *Salmonella* infection when challenged with SH, recruited macrophages to eliminate bacteria by phagocytosis, secreted cytokines to modulate immunity and presented antigens to helper T cells (SERBINA *et al*., 2008).

The relationship between chicken macrophages and *Salmonella,* as well as intracellular survival of *Salmonella* in chicken macrophages, remains poorly understood. According to Van Immerseel *et al*. (2002), the encounter between specialized epithelial cells and microorganisms quickly stimulates the release of pro-inflammatory chemokines that attract innate immune cells (i.e., granulocytes and macrophages), which are able to trigger a wide range of new immune responses such as the emergence of T helper lymphocytes (CD4+ cells). An early increase in CD4+ and CD8+ cells has been reported in chickens fed probiotics (ASHEG *et al*., 2002; LOURENÇO *et al*., 2013; MUNIZ *et al*., 2013). In some cases, *Salmonella* cells invade and multiply within the macrophages (HENSEL *et al*., 1998; RUBY *et al*., 2012; TSOLIS *et al*., 2008), and widely distribute themselves in the lymphoid and nonlymphoid tissues, facilitating their spreading to various organs of the host.

In this study, histology observations in ileum seemed atypical as reported in other *Salmonella* trials (LOURENÇO *et al*., 2013; MUNIZ *et al*., 2013) suggesting a considerable variation on ileal morphology when *Salmonella* is present. This variation in ileum histology could be associated to the fact that *Salmonella* has the cecum as target tissue.

Some alterations were observed on ileum histology due to PRO activity such that lamina propria and epithelial thickness increased along with goblet cells proliferation. Probiotics exert a range of effects on mucosal barrier function and on responses of the underlying immune tissue of the gut associated with lymphoid tissue (GALT) (HARDY *et al*., 2013). This barrier function is enforced by the ability of probiotics to influence mucin expression and mucus secretion of goblet cells. It is likely that the probiotic-mediated modulation of mucin expression is a host´s strategy to allow beneficial microbes to colonize the gut (CABALLERO-FRANCO *et al*., 2007). Furthermore, mucins may exert prebiotic-type effects as carbohydrate content can account for 90% of their weight (PEREZ-VILAR, HILL, 1999). Muniz *et al*. (2013) observed similar effects when four different probiotics increased the proliferation of goblet cells in ileum. The association of probiotics with epithelial cells might be sufficient to trigger signaling cascades at epithelium level and activate underlying immune cells in lamina propria (HOARAU *et al*., 2006). Probiotics may increase epithelial and lamina propria thickness, characterized by cell proliferation and inflammatory cells infiltration, respectively (FIGURE 2D), describing a mucosal wound repair (LEONI *et al*., 2015). In a recent publication, (Kraieski *et al*, (2016) observed a positive correlation between ileal epithelial thickness and goblet cells proliferation with BWG, and a negative correlation with FC at 21 days of age. In the present experiment, PRO fed at 250/ton improved BWG while the SH+PRO 250 group presented higher ileal ISI than the SH Control birds at 21 days along with increased epithelial thickness, goblet cells proliferation and lamina propria thickness.

The metagenomics analysis also showed a significant increase in *Bacillus* genus abundance in the ileum of birds fed PRO going from 0.004 ± 0.002 % for the Control group to 0.019 ± 0.004 % for the SH+PRO 500 animals (TABLE S1). That could be due to the presence of *Bacilli* from PRO in that organ itself or, could have been the result of gut microbial changes in *Bacilli* populations not

necessarily of PRO origin, since the *Bacillus* genus is commonly found in the ileal microbiota of broilers.

The diversity index by Shannon-Wiener revealed that cecal microbial composition of the SH+PRO 500 group was significantly more diverse compared to the Control and the SH Control groups (FIGURE 4). Pereira (2014) detected less diversity in chickens fed with *Bacillus subtilis* spores. However, it has been reported that the use of probiotics can increase the intestinal microbiota diversity in different organisms (PREIDIS *et al*., 2012; REMELY *et al*., 2015). Diversity is a combination of richness and evenness. Increasing the diversity tends to suggest more stable ecosystems with more connections within them, even though statistical differences in performance were not observed in the SH+PRO 500 treatment.

In general, the most abundant phylum in the chicken intestinal microbiota is Firmicutes followed by two minor phyla, Proteobacteria and Bacteroidetes. In addition, members of phylum Actinobacteria, Tenericutes (WAITE, TAYLOR, 2014), Cyanobacteria and Fusobacteria (QU *et al*., 2008) can be found in very low abundance. In the present study, Firmicutes was the most predominant phylum found in ileum and cecum in all groups. Proteobacteria, Cyanobacteria (ileum) and Tenericutes (cecum) were also observed but showing lower abundance (FIGURE 5A and 5B).

Enterococcus (phylum Firmicutes) is a large genus of lactic acid bacteria, commensals of animal and human gut (LEBRETON *et al*., 2014). In ileum, this genus was significantly higher ($P<0.05$) in the SH+PRO 250 rather than in the Control group (FIGURE 6A). Many enterococci species such as *E. faecium* produce bacteriocins which have been associated with growth inhibition of food-borne pathogens in the gut (FRANZ *et al*., 2007). It might be possible that increases in the relative abundance of above mentioned commensals in probiotic treated chickens reduced *Salmonella* colonization or simply contributed to intestinal health. Members of Peptostreptococcaceae family (class Clostridia) seemed to be more abundant in SH+PRO 250 broilers compared to the Control group. The Peptostreptococcaceae was isolated from various environments including clinical human and animal samples, manure, soil, marine and terrestrial sediments, and deep-sea hydrothermal vents. High percentage of

Peptostreptococcaceae was found in ileal samples from conventional broiler chickens at 7 and 41 days of age, assuming that this family might be considered a commensal bacteria group (MOHD SHAUFI *et al*., 2015). Another significant difference in ileum ($P<0.05$) is related to unidentified members of Streptophyta order, within the Cyanobacteria phylum, that could be attributed to chloroplasts, non-photosynthetic bacteria commonly found in the animal gut (DI RIENZI *et al*., 2013). This bacterium was more abundant in the SH+PRO 250 group compared to the Control and the SH+PRO 500 treatments (FIGURE 6A).

An unidentified member of RF39 order (phylum Tenericutes, class Mollicutes) was more abundant when SH was present while feeding PRO could reduce it numerically in cecal microbiota (FIGURE 6B). In past studies, it was reported that Mollicutes were enriched in birds affected by necrotic enteritis disease and this could possibly be associated with intestinal disorders for chickens (STANLEY *et al*., 2012). However, Pérez-Brocal *et al*. (2013) observed that humans with Crohn's disease (inflammatory bowel disease) showed lower abundance of bacteria from RF39 order compared to Control group. Goodrich *et al*., (2014) observed an increase of RF39 order in lean body mass adults, compared to obese individuals. Besides the lack of information in literature, it is not possible to assume correlations with those data once the genus from RF39 order was unidentified in the current experiment.

CONCLUSIONS

A probiotic composed by three strains of *Bacillus subtilis* improved animal performance when fed at 250 g/ton and reduced *Salmonella* colonization in liver and cecum at 250 and 500 g/ton when birds were orally challenged with SH strain UFPR1. The mobilization of immune cells in liver can be a relevant mode of action of PRO in birds challenged with SH. PRO can promote important histologic alterations related to activation of defense response and gut absorption. In addition, the supplementation of PRO increased the diversity of cecal microbiota, which suggests a more stable ecosystem, and increased some commensal bacterial groups in ileum, some of which are lactic-acid producing organisms.

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CHAPTER 3

NATURAL ENVIRONMENTAL SELECTION DIFFERENTIALLY MODULATES THE DIVERSITY OF GUT MICROBES AND INNATE IMMUNE RESPONSES OF FERAL AND MODERN BROILER CHICKENS

ABSTRACT

Feralization occurs when a domestic population recolonizes the wild, escaping its previous restricted environment, and has been considered as the reverse of domestication. Modern broiler chickens have been selected on growth characteristics that could adversely affect innate immune competence leaving chickens more susceptible to diseases. Understanding the feral chicken's gut microbiota and its relation with immunity may provide insights to enhance performance and the microbiological safety of poultry products. Ileal and cecal contents of 21 feral chickens from Bermuda (BFC), 28 feral chickens from Hawaii (HFC) and 12 Cobb broiler chickens (BC) were collected and analyzed by 16S rRNA high throughput sequencing. We also compared the expression profiles of chicken Toll-like receptors (TLRs) genes in ileum and liver using RT-qPCR. In the ileum, the HFC group had more diversity than BFC and BC, while BFC and HFC were significantly more diverse compared to BC in the cecum ($p<0.05$). A proposed core microbiota was defined for ileal and cecal samples. Many taxa were exclusively found in the feral birds. Some of them might have been lost during the process of domestication for broilers or acquired from the environment in feral chickens leading to differential gains and losses taxa. Other taxa were abundantly identified in the BC group, which may be correlated to better weight gain and productivity, as reported by other studies. The progressive loss of the microbial diversity by the BC group, may be correlated to a downregulation of TLRs in the ileum leading to a gut permeability and bacterial translocation to other peripheral organs. Activation of the innate immune response by TLRs may impair liver homeostasis and enhance liver inflammation. Further work with controlled experimental designs will be needed to clarify these connections and explore possible links related to concomitant evolutionary changes in the functional genes of feral and commercial chickens.

Key words: 16S rRNA, domestication, feralization, gut microbiota, Toll-like receptors.

INTRODUCTION

Chickens are considered to represent an efficient agricultural species in converting feed to lean meat, although their feed is often of low digestibility and their intestines are smaller, with shorter transit digestion times compared to those of mammals (CHOCT, 2009; MCWHORTER *et al*., 2009). The gut microbiota of a (healthy) chicken is presumed to play an important role in nutrient assimilation, vitamin and amino acid production, and prevention of pathogen colonization (APAJALAHTI, 2018). The chicken gut microbiota may also act as a reservoir of bacterial pathogens which can spread to human beings, or act as a reservoir of antibiotic-resistance determinants, which can be transferred to other microorganisms including opportunistic pathogens (ZHOU, WANG, LIN, 2012).

Economic pressure on the modern poultry industry has directed the selection process towards fast-growing broilers that have a reduced feed conversion ratio and higher growth efficiency. This selection is based heavily on genetic growth characteristics (ZHAO *et al*., 2013), balanced diets (TOROK *et al*., 2008), housing condition (NORDENTOFT *et al*., 2011), floor litter (CRESSMAN *et al*., 2010), and stocking density (GUARDIA *et al*., 2008). All these factors could adversely shape the gut microbiota and affect the immune competence leaving chickens more susceptible to disease.

Feralization is a process by which domesticated species have escaped their controlled environment and colonized new habitats. It is also called “domestication in reverse (GERING *et al*., 2015; JOHNSSON *et al*., 2016), as it involves the removal of direct anthropogenic control over natural and sexual selection regimes. Studying chickens exposed to a feralization process could be useful in elucidating the impact of human directed breeding and management on the development of gut microbiota.

The aim of this study was to report an in-depth cataloguing of the gut microbiota composition of feral (i.e. formerly domesticated, wild-living) chickens

from Hawaii and Bermuda (HF and BF, respectively) and broiler chickens (BC) using high throughput sequencing, and also comparing the innate immune response by Toll-like receptors (TLRs) genes in liver and ileum using RT-qPCR. The results allowed us to understand what happens to the gut microbiota of domesticated chickens during the process of feralization, and to offer some insights if rearing animals in more natural type of environments might improve genetic, microbial and immunological aspects leading to safer food supply.

MATERIAL AND METHODS

ETHICAL STATEMENT

Collection and export of samples derived from feral chickens was approved by the University of Hawaii Center for Tropical and Agricultural Research Station and the Bermuda Department of Environment and Natural Resources in support of the Bermuda Biodiversity Project. In addition, this study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the Michigan State University, under permit number 06/17-093-00.

ANIMALS

A total of 21 feral chickens from St. George's island, Bermuda, 28 feral chickens from Kauai, Hawaii, and 11 broiler chickens (BC) from the Poultry Farm at University of Illinois at Urbana-Champaign, were investigated. All feral birds were the result of natural conditions, without human control or interference, and therefore categorized as Bermuda feral chickens (BFC) and Hawaii feral chickens (HFC). Bermuda and Hawaii support a large, self-sustaining population of feral chickens that have been living in the wild since at least the mid-1980s. Based on preliminary genetic and morphological analyses, these birds appear to be an admixed flock originating from several breeds that are popular sources of meat and eggs in the Western hemisphere. Our ad hoc qualitative observations of gut

content suggest a highly variable diet including locally-occurring invertebrates (e.g., snails and insects), local ornamental and/or natural vegetation (e.g., seeds and shoots) and garbage from Bermuda and Hawaii households and businesses. We also sampled eleven Cobb 500 male broiler chickens from the University of Illinois (UIUC), at Poultry Farm from Animal Sciences Department, raised in the same house and provided with food and water *ad libitum*. The detailed diets from the three evaluated groups are presented in table 1. All birds were euthanized and fecal and tissue samples were collected, stored in proper conditions and transported to University of Illinois at Urbana-Champaign for further analysis.

TABLE 1 – CHICKEN SAMPLES COLLECTED IN THIS STUDY. ORIGIN OF SAMPLES, ANTIBIOTIC TREATMENTS AND FEED COMPOSITION ARE REPORTED.

Samples - Ileum	Samples - Cecum	Groups	Origin	Feed	Antibiotics
I.HA.5809	C.HAE.528	Bermuda Feral Chickens (BFC)	St. George (Bermuda)	Invertebrates, seeds, shoots, household and business garbage.	No.
I.HAE.525	C.HAE.1425				
I.HAE.618	C.HAHAE.129				
I.HAE.1425	C.HAHAE.435				
I.HAHAE.927	C.HAHAE.927				
I.HAHAE.1226	C.HAHAE.933				
I.HAHAS.129	C.HAHAE.1226				
I.HAHAS.435	C.HAK.2029				
I.HAR.2024	C.HAS.120				
I.HAS.1208	C.HAS.236				
I.HAW.125	C.HAS.1213				
I.HAW.231	C.HAS.1215				
I.HAW.1414	C.HAW.125				
I.KP.521	C.HAW.231				
I.KPCH.6004	C.HAW.1414				
I.KPMP.5005	C.KP.521				
I.KPRT.122	C.KPCH.6084				
I.MK.0225	C.KPMP.005				
I.MK.01001	C.MK.01001				
I.MK.01002	C.MK.01002				
I.MK.01003	C.MK.01003				
I.MK.01006	C.MK.01005				
I.MK.01008	C.MK.01006				
I.MK.01009	C.MK.02002				
I.MK.02002	C.MK.02003				
I.MK.02003	C.MK.02005				
I.MK.02004	C.ML.01008				
	C.NTBG.01001				

22I, 23I, 24I, 25I, 26I, 27I, 30I, 40I, 41I, 42I, 43I, 45I, 46I, 47I, 50I, 52I, 53I, 54I, 56I, 57I, 58I.	20C, 22C, 23C, 24C, 25C, 26C, 41C, 42C, 43C, 45C, 46C, 50C, 52C, 53C, 54C, 56C, 57C, 58C.	Hawaii Feral Chickens (HFC)	Kauai (Hawaii)	Invertebrates, seeds, shoots, household and business garbage.	No.
I2, I3I, I4, I5, I6, I7, I8, I9, I10, I11.	C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11.	Broiler Chickens (BC)	Poultry Farm – floor pens (litter), University of Illinois at Urbana-Champaign	Cereals (wheat, corn), protein flour (soy, sunflower), vegetables oils (soy), mineral	No.

GENOMIC DNA EXTRACTION AND AMPLIFICATION OF 16S RRNA

Ileal and cecal luminal contents were obtained, frozen in liquid nitrogen and stored at -80ºC until analysis. Samples were subjected to DNA extraction using the Power Soil DNA Extraction Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). Amplification of 16S rRNA genes was conducted at the University of Illinois at Urbana-Champaign Biotech Center using the Fluidigm system, which permits parallel amplification of a specific region from a target gene prior to high throughput sequencing. Amplicon libraries for the V4 region of the 16S rRNA gene were generated using the primer pair 515F (5´GTGYCAGCMGCCGCGGTAA 3´) and 806R (5´ GGACTACNVGGGTWTCTAAT 3´), delivering an amplified region of 292 base pairs. Following sequencing of amplicons was performed using the Illumina MiSeq V3 platform with paired read (2x300) sequencing to ensure recovery of the integral V4 region.

BIOINFORMATIC ANALYSIS OF SEQUENCING RESULTS

The raw paired-reads were quality checked with FastQC (ANDREWS, 2010), trimmed if necessary using the FASTX-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), and paired merger with PEAR (ZHANG *et al*., 2014). Resulting amplicons were processed using the QIIME pipeline

(CAPORASO *et al*., 2011). Reads with quality scores under 25 and chimeric sequences predicted with usearch (EDGAR, 2010) were excluded from following analyses. Resulting quality-controlled sequences were binned into operational taxonomic units (OTUs) at a 97% similarity cutoff (KUNIN *et al*., 2010) using uclust (EDGAR, 2010). We used the *open reference* OTU picking method, where reads are clustered against the reference database collection, and any unmatched reads are subsequently clustered *de novo*. The cluster seeds were used as representative sequences. These non-chimeric representatives were aligned with the PyNAST algorithm (CAPORASO *et al*., 2011) using as reference the Greengenes core s*et al*ignment (DESANTIS *et al*., 2006). Taxonomy assignations were inferred through comparisons with both the RDP (COLE *et al*., 2009) and BLASTn (ALTSCHUP *et al*., 1990) databases. Rarefaction analysis was performed in order to remove the heterogeneity of the number of sequences per sample prior to calculation of alpha and beta diversity statistics. Alpha and beta diversity metrics were calculated using QIIME.

RNA EXTRACTION AND REVERSE TRANSCRIPTION (RT)-PCR

Samples of ileal and liver from BFC and BC were collected and extracted RNA by TriZol (MBI Fermentas, MD, USA) according to the manufacturer's instructions. To avoid the possible traces of genomic DNA, 5 mg of each RNA sample was incubated at 37°C for 10 min with 5U of RNase free DNase. Following this DNase was inactivated by incubation at 65°C for 10 min. Subsequently, total RNA (5 mg) from each sample was reverse transcribed into cDNA by using the RevertAid First strand cDNA synthesis kit (MBI Fermentas, MD, USA) according to the manufacturer's instructions. The resultant cDNA was stored frozen at -20°C until further analysis.

RELATIVE QUANTIFICATION OF TOLL-LIKE RECEPTORS MRNA BY REAL TIME PCR

The relative expression levels of TLR mRNA (TLR1, TLR2, TLR3, TLR4, TLR5, TLR15 and TLR21) were quantified by real time PCR performed in

Mx3000P system (Stratagene, CA, USA). Each reaction was carried out in triplicates in a total volume of 25 mL containing 1X Maxima SYBR Green=ROX qPCR master mix (MBI Fermentas, MD, USA), 10pmol concentration of each gene specific primers (TABLE 2), and 1 mL of cDNA template (100 ng=mL). PCR cycling conditions were: 95°C for 10 min followed by 40 cycles of denaturation at 94°C for 30 s; annealing at 55–60°C for 30 s and extension at 72°C for 30 s. In each PCR reaction, a no template control was included to check contamination of master mix. Nonreverse transcribed RNA (10 ng) of each sample was used instead of cDNA to check contamination of samples with genomic DNA; failure of the amplification confirmed the purity of the sample. To assess the efficiency of primers, standard curves for each primer pair were generated using serially diluted transcribed RNA samples. PCR efficiency was calculated from the slope of standard curves. The resulting threshold cycle [Ct, a fractional PCR cycle number at which the change in reporter dye (DRn) passes the significant threshold] values were normalized to the endogenous control, beta actin (DCt¼Ct value of target gene-Ct value of beta actin).

TABLE 2 – SEQUENCE OF PRIMERS USED IN RT-PCR

Gene	Ligand	Forward primer (5'–3')	Reverse primer (5'–3')
TLR1	Triacylated lipopeptides	AGTCCATCTTTGTGTTGTCGCC	ATTGGCTCCAGCAAGATCAGG
TLR2	Diacylated lipopeptides	GATTGTGGACAACATCATTGACTC	AGAGCTGCTTTCAAGTTTTCCC
TLR3	dsRNA	TCAGTACATTTGTAACACCCCGCC	GGCGTCATAATCAAACACTCC
TLR4	LPS	AGTCTGAAATTGCTGAGCTCAAAT	GCGACGTTAAGCCATGGAAG
TLR5	Flagellin	CCTTGTGCTTTGAGGAACGAGA	CACCCATCTTTGAGAAACTGCC
TLR21	DNA	GTTCTCTCTCCCAGTTTTGTAAATAGC	GTGGTTCATTGGTTGTTTTTAGGAC
TLR15	Protease	TGCCCCTCCCACTGCTGTCCACT	AAAGGTGCCTTGACATCCT

EVALUATION OF INTESTINAL HEALTH – HISTOLOGY BY ISI (I SEE INSIDE METHODOLOGY)

Samples of liver and ileum from BFC and BC were collected and further microscopic evaluation of the gastrointestinal tract was performed using the ISI

Methodology (“I See Inside”; Pat. INPI-BR10201500360l9) as published by KRAIESKI *et al*. (2016) and BELOTE *et al* (2018). Shortly, this methodology was developed based on a numeric score of histological alterations. For each alteration observed during microscopic analysis, an impact factor (IF) is defined according to its importance in affecting organ functional capacity based on previous knowledge of literature and background research (e.g. necrosis has the highest IF because the functional capacity of affected cells is totally lost). The IF ranges from 1 to 3, where 3 represents an IF of the greatest significance in terms of the organ function. In addition, the extent of each alteration (intensity or observed frequency compared to non-affected tissue) is evaluated per field (liver) or per villi (intestine) and scored ranging from 0 to 3. To reach the final ISI value, the IF of each alteration is multiplied by the respective score number, and the results of all alterations are summed.

STATISTICAL ANALYSIS

All statistical analyses were performed with software Statistix 9. Data were evaluated by the Shapiro-Wilk normality test. Parametric data were subjected to analysis of variance (ANOVA) and Tukey's test to establish differences among treatment means. Nonparametric data were submitted to the Kruskal-Wallis test at a 5% probability value. Changes in the populations of individual bacteria with abundance higher than 1% were analyzed by ANOVA or PERMANOVA and Tukey's test accordingly.

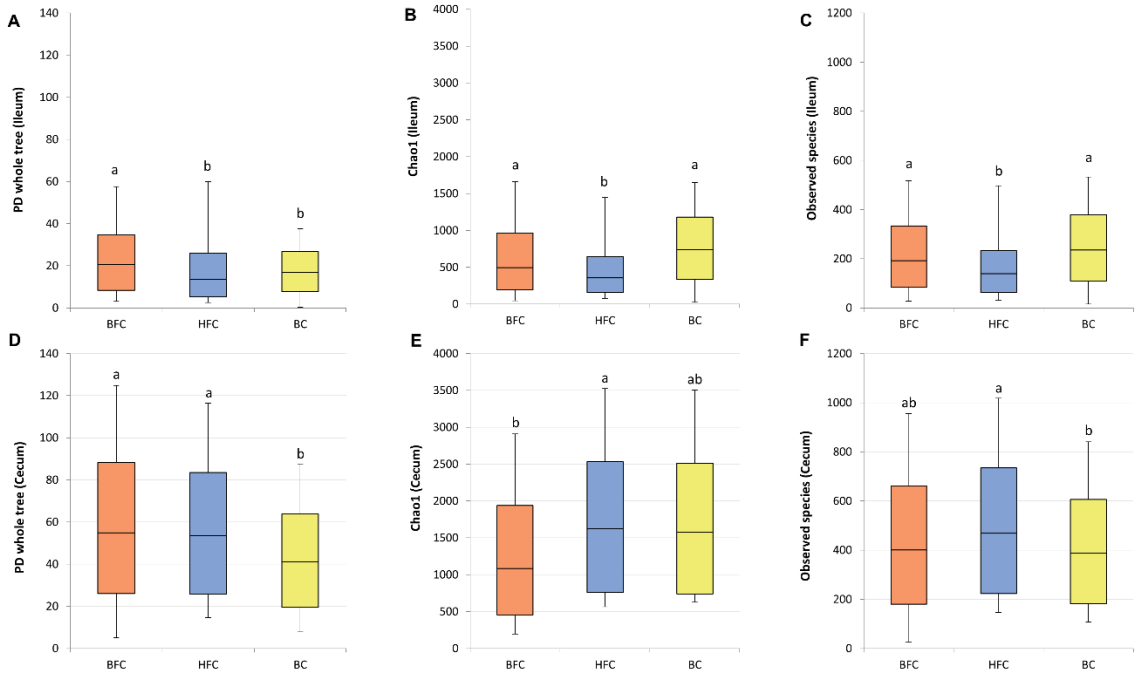
RESULTS AND DISCUSSION

Illumina-based 16S rRNA microbial profiling produced a total of 1.742.420 sequencing reads with an average of filtered 14.282 reads per sample. The microbiota analysis of BFC, HFC and BC were compared. We employed three indices (Phylogenetic Diversity (PD) Whole Tree, Chao1 and Observed Species) to estimate the alpha diversity of the ileal and cecal populations. Moreover, boxplots reveal differences between the ileal and cecal microbiota of BFC, HFC and BC (FIGURE 1). Specifically, in the ileum, the BFC group had more diversity

(PD Whole Tree) than HFC and BC ($p<0.05$, FIGURE 1A). The HFC presented less richness (Chao1) ($p<0.05$, FIGURE 1B) and less observed species than other groups in ileal microbiota ($p<0.05$, FIGURE 1C). For cecal microbiota, PD Whole Tree revealed that microbial composition of the BFC and HFC were significantly more diverse compared to BC ($p <0.05$, FIGURE 1D). The HFC had significant higher richness (Chao1) ($p<0.05$, FIGURE 1E) compared to BFC group and higher observed species ($p<0.05$, FIGURE 1F) compared to BC group in cecal microbiota.

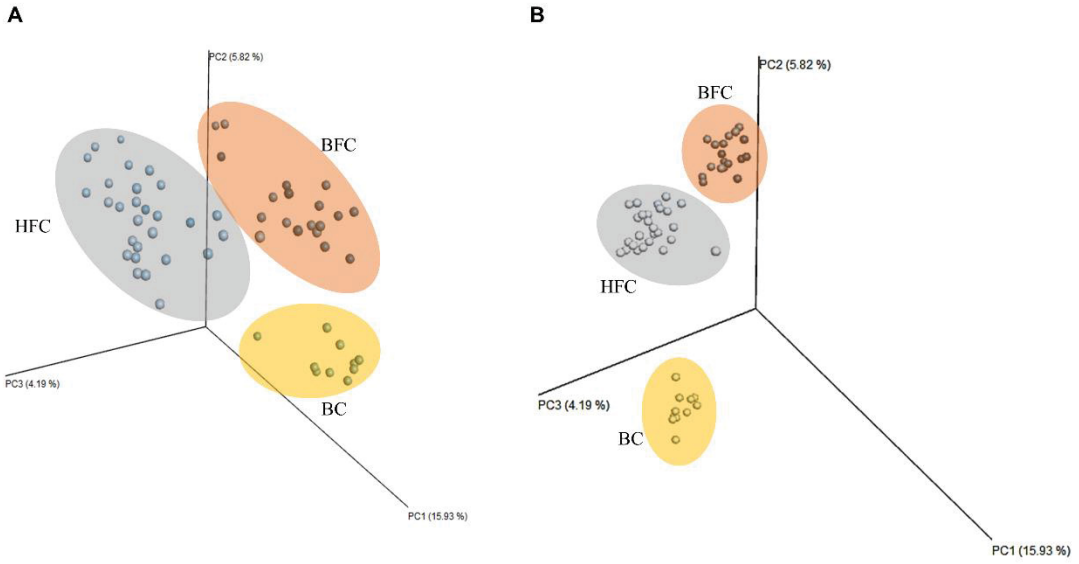
Despite the limitations of different diversity indices, in many cases microbiome diversity may be positively correlated with the proportion of competitive interactions and so a diverse microbiome may also indicate a more stable and resilient one (JOHNSON, BURNET, 2016). However, as demonstrated by other studies (FALONY *et al*., 2016; MANICHANH *et al*., 2006), diversity does not necessarily equate to a stable microbiome since a large number of interacting species could also have a destabilizing effect.

FIGURE 1 - BETA DIVERSITY MEASURES OF BERMUDA FERAL CHICKENS (BFC), HAWAII FERAL CHICKENS (HFC) AND BROILER CHICKENS (BC). A) Box plots of PD Whole Tree in the ileum. B) Box plots of Chao1 in the ileum. C) Box plots of Observed species in the ileum. D) Box plots of PD Whole Tree in the cecum. E) Box plots of Chao1 in the cecum. E) Box plots of Observed species in the cecum. ^{a,b} Different letters indicate significant differences at p-value<0.05 at Tukey test.



In order to determine how similar microbial communities are between BFC, HFC and BC groups, we analyzed the β-diversity based on unweighted UniFrac, after which the UniFrac distance matrix was represented through Principal Coordinate Analysis (PCoA), divided in ileal and cecal graphics (FIGURES 2A and 2B, respectively). As would be predicted these analyses showed that the feral chickens groups from Bermuda and Hawaii, being clearly separated from commercial broiler chickens in both the ileum and cecum. In addition, when we compared the two feral groups, it was also possible to observe two different clusters.

FIGURE 2 – A) PRINCIPAL COORDINATE ANALYSIS (PCOA) ENCOMPASSING ALL ILEAL BFC, HFC AND BC DATASETS THROUGH THREE-DIMENSIONAL IMAGES, BASED ON UNWEIGHT UNIFRAC METRIC DISTANCE. B) PRINCIPAL COORDINATE ANALYSIS (PCOA) ENCOMPASSING ALL CECAL BFC, HFC AND BC DATASETS THROUGH THREE-DIMENSIONAL IMAGES, BASED ON UNWEIGHT UNIFRAC METRIC DISTANCE.



Such findings were shown to be statistically validated by a $p<0.05$, as obtained by Permanova test, when the data sets of the three clusters were compared in ileal and cecal microbiota.

Exploring the predicted taxonomic profiles at phylum level for the analyzed samples clearly shows that the BFC, HFC and BC groups possess a distinct microbiota composition (FIGURE 3 and TABLES 3 and 4).

The ileal microbiota of BFC, HFC and BC was shown to be dominated by Firmicutes phylum, though a significantly different level ($p<0.05$, with an average relative abundance of 69.14%, 63.5% and 85.8%, respectively), followed by Proteobacteria phylum ($p<0.05$, relative abundance of 21.38%, 19.51% and 0.17%, respectively). The BC group had Actinobacteria as the second most dominant phylum ($p<0.05$, relative abundance of 13.32%). Still, feral chickens from Bermuda presented significant higher populations of the Cyanobacteria phylum compared to HFC and BC ($p<0.05$) (FIGURE 3 and TABLE 3).

TABLE 3 – RELATIVE ABUNDANCE AT PHYLUM LEVEL OF BACTERIAL AND ARCHEAL POPULATION IN ILEAL MICROBIOTA OF BERMUDA FERAL CHICKENS (BFC), HAWAII FERAL CHICKENS (HFC) AND BROILER CHICKENS (BC). ^{a,b} DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES AT $P<0.05$ AT TUKEY TEST.

Phylum	BFC	HFC	BC	p-value
Other	0.23±0.22	0.74±0.19	0.05±0.32	0.105
Euryarcheota	0.08±0.03	0.003± 0.02	0.01±0.04	0.116
Actinobacteria	**4.67±1.27 b**	**1.15±1.12 b**	**13.32±1.85 a**	**0.000**
Bacteroidetes	2.76±1.51	3.61±1.33	0.33±2.19	0.447
Cyanobacteria	**5.92±1.10 a**	**0.04±0.97 b**	**0.30±1.59 b**	**0.001**
Elusimicrobia	0.0±0.0	0.0148±0.003	0.0±0.0	0.209
Firmicutes	**69.14±4.43 b**	**63.5±5.02 ab**	**85.80±7.28 a**	**0.048**
Fusobacteria	0.03±2.40	4.88±2.11	0.0±0.0	0.253
Proteobacteria	**21.38±4.49 a**	**19.51±3.96 a**	**0.17±6.5 b**	**0.023**
Spirochaetes	0.09±0.06	0.10±0.06	0.0±0.0	0.649
Synergistetes	0.02±0.01	0.007± 0.01	0.0±0.0	0.282
Tenericutes	0.74±0.29	0.57 ±0.25	0.02±0.42	0.369
Verrucomicrobia	0.10±0.08	0.13±0.07	0.0±0.0	0.635

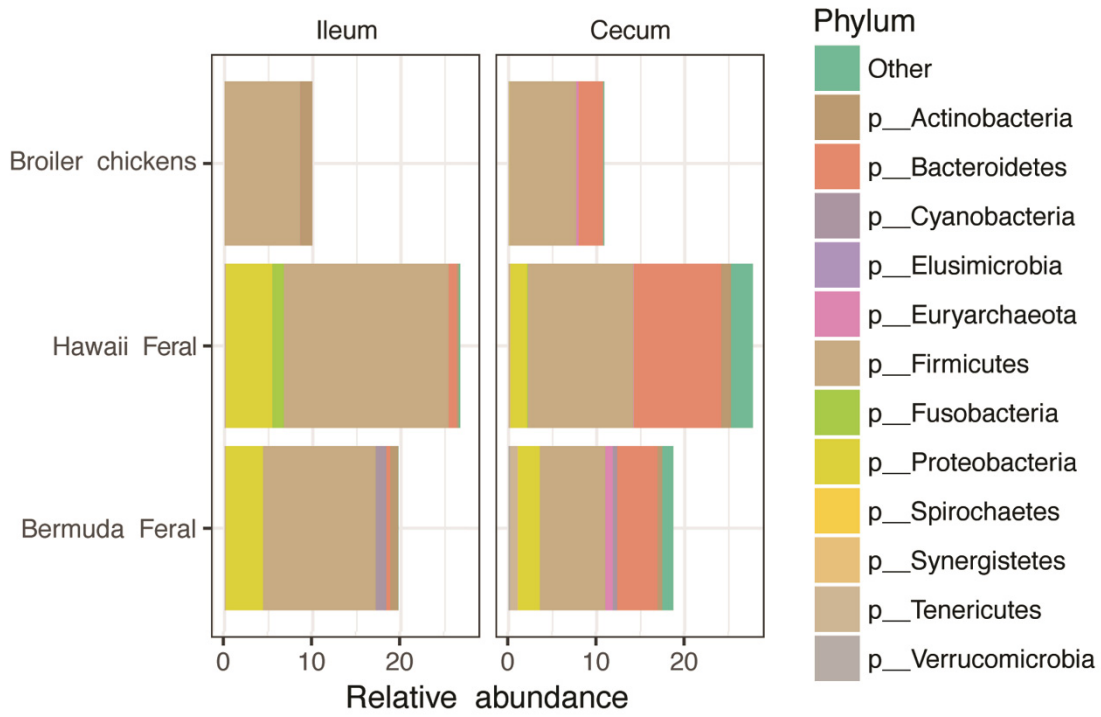
The cecal microbiota of BFC, HFC and BC was also dominated by the Firmicutes phylum ($p<0.05$, with an average relative abundance of 38.11%, 42.84% and 68.47%, respectively), outnumbering the Bacteroidetes ($p<0.05$, with an average relative abundance of 24.98%, 35.36% and 25.22%, respectively). The Proteobacteria phylum were also observed in higher abundance in BFC and HFC compared to BC ($p<0.05$). Interestingly, BFC had higher abundance of Tenericutes, Cyanobacteria and Verrucomicrobia ($p<0.05$) in comparison to the other groups. Feral chickens from Bermuda and Hawaii also presented higher abundance of Actinobacteria and others non-identified communities in the ceca ($p<0.05$) (FIGURE 3 and TABLE 4).

TABLE 4 – RELATIVE ABUNDANCE AT PHYLUM LEVEL OF BACTERIAL AND ARCHEAL POPULATION IN CECAL MICROBIOTA OF BERMUDA FERAL CHICKENS (BFC), HAWAII FERAL CHICKENS (HFC) AND BROILER CHICKENS (BC). [a,b] DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES AT P<0.05 AT TUKEY TEST.

Phylum	**BFC**	**HFC**	**BC**	**p-value**
Other	**6.94±1.13 a**	**8.86±0.90 a**	**1.52±1.44 b**	**0.001**
Euryarcheota	**4.78±0.83 a**	**0.28±0.67 b**	**2.40±1.07 ab**	**0.001**
Actinobacteria	**2.86±0.67 a**	**3.98±0.54 a**	**0.15±0.86 b**	**0.001**
Bacteroidetes	**24.98±1.48 b**	**35.36±1.98 a**	**25.22±3.17 b**	**0.002**
Cyanobacteria	**2.72±0.43 a**	**0.01±0.34 b**	**0.04±0.55 b**	**0.000**
Elusimicrobia	0.12±0.04	0.07±0.03	0.0±0.0	0.169
Firmicutes	**38.11±3.20 b**	**42.84±2.57 b**	**68.47±4.10 a**	**0.000**
Fusobacteria	0.005±0.24	0.30±0.19	0.0±0.0	0.564
Proteobacteria	**12.40±2.54 a**	**6.87±2.04 ab**	**0.60±3.25 b**	**0.021**
Spirochaetes	0.03±0.02	0.06±0.01	0.0±0.0	0.154
Synergistetes	0.51±0.15	0.23±0.12	0.0±0.0	0.128
Tenericutes	**4.73±0.55 a**	**0.90±0.44 b**	**1.30±0.71 b**	**0.000**
Verrucomicrobia	**1.15±0.22 a**	**0.10±0.17 b**	**0.23±0.28 b**	**0.001**

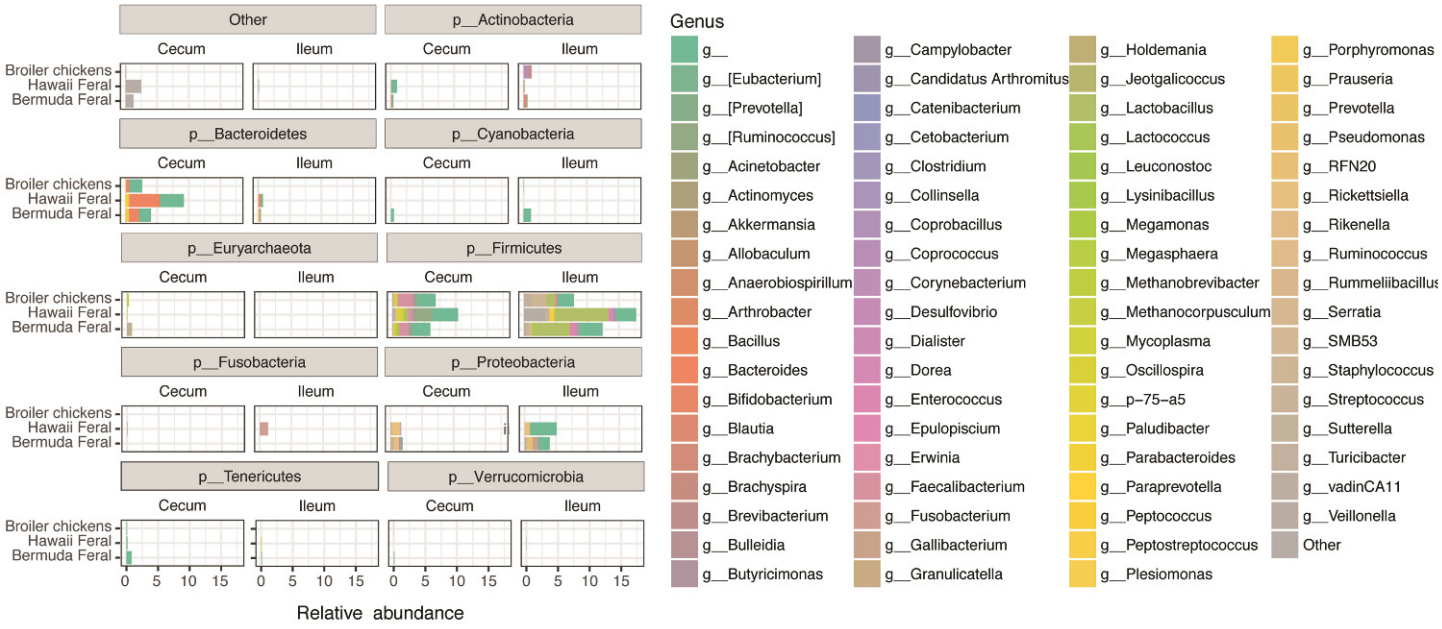
An analysis of all publically available 16S rRNA gene sequences that were generated with the Sanger DNA sequencing technology from intestinal samples of chicken was performed by Wei, Morrison and Yu (2013). In this bacterial census, Firmicutes (70% of the bacterial sequences), Bacteroidetes (12.3% of bacterial sequences), Proteobacteria (9.3% of the bacterial sequences) and Actinobacteria (3%) were the most dominants phyla in broiler chickens. Other minor phyla, including Cyanobacteria, Spirochaetes, Synergistetes Fusobacteria, Tenericutes, and Verrucomicrobia, were represented by no more than several sequences, suggesting their low abundance or prevalence in the gut of chickens.

FIGURE 3 – RELATIVE ABUNDANCE AT PHYLUM LEVEL OF BACTERIA POPULATION IN CECAL AND ILEAL MICROBIOTA OF GROUPS BROILER CHICKENS (BC), HAWAII FERAL CHICKENS (HFC) AND BERMUDA FERAL CHICKENS (BFC), ANALYZED BY SEQUENCING USING ILLUMINA MISEQ SYSTEM.



To determine the differences in the composition and relative abundance of the microbiota of these three groups at the genus level, we considered a difference in relative abundance to exist if at least one sample group was higher than 1% (FIGURE 4).

FIGURE 4 - RELATIVE ABUNDANCE AT GENUS LEVEL OF BACTERIAL AND ARCHEAL POPULATION IN CECAL AND ILEAL MICROBIOTA OF BROILER CHICKENS (BC), HAWAII FERAL CHICKENS (HFC) AND BERMUDA FERAL CHICKENS (BFC), ANALYZED BY SEQUENCING USING ILLUMINA MISEQ SYSTEM.



The core microbiota, defined as stable and permanent (and sometimes abundant) members of a microbial community that are present in all samples in a given sample set (Astudillo-Garcia *et al*. 2017), also revealed particular differences between feral and broiler chickens. In ileal microbiota, only two taxa of core microbiota were observed: *Lactobacillus* and *Enterococcus* (TABLE 5). In the ceca, *Bacteroides, Parabacteroides, Lactobacillus, Blautia, Coprococcus, Dorea, [Ruminococcus], Peptococcus, Faecalibacterium, Oscillospira, Ruminococcus* and *Coprobacillus* were present in all three groups, composing the cecal core microbiota (Supplementary table 4). Similarly, Ferrario *et al*. (2017) also found that the cecal core microbiota from feral, free range and broiler chickens were basically belonging to Firmicutes phylum (18 taxa).

Feralization, process which involves the breeding within and between wild and domestic birds, as well as the environmental exposure has led to an increase in microbiome diversity, giving a large degree of variation on which selection can

act. We observed numerous bacterial taxa that may have been lost in the modern broiler chickens, only being identified in the feral chickens group. Ileal samples from feral birds (BFC and HFC) showed an exclusive presence of: *vadinCA11* (Archea, phylum Euryarcheota); *Actinomyces* (phylum Actinobacteria); Bacteroidetes members such as *Parabacteroides, Prevotella* and *Paraprevotella*; Firmicutes members as *Lactococcus, Epulopiscium, Peptostreptococcus, Dialister, Megamonas, Megasphaera, Veillonella and Coprobacillus*; Fusobacteria members as *Cetobacterium* and *Fusobacterium*, Proteobacteria members as *Sutterella, Desulfovibrio, Erwinia, Plesiomonas, Serratia, Rickettsiella, Gallibacterium, Acinetobacter* and *Pseudomonas*; *Brachyspira* (Spirochaetes phylum) and *Mycoplasma* (Tenericutes phylum) (TABLE 5).

TABLE 5. RELATIVE ABUNDANCE AT GENUS LEVEL OF BACTERIAL AND ARCHEAL POPULATION IN ILEAL MICROBIOTA OF BERMUDA FERAL CHICKENS (BFC), HAWAII FERAL CHICKENS (HFC) AND BROILER CHICKENS (BC). [a,b] DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES AT P<0.05 AT TUKEY TEST.

Genus	BFC	HFC	BC	p value	Core microbiota	Exclusive in feral	Exclusive in broilers
Other	0.23±0.22	0.74±0.19	0.05±0.32	0.105			
vadinCA11	0.07±0.02	0.003± 0.02	0.00±0.00	0.153		*	
Actinomyces	0.11±0.06	0.007±0.05	0.00±0.00	0.425		*	*
Brevibacterium	**0.00±0.00 b**	**0.00±0.00 b**	**1.75±0.231 a**	**0.000**			*
Corynebacterium	**0.44±0.60 b**	**0.00±0.00 b**	**9.01±0.87 a**	**0.000**			*
Brachybacterium	**0.01±0.10 b**	**0.00±0.00 b**	**1.85±0.15 a**	**0.000**			
Arthrobacter	0.33±0.18	0.003±0.16	0.03±0.26	0.377			*
Prauseria	**0.00±0.00 b**	**0.00±0.00 b**	**0.17±0.06 a**	**0.043**			
Bifidobacterium	1.51±0.63	0.48±0.56	0.06±0.92	0.337			
Bacteroides	0.93±0.57	1.61±0.50	0.03±0.83	0.260			
Parabacteroides	0.11±0.06	0.16±0.05	0.00±0.00	0.356		*	
Prevotella	0.81±0.45	0.03±0.39	0.00±0.00	0.380		*	
Paraprevotella	0.08±0.03	0.02±0.03	0.00±0.00	0.387		*	
[Prevotella]	0.05±0.02	0.04±0.02	0.00±0.00	0.511		*	
Bacillus	**0.15±0.05 b**	**0.003±0.04 b**	**1.03±0.07 a**	**0.000**			
Lysinibacillus	0.21±0.07	0.00±0.00	0.03±0.10	0.079			
Rummeliibacillus	**0.019±0.03 b**	**0.00±0.00 b**	**0.22±0.05 a**	**0.002**			
Jeotgalicoccus	**0.00±0.00 b**	**0.00±0.00 b**	**0.79±0.08 a**	**0.000**			
Staphylococcus	**0.004±0.85 b**	**0.09±0.97 b**	**21.09±1.41 a**	**0.000**			
Granulicatella	**0.00±0.00 b**	**0.00±0.00 b**	**0.31±0.03 a**	**0.000**			*
Enterococcus	3.18±0.98	2.74±0.86	2.79±1.42	0.942	*		
Lactobacillus	26.51±6.20	31.60±5.47	13.66±8.99	0.234	*		
Leuconostoc	**0.77±0.17 a**	**0.00±0.00 b**	**0.00±0.00 b**	**0.004**			
Lactococcus	0.25±0.11	0.12±0.09	0.00±0.00	0.421		*	
Streptococcus	1.20±0.86	2.34±0.76	0.98±1.25	0.506			
Turicibacter	**1.92±0.96 b**	**0.05±0.85 b**	**11.28±1.39 a**	**0.000**			
Candidatus Arthromitus	0.07±0.04	0.003±0.04	0.18±0.06	0.097			
Clostridium	**0.50±0.33 ab**	**1.56±0.29 a**	**0.02±0.47 b**	**0.010**			
SMB53	**2.37±0.34 a**	**0.24±0.30 b**	**1.84±0.50 a**	**0.000**			
Blautia	0.12±0.07	0.05±0.06	0.06±0.11	0.741			
Coprococcus	0.03±0.03	0.08±0.03	0.01±0.05	0.495			
Dorea	0.004±0.02	0.09±0.02	0.03±0.04	0.054			
Epulopiscium	**2.27±0.53 a**	**0.06±0.47 ab**	**0.00±0.00 b**	**0.006**		*	
[Ruminococcus]	0.07±0.22	0.65±0.19	0.09±0.32	0.113			

Peptostreptococcus	**0.004±1.10 b**	**2.86±0.97 a**	**0.00±0.00 b**	**0.010**	*
Faecalibacterium	0.23±0.06	0.11±0.06	0.16±0.10	0.468	
Oscillospira	0.05±0.10	0.26±0.09	0.01±0.15	0.205	
Dialister	0.32±0.18	0.01±0.16	0.00±0.00	0.417	*
Megamonas	0.61±0.34	0.02±0.30	0.00±0.00	0.377	*
Megasphaera	0.74±0.35	0.12±0.31	0.00±0.00	0.352	*
Veillonella	0.19±0.07	0.20±0.06	0.00±0.00	0.306	*
Allobaculum	0.13±0.06	0.00±0.00	0.00±0.00	0.265	
Catenibacterium	0.10±0.06	0.00±0.00	0.00±0.00	0.421	
Coprobacillus	0.01±0.01	0.12±0.04	0.00±0.00	0.152	*
Cetobacterium	0.01±0.01	0.04±0.03	0.00±0.00	0.571	*
Fusobacterium	0.03±2.40	4.83±2.11	0.00±0.00	0.260	*
Sutterella	0.65±0.18	0.42±0.16	0.00±0.00	0.145	*
Desulfovibrio	0.20±0.10	0.15±0.09	0.00±0.00	0.573	*
Erwinia	0.11±0.04	0.001±0.03	0.00±0.00	0.095	*
Plesiomonas	0.03±0.15	0.20±0.13	0.00±0.00	0.629	*
Serratia	0.48±0.18	0.014±0.16	0.00±0.00	0.131	
Rickettsiella	2.21±1.27	2.88±1.12	0.00±0.00	0.417	*
Gallibacterium	4.15±1.76	0.040±1.55	0.00±0.00	0.183	*
Acinetobacter	0.12±0.04	0.001±0.002	0.00±0.00	0.123	*
Pseudomonas	2.23±0.73	0.001±0.65	0.00±0.00	0.062	*
Brachyspira	0.09±0.06	0.09±0.06	0.00±0.00	0.697	*
Mycoplasma	0.15±0.20	0.43±0.18	0.00±0.00	0.386	*

In the cecal samples, feral birds also demonstrated the unique presence of some bacteria as: *Methanocorpusculum* and *vadinCA11* (Archea, phylum Euryarcheota); Firmicutes members as *Megamonas, Megasphaera, Bulleidia, RFN20, [Eubacterium], p-75-a5*; Fusobacteria members as *Fusobacterium* and Proteobacteria members as *Campylobacter, Anaerobiospirillum* and *Rickettsiella* (TABLE 6).

TABLE 6. RELATIVE ABUNDANCE AT GENUS LEVEL OF BACTERIAL AND ARCHEAL POPULATION IN CECAL MICROBIOTA OF BERMUDA FERAL CHICKENS (BFC), HAWAII FERAL CHICKENS (HFC) AND BROILER CHICKENS (BC). [a,b] DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES AT $P < 0.05$ AT TUKEY TEST.

Genus	BFC	HFC	BC	p value	Core microbiota	Exclusive in feral	Exclusive in broilers
Other	**6.94±1.13 a**	**8.86±0.90 a**	**1.52±1.44 b**	**0.000**			
Methanobrevibacter	**0.42±0.34 b**	**0.17±0.27 b**	**2.40±0.43 a**	**0.001**			
Methanocorpusculum	0.36±0.13	0.09±0.10	0.00±0.00	0.189		*	
vadinCA11	**4.00±0.76 a**	**0.007±0.61 b**	**0.00±0.00 b**	**0.000**		*	
Corynebacterium	**0.005±0.01 b**	**0.00±0.00 b**	**0.06±0.01 a**	**0.004**			*
Brachybacterium	**0.00±0.00 b**	**0.00±0.00 b**	**0.04±0.01 a**	**0.042**			*
Bifidobacterium	**1.22±0.27 a**	**0.26±0.22 b**	**0.009±0.35 b**	**0.009**			
Collinsella	**0.03±0.09 b**	**0.52±0.07 a**	**0.00±0.00 b**	**0.000**			
Bacteroides	**8.71±1.63 b**	**17.35±1.31 a**	**4.26±2.09 c**	**0.000**	*		
Paludibacter	0.15±0.04	0.04±0.03	0.00±0.06	0.121	*		
Parabacteroides	1.94±0.30	2.11±0.24	1.11±0.38	0.097			
Porphyromonas	0.32±0.09	0.26±0.07	0.00±0.00	0.122	*		
Prevotella	**1.35±0.25 a**	**0.30±0.20 b**	**0.01±0.32 b**	**0.002**			
Rikenella	0.11±0.04	0.02±0.03	0.00±0.00	0.237	*		
Butyricimonas	**0.005±0.05 b**	**0.06±0.04 b**	**0.83±0.07 a**	**0.000**			
Paraprevotella	0.27±0.07	0.14±0.05	0.00±0.00	0.065	*		
[Prevotella]	0.29±0.09	0.42±0.11	0.00±0.00	0.080	*		
Bacillus	**0.00±0.00 b**	**0.00±0.00 b**	**0.20±0.04 a**	**0.001**			*
Staphylococcus	**0.00±0.00 b**	**0.00±0.00 b**	**0.23±0.05 a**	**0.001**			*
Enterococcus	**0.01±0.03 b**	**0.14±0.02 a**	**0.06±0.04 ab**	**0.017**			
Lactobacillus	0.92±2.15	3.05±1.72	0.40±2.75	0.624	*		
Streptococcus	0.57±0.23	0.01±0.29	0.60±0.37	0.276			
Turicibacter	**0.00±0.00 b**	**0.003±0.008 b**	**0.14±0.01 a**	**0.000**			
Clostridium	**0.03±0.06 b**	**0.19±0.05 ab**	**0.30±0.08 a**	**0.028**			
SMB53	**0.00±0.00 b**	**0.003±0.008 b**	**0.13±0.01 a**	**0.000**			
Blautia	**0.13±0.10 b**	**0.43±0.08 ab**	**0.78±0.13 a**	**0.001**	*		
Coprococcus	**0.56±0.22 b**	**0.47±0.17 b**	**2.08±0.28 a**	**0.000**	*		
Dorea	**0.12±0.19 b**	**1.29±0.15 ab**	**1.06±0.25 a**	**0.000**	*		
[Ruminococcus]	**2.02±1.14 b**	**10.05±0.92 a**	**4.02±1.46 b**	**0.000**	*		
Peptococcus	**0.15±0.08 b**	**0.47±0.07 a**	**0.20±0.11 ab**	**0.012**	*		

Faecalibacterium	**8.42±1.56 b**	**1.89±1.25 c**	**21.06±1.99 a**	**0.000**	*	
Oscillospira	**2.03±0.50 b**	**3.56±0.40 ab**	**4.79±0.64 a**	**0.004**	*	
Ruminococcus	0.48±0.20	1.02±0.16	1.08±0.26	0.088	*	
Megamonas	**2.01±0.40 a**	**0.02±0.32 b**	**0.00±0.00 b**	**0.001**		*
Megasphaera	**0.64±0.17 a**	**0.02±0.14 b**	**0.00±0.00 b**	**0.020**		*
Bulleidia	**0.39±0.06 a**	**0.03±0.05 b**	**0.00±0.00 b**	**0.000**		*
Coprobacillus	0.11±0.04	0.20±0.03	0.21±0.05	0.222	*	
Holdemania	**0.00±0.00 b**	**0.007±0.03 b**	**0.18±0.04 a**	**0.008**		
RFN20	0.93±0.30	0.11±0.24	0.00±0.00	0.071		*
[Eubacterium]	0.26±0.61	1.46±0.49	0.00±0.00	0.176		*
p-75-a5	0.18±0.10	0.20±0.08	0.00±0.00	0.422		*
Fusobacterium	0.005±0.24	0.30±0.19	0.00±0.00	0.564		*
Sutterella	**2.87±0.32 a**	**0.87±0.26 b**	**0.04±0.42 b**	**0.000**		
Desulfovibrio	**1.78±0.18 a**	**0.65±0.15 b**	**0.01±0.23 b**	**0.000**		
Campylobacter	0.08±0.04	0.03±0.03	0.00±0.00	0.530		*
Anaerobiospirillum	**0.32±0.10 a**	**0.007±0.08 ab**	**0.00±0.00 b**	**0.035**		*
Rickettsiella	4.80±2.53	5.00±2.03	0.00±0.00	0.399		*
Akkermansia	0.18±0.10	0.02±0.08	0.23±0.13	0.310		

The taxa *Actinomyces, Fusobacterium, RFN20, p-75-a5* and *Anaerobiospirillum* detected in feral chicken cecal samples have never been identified in previous microbiome studies in commercial chickens. They may have been lost during the generations or even extinct in modern broiler birds. Interestingly, many of those exclusive genera identified only in feral birds, could have positive effects on the host. Representatives of *Veillonellaceae* family such as *Diallister, Megamonas, Megasphaera* and *Veillonella* consume lactate and produce high amounts of the short chain fatty acids (SCFA) acetate and propionate (WATANABE, NAGAI, MOROTOMI, 2012). The majority of SCFA in the gut are derived from the fermentation by bacterial species of complex carbohydrates present in intestinal content such as soluble dietary fibers or resistant starch. The main SCFA present in the gut (acetate, propionate and butyrate) have been shown to improve epithelial barrier integrity, increase mucin production, regulate epithelial cell proliferation and induce the upregulation and/or assembly of tight junctions (DEN BESTEN *et al*., 2013). Similarly, *Parabacteroides* and *Eubacterium*, other bacterial genera from the feral chicken' gut, produce succinate and butyrate, respectively (LOUIS *et al*., 2004). Other taxa

should also be explored. *Sutterella*, for example, is a potential probiotic present in pigeon “milk” that can improve the rate of growth and feed conversion ratio in chickens (GILLESPIE *et al*., 2012)

Our analysis also highlights which genera present in BC animals overlapped with feral ones. Actinobacteria members (*Brevibacterium* and *Corynebacterium*, in both intestinal segments) and Firmicutes (*Bacillus, Rummeliibacillus, Jeotgalicoccus, Staphylococcus, Granulicatella*, and *Turicibacter*) had higher abundance in comparison with feral groups in the ileum ($p<0.05$) (TABLE 5). In the cecum, the BC group also showed a significant higher abundance of *Methanobrevibacter, Butyricimonas* (Bacteroidetes), *Bacillus, Staphylococcus, Turicibacter, Clostridium, SMB53, Blautia, Coprococcus, Dorea, Faecalibacterium* and *Oscillospira* (Firmicutes) compared to the feral chicken’s groups ($p<0.05$) (TABLE 6).

During the early domestication process, *Gallus gallus domesticus* would have become adapted to a domestic environment in which food, water and protection from predators were provided by humans (AL-NASSER *et al*., 2007; TIXIER-BOICHARD, BED’HOM, ROGNON, 2011). Breeding programs became highly specialized during the 1900s and commercially desirable traits (e.g. meat production for ‘broilers’ or egg production for ‘layers’) became important (AL-NASSER *et al*., 2007; TIXIER-BOICHARD, BED’HOM, ROGNON, 2011). As a result, domesticated poultry have lost some genetic diversity and display behavioral, physiological and physical characteristics distinct from their wild ancestor (AL-NASSER *et al*., 2007; CHENG, 2010). Currently, the interactions in the intestinal microbiota have adapted to the changing environment and hosts and may have impacted the domestic chickens’ health and performance. In a recent study by Ferrario *et al*. (2017), the functional microbiome of broilers was more enriched of genes related to carbohydrate metabolism and energy recovery (or host weight gain) from food than in feral chickens. Thus, one may argue that the cecal microbiota of BC has been selected toward an enrichment of those microorganisms that perform efficient energy recovery from the diet (LEY, HAMADY, LOZUPONE, 2008a)

In this study, many genera found in higher abundances in the BC group, have been reported and related to an improved gut integrity and health. *Bacillus*

(HAYASHI *et al*., 2018; LIN *et al*., 2017), *Blautia* (EREN *et al*., 2015), *Faecalibacterium* (GANGADOO *et al*., 2017; MIQUEL *et al*., 2013), *Oscillospira* (KOHL *et al*., 2014), have been mentioned as commensals and responsible for important interactions with the host.

Another interesting bacterium more abundant in BC group is the methanogenic Archea, in particular *Methanobrevibacter*, which participate in regulating gut metabolism by removing excessive bowel hydrogen, improve acetate and butyrate production which are vital carbon sources for gut epithelium cells. This syntrophic interaction between the gut bacteria and archaea may raise energy extraction when high levels of polysaccharide are given in diets (HOU *et al*., 2016). The higher *Methanobrevibacter* abundance and others previously mentioned in the BC group may have increased concomitant with the selection of higher performance of the modern poultry production.

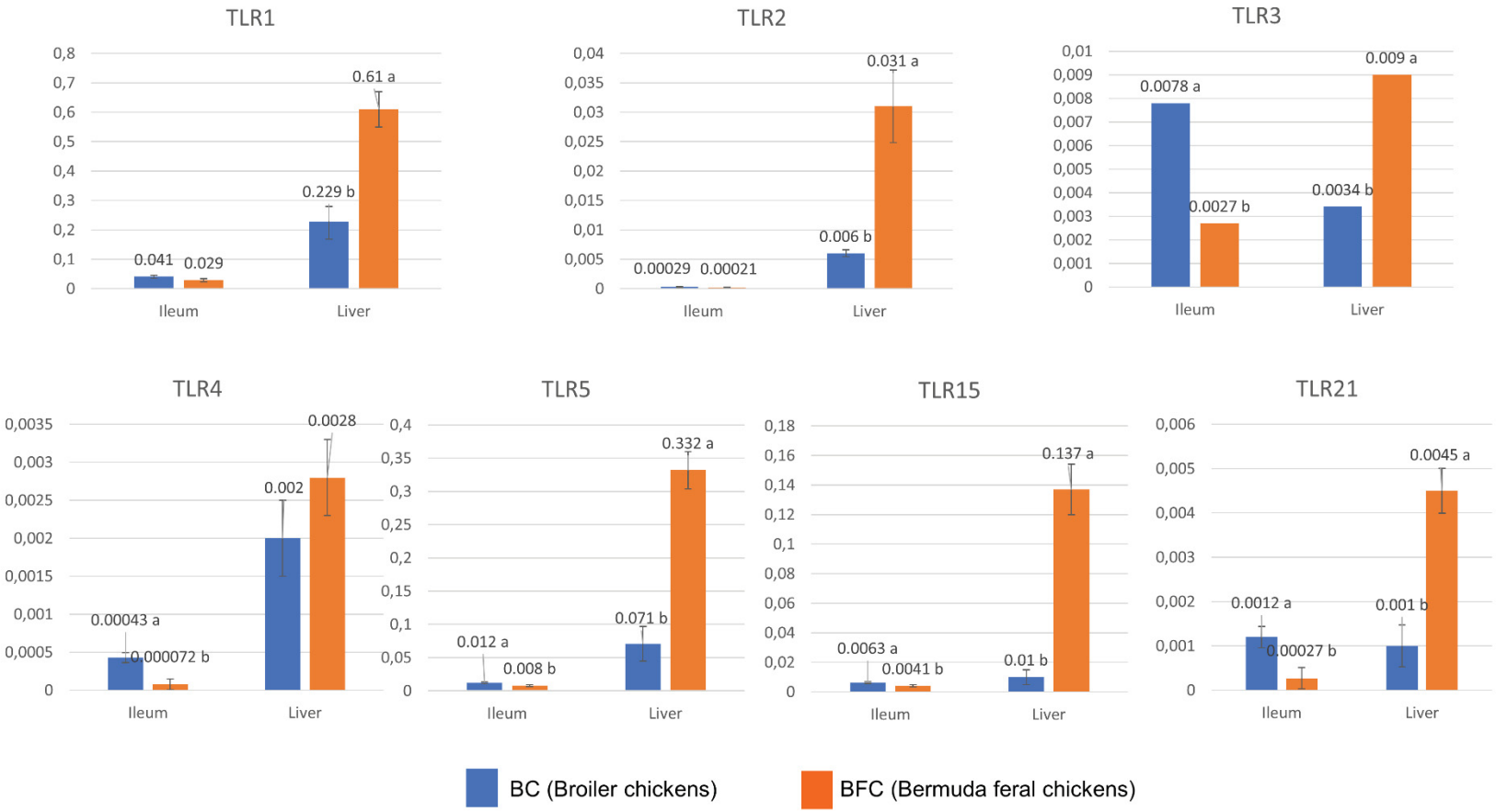
Several studies have shown that individual species of the microbiota modulate the ratio among the different types of immune cells, suggesting that the composition of the microbiota may have an important influence on the host immune response. The host immune defense system requires a careful surveillance able to distinguish microbes with pathogenic potential (pathobionts) from non-pathogenic microorganisms (mainly symbionts) (TANOUE, UMESAKI, 2010).

The ability of the host cells to discriminate pathogens from commensals is mediated by pattern recognition receptors (PRRs) that include the families of TLRs, nucleotide-binding oligomerization domain (NOD-) like receptors (NLRs), C-type lectin receptors (CLRs), cytosolic DNA receptors (CDRs), and RIG-I-like receptors (RLRs). In particular, TLRs are mostly (but not exclusively) present on the membrane of immune and epithelial cells (KAWAI; AKIRA, 2006) and NODs are present in the cytoplasm of enteric cells (LAVELLE *et al*., 2010). TLRs and NODs are capable of recognizing conserved molecular motives, generally divided in microbe-associated molecular patterns (MAMPS, expressed by resident microbiota) and pathogen associated molecular patterns (PAMPS, produced by microbial invaders). Their engagement induces several intracellular signaling cascades resulting in the production of cytokines, chemokines, and transcription factors that are essential for the maintenance of the gut homeostasis and/or

infection control (AKIRA, UEMATSU, TAKEUCHI, 2006). Therefore, TLRs play an important role in suppressing the activation of the inflammatory cascade to maintain the balance of intestinal homeostasis and in promoting inflammatory responses to pathogens (BAILEY, 2012). Chickens have ten different transmembrane proteins belong to the TLR family. Although they are constantly exposed to a significant charge of commensal bacteria, they are able to restrain inflammation to a steady-state condition, keeping a tone of hyporesponsiveness against the intestinal microbiota (ICHINOHE *et al*., 2011).

The results of the expression analysis of the TLR genes are presented in Figure 5. The expression level of TLRs showed a general trend of upregulation in the ileum of BFC compared to BC, with significant difference for TLR3, TLR4, TLR5, TLR15 and TLR21 ($p<0.05$). In this study, we assume that the higher microbiota diversity in BFC group is related to a higher TLR expression in the ileum compared to BC group. The higher expression of TLR4 in the ileum of BFC than BC groups (p-value<0.05), plays an important role in the intestinal mucosal defense against Gram-negative bacteria, which was also measured by our sequence-based analysis (BFC=31%, BC=8,2%). TLR5 was also upregulated in ileal samples from BFC and has been linked to the innate immune receptor for bacterial flagellin. Mucosal barrier breakdown and inflammation in the gut has been associated with high levels of flagellin, the principal protein comprising bacterial flagella (SANDERS, 2005) A wide diversity of gut commensals including members of the phyla Proteobacteria (significative higher abundance in BFC, $p<0.05$), have flagella for motility (LOZUPONE *et al*., 2012).

FIGURE 5 – TISSUE PROFILING IN TOLL-LIKE RECEPTORS (TLR)1, TLR2, TLR3, TLR4, TLR5, TLR15 AND TLR21 MRNA EXPRESSION LEVEL IN LIVER AND ILEUM OF BROILER CHICKENS (BC) AND BERMUDA FERAL CHICKENS (BFC). [a,b] DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES AT P<0.05 AT KRUSKAL-WALLIS.



Additionally, we evaluated histological alterations of BFC and BC groups, performing the ISI approach, summarized in Figure 6. In the ileum, the BFC group had increased ISI ileal scores compared to the BC group (FIGURE 6A). The main alterations observed in feral chickens from Bermuda were an increase in lamina propria thickness, epithelial plasma infiltration, mixed inflammatory infiltration in the lamina propria, proliferation of goblet cells, congestion and presence of oocysts ($P<0.05$, FIGURES 6A and 7A), while BC presented lower scores ($P<0.05$), showing a normal intestinal mucosa without severe alterations (FIGURES 6A and 7B).

FIGURE 6 - HISTOLOGICAL ALTERATIONS (ISI) BETWEEN BFC (BERMUDA FERAL CHICKENS) AND BC (BROILER CHICKENS). A) LIVER (SCORE PER FIELD). B) ILEUM (SCORE PER VILLI). * INDICATE SIGNIFICANT DIFFERENCE BETWEEN TOTAL ISI SCORE (SUM OF ALL PARAMETERS) AT $P<0.05$ AT KRUSKAL-WALLIS. ** INDICATE SIGNIFICANT DIFFERENCE ON A HISTOLOGICAL PARAMETER.

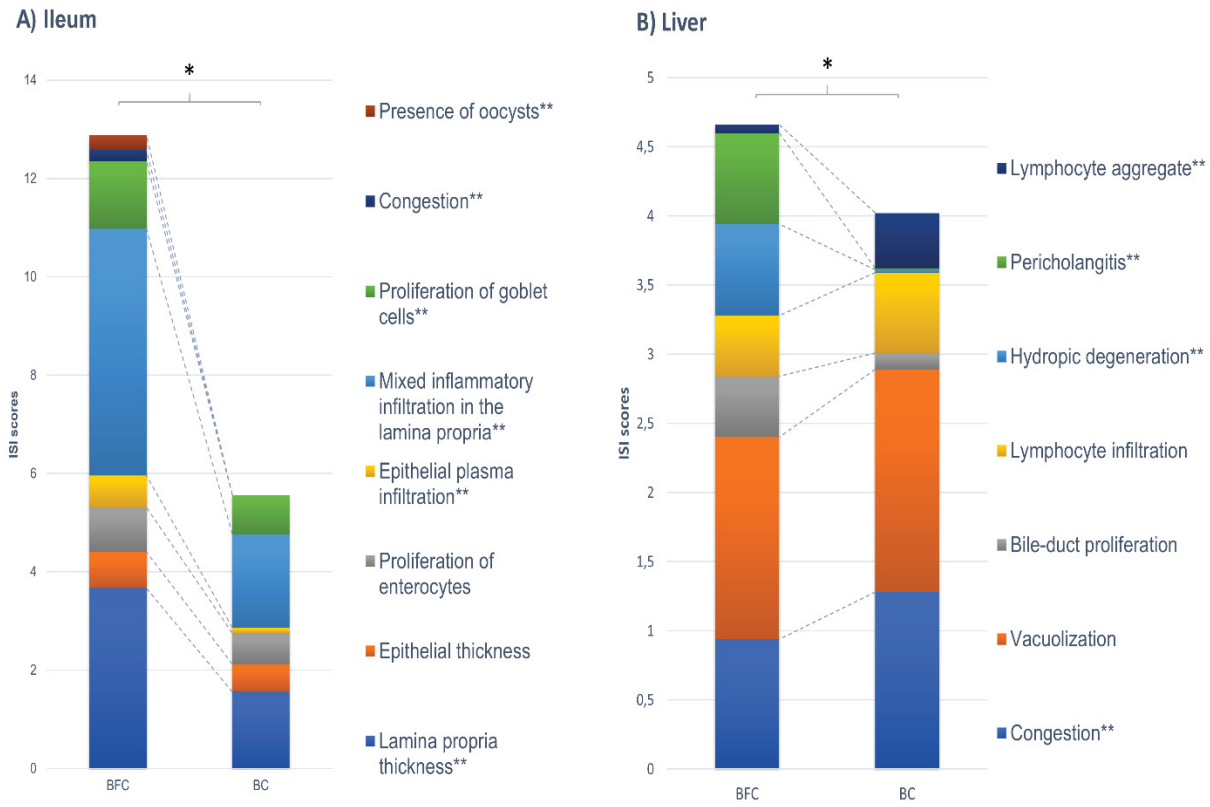
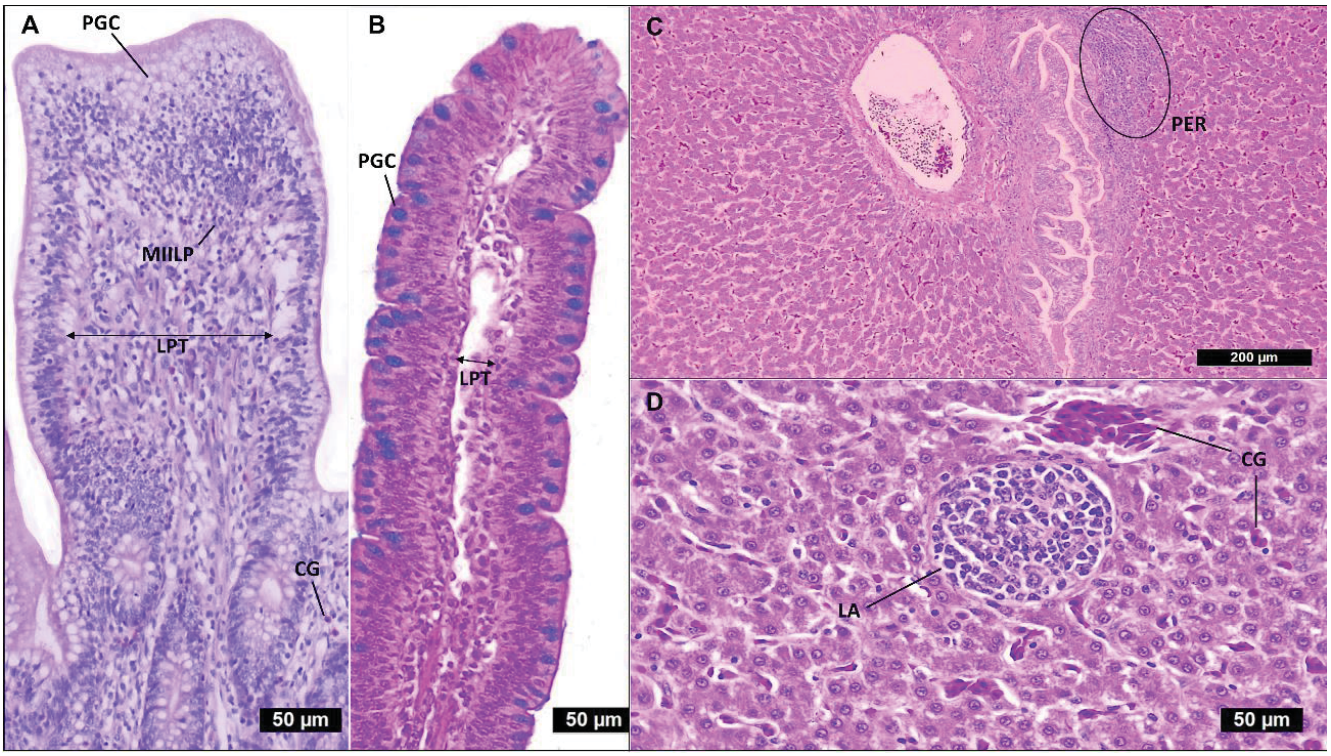


FIGURE 7 – HISTOLOGICAL ALTERATIONS IN ILEUM (A, B) AND LIVER (C, D) ACCORDING TO I SEE INSIDE (ISI) SCORING METHODOLOGY. A) Ileal villus from BFC (Bermuda Feral Chickens), presenting score 1 of congestion (CG), score 2 of proliferation of goblet cells (PGC), score 2 of mixed inflammatory infiltration in the lamina propria (MIILP) and score 2 of lamina propria thickness (LPT), 400X. B) Ileal villus from BC (Broiler Chickens) presenting score 1 of proliferation of goblet cells (PGC), score 0 of mixed inflammatory infiltration in the lamina propria (MIILP) and score 0 of lamina propria thickness (LPT), 400X. C) Liver from BFC (Bermuda Feral Chickens), presenting score 2 of pericholangitis (PER), 100X. D) Liver from BC (Broiler Chickens) presenting score 2 of lymphocyte aggregation (LA) and score 2 of congestion (CG), 400X.



It seems that a more diverse microbiota and its bacterial products might be responsible for TLR activation and upregulation of pro-inflammatory mediators that facilitate host immune responses. Also, the diet variation on feral birds (insects, invertebrates, seeds, shoots, household and business garbage) might be a strong factor of shaping the gut microbiota and provoking an intestinal mucosal inflammation, which may be an evolutionary protective mechanism against food-borne pathogens

particularly derived from diets and the external environment (LEY; HAMADY; LOZUPONE, 2008). However, it was observed that gut inflammation enhances the transmission and growth of pathogenic bacteria such as *Salmonella* through the increase of the growth factor tetrathionate (WINTER *et al*., 2010).

In the liver, we also measured the expression of TLRs (FIGURE 5). Interestingly, the data showed an opposite expression by TLR1, TLR2, TLR3, TLR5, TLR15 and TLR21, characterized by a significant upregulation of BC compared to BFC group ($p<0.05$).

The less ileal microbiota diversity, characterized by downregulation of TLRs and low-grade mucosal inflammation observed in BC group by histology compared feral chickens, may have increased gut permeability and promoted bacterial translocation. Bacterial translocation is defined as the migration of viable bacteria or bacterial products from the intestinal lumen to other extra intestinal organs and sites like the liver (WIEST, GARCIA-TSAO, 2005). Increased translocation of bacteria and bacterial products from the intestine may impair liver homeostasis and enhance liver inflammation through activation of the innate immune system. (CULLENDER *et al*., 2013). In particular, translocated bacterial products augment the activation of hepatic immune cells through pattern recognition receptors including TLRs. In our study, we observed this effect when BC presented more activation of TLRs in the liver when compared to BFC (FIGURE 5). As we observed in the liver histological analysis, BC had a higher prevalence of inflammation (lymphocyte aggregates and congestion, $P<0.05$, FIGURE 6B and 7D) while BFC presented significant alterations such as pericholangitis and hydropic degeneration (inflammation of the tissues surrounding the bile ducts and intracytoplasmic fluid accumulation, respectively) (FIGURE 6B and 7C).

Feralization offers a unique opportunity to observe how natural selection acts on a domestic population returned to natural conditions, and especially how the microbiome responds to the reintroduction of such strong selective forces. The hybridization between wild and domestic birds, as well as the environmental exposure has therefore led to a large increase in microbiome diversity, giving a large degree of variation on which selection can act. On the other hand, we observed that modern commercial broiler chickens demonstrated a considerable reduction in gut microbiota diversity and an interesting systemic innate immune response. The modern poultry industry has witnessed an acquisition of some genotypic and phenotypic characteristics which are more and more distant from their ancestors (use of antibiotics,

genetic selection, nutrition, controlled environments and biosecurity) toward maximum increase in weight gain and growth rate. Selection for high production potential in poultry, however, is not without negative consequences. Whereas the process of natural selection enables an individual to allocate resources according to demands for growth, reproduction, maintenance, and well-being, artificial selection for production potential can disturb genetic homeostasis, leading to deficient resources for the well-being and sustainability of the individual (BEILHARZ; LUXFORD; WILKINSON, 1993). This artificial process for improved broiler performance also has had a negative impact on the immune response (CHEEMA *et al*., 2007). The less diverse microbiota, and the upregulation of TLRs associated with immune histological alteration observed in the liver shows us a state of vulnerability of the immune response in the commercial and modern broiler chicken. All factors discussed in this study make us think if rearing broiler chickens in more natural type of environments might improve animal welfare, genetic selection, reduce infectious diseases without reliance on subtherapeutic antibiotics leading to safer food supply and higher productivity.

CONCLUSIONS

Commercial broilers and feral chickens from Hawaii and Bermuda have a distinct gut microbiome and innate immune response represented by TLRs. Feral chickens showed a significantly more diverse microbiota compared to broiler chickens, mainly in the ceca and less so in the ileum. The core microbiota was composed by 2 ileal taxa and 16 in the cecal samples. Many taxa were found exclusively in the feral birds. Some of them might have been lost during the process of domestication or gained from the environment. Other taxa were identified in the BC group, which may be correlated with higher weight gain and productivity, as reported by literature. The progressive loss of the microbial diversity by the BC group, could be related to a downregulation of TLRs in the ileum leading to gut permeability and bacterial translocation to other peripheral organs. Activation of the innate immune response by TLRs may impair liver homeostasis and enhance liver inflammation. While the roles of different types of microbiota diversity in host health remain to be defined, the exposure to a more natural environment, return to the natural state or bioengineering the modern gut microbiota with diversified species may be necessary to understand the evolution of functionality, leading to better animal welfare, higher productivity and safer products. Further work with better experimental designs will be needed to clarify these connections and explore possible links related to concomitant evolutionary changes in the functional genes of feral and commercial chickens.

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FINAL CONSIDERATIONS

Salmonella continues to be one of the major foodborne pathogens of great concern for public health, for the food industry, and in general all around the world. Understanding the basic growth, mechanisms, pathogenesis, antimicrobial resistance and relation with the host of this pathogen have great importance in order to be able to develop effective, accurate and rapid ways for detection and isolation, and being able to find ways to reduce the *Salmonella.* SH frequently causes food-borne illness in humans and frequently found in broiler operations in several countries, causing economic losses and health-related concerns.

Different studies have shown a considerable genetic variation between closely related strains such as several isolates of the same genotype. With the increasing number of available *Salmonella* genome sequences, when these genomes are compared, the genetic variation within bacterial strains is greater than previously predicted. Several molecular methods are used to differentiate *Salmonella* isolates, being essential for identification of outbreaks, monitoring of trends and to elucidate their evolutionary relationships.

The SH UFPR1 strain was isolated from commercial broiler carcasses in South of Brazil and different feed additives and vaccines were not efficient in previous trials. Whole-genome sequencing followed by comparative genome analysis were used to elucidate the evolutionary relationships of UFPR1 and a multidrug resistant strain. The UFPR1 strain revealed 11 missing genomic fragments which explains the high susceptibility to antibiotics and SCOA resistance.

Due to the ban of antibiotics growth promoters, a number of replacements have been proposed. They are antibacterial vaccines, immunomodulatory agents, bacteriophages, antimicrobial peptides, organic acids, pro-, pre-, and symbiotic, plant extracts, feed enzymes, etc. In order to find an efficient product to control the UFPR1 strain, a probiotic (*Bacillus subtilis*) was successfully tested. It improved performance, reduced *Salmonella* colonization, promoted alterations related to defense response and gut absorption, also changed the microbiota composition.

There is a fast-growing collection of data describing the structure and functional capacity of the chicken microbiome in a variety of conditions available to the research community for consideration and further exploration. Ongoing efforts to further characterize the functions of the microbiome and the mechanisms underlying host-

microbe interactions will provide a better understanding of the role of the microbiome in health/disease and productivity. Studying chickens on a feralization process such as feral birds in Bermuda and Hawaii, offered us insights to better understand the gut microbiome evolution and functionality in order to improve productivity in the modern chicken. Further work with controlled experimental designs will be needed to clarify these connections and explore possible links related to concomitant evolutionary changes in the functional genes of feral and commercial chickens.

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APPENDIX 1

frontiers in Veterinary Science

ORIGINAL RESEARCH
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Phenotypic and Genotypic Features of a *Salmonella* Heidelberg Strain Isolated in Broilers in Brazil and Their Possible Association to Antibiotics and Short-Chain Organic Acids Resistance and Susceptibility

Elizabeth Santin[1], Ricardo Mitsuo Hayashi[1], Jessica Carolino Wammes[1], Ricardo Gonzalez-Esquerra[2], Marcelo Falsarella Carazzolle[3], Caio César do Melo Freire[4], Paulo Sérgio Monzani[5] and Anderson Ferreira da Cunha[4]*

[1] Laboratório de Microbiologia e Ornitopatologia, Universidade Federal do Paraná, Curitiba, Brazil, [2] Novus International, Inc., Indaiatuba, Brazil, [3] Laboratório de Genômica e Expressão, Universidade Estadual de Campinas, Campinas, Brazil, [4] Laboratório de Bioquímica e Genética Aplicada, Departamento de Genética e Evolução, Centro de Ciências Biológicas e da Saúde, Universidade Federal de São Carlos, São Carlos, Brazil, [5] Faculdade de Zootecnia e Engenharia de Alimentos, Departamento de Medicina Veterinária, Universidade de São Paulo, Pirassununga, Brazil

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***Correspondence:**
Elizabeth Santin
Elsantin@hotmail.com

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Salmonella enterica serovar Heidelberg is a human pathogen also found in broilers. A strain (UFPR1) has been associated with field reports of resistance to short-chain organic acids (SCOA) in broilers in the South of Brazil, but was susceptible to a *Bacillus subtilis*-based probiotic added in feed in a related study. This work aimed to (I) report clinical symptoms caused by SH UFPR1 in broilers, (II) study its susceptibility to some antibiotics *in vitro*, and (III) SCOA *in vivo*; and (IV) relate these phenotypic observations with its genome characteristics. Two *in vivo* trials used 1-day-old chicks housed for 21 days in 8 sterilized isolated negative pressure rooms with 4 battery cages of 12 birds each. Birds were challenged or not with 10^7 CFU/bird of SH UFPR1 orally and exposed or not to SCOA in a 2 × 2 factorial design. Zootechnical parameters were unaffected ($P > 0.05$), no clinical signs were observed, and few cecal and hepatic histologic and immune-related alterations were seen, in birds challenged with SH. Formic and propionic acids added together in drinking water, fumaric and benzoic acid in feed (Trial 1), and coated calcium butyrate in feed (Trial 2) did not reduce the SH isolation frequencies seen in cecum and liver in broilers after SH challenge ($P > 0.05$). SH UFPR1 was susceptible to amikacin, amoxicillin + clavulanate, ceftiofur, cephalexin, doxycycline and oxytetracycline; and mildly susceptible to ampicillin + sulbactam, cephalothin, ciprofloxacin, enrofloxacin, and gentamycin in an *in vitro* minimum inhibitory concentration model using Mueller–Hinton agar. The whole genome of SH UFPR1 was sequenced and consisted of a circular chromosome, spanning 4,760,321 bp with 52.18% of GC-content encoding 84 tRNA, 22 rRNA, and 4,427 protein-coding genes. The comparison between SH UFPR1 genome and a multidrug-resistant SL476 strain revealed 11 missing genomic fragments and 5 insertions related to *bgt*, *bgr*, and *rpoS* genes. The deleted genes codify proteins associated with cell cycle regulation, virulence, drug resistance, cellular adhesion, and

APPENDIX 2

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Effect of Feeding *Bacillus subtilis* Spores to Broilers Challenged with *Salmonella enterica* serovar Heidelberg Brazilian Strain UFPR1 on Performance, Immune Response, and Gut Health

*Ricardo Mitsuo Hayashi[1], Mariana Camargo Lourenço[1], Antônio Leonardo Kraieski[1], Raquel Bighetti Araujo[2], Ricardo Gonzalez-Esquerra[2], Eduardo Leonardecz[3], Anderson Ferreira da Cunha[3], Marcelo Falsarella Carazzolle[4], Paulo Sérgio Monzani[5] and Elizabeth Santin[1]**

[1] Laboratório de Microbiologia e Ornitopatologia, Universidade Federal do Paraná, Curitiba, Brazil, [2] Novus International, Inc., Indaiatuba, Brazil, [3] Laboratório de Bioquímica e Genética Aplicada, Departamento de Genética e Evolução, Centro de Ciências Biológicas e da Saúde, Universidade Federal de São Carlos, São Carlos, Brazil, [4] Laboratório de Genômica e Expressão, Universidade de Campinas, Campinas, Brazil, [5] Departamento de Medicina Veterinária, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, Brazil

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Michael Kogut, Agricultural Research Service (USDA), United States

Reviewed by:
Guillermo Tellez, University of Arkansas, United States
Morgan Brian Farnell, Texas A&M University, United States

***Correspondence:**
Elizabeth Santin
bsantin@hotmail.com

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Salmonellosis is a poultry industry and public health concern worldwide. Recently, *Salmonella enterica* serovar Heidelberg (SH) has been reported in broilers in Brazil. The effect of feeding a blend of three strains of *Bacillus subtilis* (PRO) was studied in broilers orally challenged (10^7 CFU/chick) or not with a SH isolated in south of Brazil (UFPR1 strain). Twelve male Cobb 500 broilers per pen were randomly assigned to six treatments in a 3 × 2 factorial experiment where PRO was added at 0, 250, or 500 g/ton of broiler feed and fed to either SH-challenged (SH Control, SH + PRO 250, and SH + PRO 500) or non-challenged birds (Control, PRO 250, and PRO 500). Broiler performance, histologic alterations in intestinal morphology, *Salmonella* quantification and immune cells counts in liver (macrophages, T CD4+ and T CD8+) were analyzed. Changes in the intestinal microbiota of broilers were also studied by metagenomics for Control, SH Control, SH + PRO 250, and SH + PRO 500 only. Feeding PRO at 250 or 500 g/ton reduced SH counts and incidence in liver and cecum at 21 days of age. It was observed that PRO groups increased the macrophage mobilization to the liver in SH-challenged birds ($P < 0.05$) but reduced these cells in the liver of non-challenged birds, showing an interesting immune cell dynamics effect. PRO at 250 g/ton did not affect gut histology, but improved animal performance ($P < 0.05$) while PRO at 500/ton did not affect animal performance but increased histologic alteration related to activation of the defense response in the ileum in SH challenged birds compared to control birds ($P < 0.05$). SH + PRO 500 group presented a more diverse cecal microbiota (Shannon–Wiener index; $P < 0.05$) compared to Control and SH Control groups; while SH + PRO 250 had greater ileal richness (JackkNife index) compared to Control ($P < 0.05$). PRO