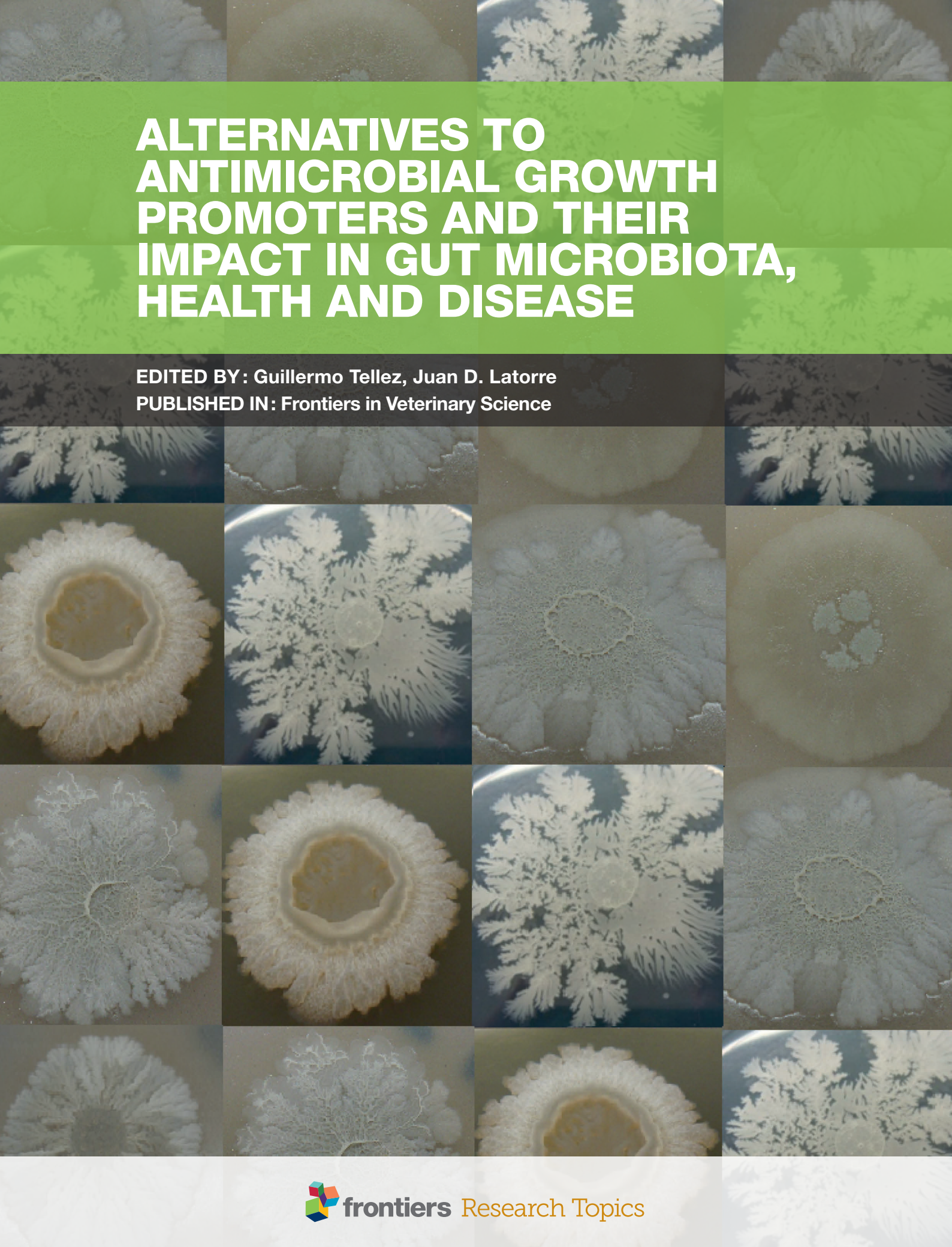

ALTERNATIVES TO ANTIMICROBIAL GROWTH PROMOTERS AND THEIR IMPACT IN GUT MICROBIOTA, HEALTH AND DISEASE

EDITED BY: Guillermo Tellez, Juan D. Latorre

PUBLISHED IN: Frontiers in Veterinary Science

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88945-375-7
DOI 10.3389/978-2-88945-375-7

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ALTERNATIVES TO ANTIMICROBIAL GROWTH PROMOTERS AND THEIR IMPACT IN GUT MICROBIOTA, HEALTH AND DISEASE

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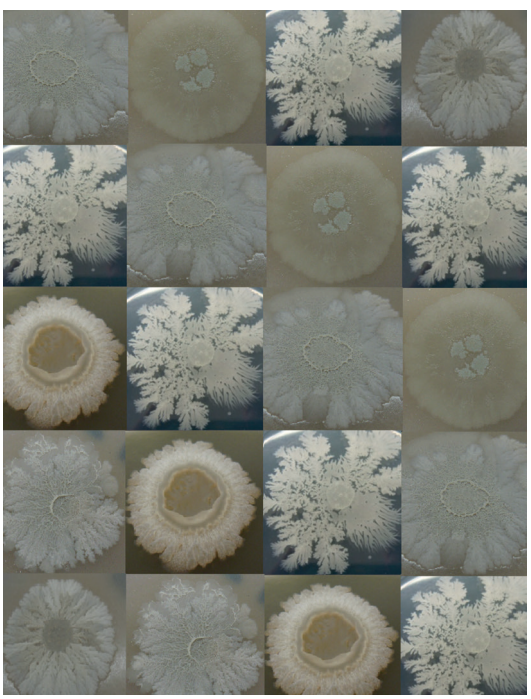

Selected bacterial as direct-fed microbials (DFM) from the genus Bacillus are one of the alternative candidates to replace the use of antimicrobial growth promoters.

Cover image by Juan D. Latorre

In the context of disease pathogenesis, it has been observed that after inadequate administration of antibiotics, animals become more susceptible to intestinal colonization and organ invasion by enteropathogens, these could be related to changes caused in the gastrointestinal microbial community. Therefore, we must reconsider the negative consequences that disruption of the microbiome has in the biology of metazoans (dysbacteriosis). Alternations of the intestinal microbiota composition in animals can be caused by multiple factors, including the misuse of antibiotics, having as a result a negative impact on the development and function of the immune, endocrine, nervous, and digestive systems. For this reason, social concerns regarding the development of antibiotic-resistant microorganisms have resulted in an urgent necessity to find feasible alternatives to maintain animal health and performance without the use of antibiotic growth promoters (AGP), in order to sustain livestock production as an economically viable source of food for human consumption. Hence, research

about AGP alternatives such as probiotics, prebiotics, phytochemicals, organic acids, enzymes, and vaccines has become a priority for many scientists around the world.

Citation: Tellez, G., Latorre, J. D., eds. (2017). Alternatives to Antimicrobial Growth Promoters and Their Impact in Gut Microbiota, Health and Disease. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-375-7

Table of Contents

frontiers in Veterinary Science

EDITORIAL
published: 10 November 2017
doi: 10.3389/fvets.2017.00196

Editorial: Alternatives to Antimicrobial Growth Promoters and Their Impact in Gut Microbiota, Health and Disease

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Keywords: probiotics, prebiotics, phytoadditives, vector vaccine, intestinal health, animal production

Editorial on the Research Topic

Alternatives to Antimicrobial Growth Promoters and Their Impact in Gut Microbiota, Health and Disease

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *10 August 2017*
Accepted: *31 October 2017*
Published: *10 November 2017*

Citation:
Tellez G and Latorre JD (2017) Editorial: Alternatives to Antimicrobial Growth Promoters and Their Impact in Gut Microbiota, Health and Disease. Front. Vet. Sci. 4:196. doi: 10.3389/fvets.2017.00196

It has been estimated that foodborne infections in the USA cause over 76 million illnesses responsible for 5,000 fatalities each year (1). In addition, the annual economic loss attributed to the four most common enteropathogens (*Salmonella* spp., *Campylobacter* spp., *E. coli*, and *Shigella* spp.) has been estimated to reach $7 billion dollars (2). Hence, elimination of these pathogens from animal products has become a priority due to the increased numbers of human foodborne cases and governmental regulations (3). As a result, several methods to control foodborne pathogens have been implemented, including the use of antibiotics. Nevertheless, history has confirmed that the widespread use of even new antibiotics is ultimately followed, by the appearance of resistance to those drugs, creating issues at a global scale. In recent years, substantial scientific evidence has shown that the use of certain antibiotics increases enteric colonization of antibiotic-resistant strains of enteric pathogens not only in humans but also in domestic animals (4, 5). Some of these pathogens have been shown to be extremely resistant to all antibiotics commonly used, or are capable of rapidly develop resistance when exposed to antibiotic prophylaxis or treatment. As a result, an increase in the rate and severity of these infections in food-producing animals as well as in humans has been reported in many countries around the world (6–9). Antibiotics are ineffective in the treatment of multidrug resistant bacteria. Equally frighteningly, is the fact that indiscriminate use of antibiotics can actually induce disruption of the intestinal microbiome (10, 11), reducing the production of short chain fatty acids (12) and increasing luminal pH in the distal gastrointestinal tract (13). Therefore, we must reconsider the negative consequences that disruption of the microbiome has in the biology of metazoans (dysbacteriosis). A common inclination is to classify all bacteria as "harmful" entities. Nothing could be further from the truth. The number of valuable bacterial species far exceeds the number of pathogenic species and are, in reality, essential for life. After millions of years of evolution, prokaryotes established diverse interactions with eukaryotes (14) and then life on earth change. These cooperative interactions between kingdoms (mutualism) have a fundamental role in the generation and conservation of life (15, 16). One example is the gut microbiome, estimated to contain 500–1,000 different bacterial species and clearly outnumbering the total number of genes and cells of the host by an estimated of 10-fold (17). Collectively, the intestinal microbiome represents a "forgotten organ," responsible for orchestrating major physiological tasks. Contrast with control animals, gnotobiotic animals have numerous host functions affected by the lack of intestinal microbiome, therefore affecting their immune, endocrine, nervous, and digestive systems (18–22). In simple words, both animal

and plant life depend on the mutualism relationships with their related cousins, prokaryotes. And yet, the fragile composition of the microbiome is influenced by many factors such as mode of delivery, age, dietary nutrient composition, infections, inflammation, stress, and of course, medication (23, 24). It is, therefore, not surprising to see that as a result of the indiscriminate use and abuse of antibiotics, the incidence of some foodborne pathogens such as *Salmonella* and *Campylobacter* are increasing worldwide, with reports of antibiotic resistance in clinical isolates of these and other enteric pathogens (25–27). Consequently, the World Health Organization (WHO) published a list of antibiotics that should be reserved for human use only (28). Interestingly, soon after the publication of the WHO report, and with growing consumer and scientific pressures, the European Union went one step further, creating new legislations banning the use of all antibiotics as growth promoters as of January 2006 (29–31). However, in some countries, the indiscriminate use and misuse of antibiotics are still a sad reality, particularly where there is no legislation regulating the use of antibiotic in animal agriculture. Particularly in those countries, is remarkable to confirm the alarming incidence of certain enteric pathogens associated with the indiscriminate use of some antibiotics by food-producing companies (10, 32–34). Antibiotics should be limited to infections of specific bacteria with known antibiotic sensitivity.

Over a century ago, Metchnikoff (35) proposed the revolutionary idea to consume viable bacteria to promote health by modulating the intestinal microflora. The idea is more applicable now than ever since bacterial antimicrobial resistance has become a serious worldwide problem both in medical and agricultural fields. It looks like finally, we humans have learned that this is a lost war against bacterial pathogens, especially, if we keep abusing of antibiotics. Bacteria are equipped with the biological mechanisms to evolve and find mechanisms of resistance against any chemical. Hence, antibiotic alternatives such as probiotics, prebiotics, phytochemicals, enzymes, organic acids, and vaccines to improve disease resistance in highly intense/stress food animal production systems have become a priority for many scientists around the world (36, 37). Evidently, there is no such thing as a silver bullet. Rather, the combination of several of these nutraceuticals, accompanied with good husbandry and management practices, oriented to improve biosecurity programs are becoming the new strategies incorporated in many companies. In this research topic, we present 10 original research articles and 1 general commentary article included in 5 different chapters, evaluating multiple alternatives to antibiotic growth promoters to be used in animal production.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 31 August 2016
doi: 10.3389/fvets.2016.00072

Risks Involved in the Use of Enrofloxacin for *Salmonella* Enteritidis or *Salmonella* Heidelberg in Commercial Poultry

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OPEN ACCESS

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 06 June 2016
Accepted: 19 August 2016
Published: 31 August 2016

Citation:
Morales-Barrera E, Calhoun N, Lobato-Tapia JL, Lucca V, Prado-Rebolledo O, Hernandez-Velasco X, Merino-Guzman R, Petrone-García VM, Latorre JD, Mahaffey BD, Teague KD, Graham LE, Wolfenden AD, Baxter MFA, Hargis BM and Tellez G (2016) Risks Involved in the Use of Enrofloxacin for Salmonella Enteritidis or Salmonella Heidelberg in Commercial Poultry. Front. Vet. Sci. 3:72. doi: 10.3389/fvets.2016.00072

The objectives of the present study were to evaluate the risks involved in the use of Enrofloxacin for *Salmonella* Enteritidis (SE) or *Salmonella* Heidelberg (SH) in commercial poultry and determine the effects of a probiotic as an antibiotic alternative. Two experiments were conducted to evaluate the risks involved in the use of Enrofloxacin for SE or SH in commercial poultry. Experiment 1 consisted of two trials. In each trial, chickens were assigned to one of three groups; control + SE challenged; Enrofloxacin 25 mg/kg + SE; and Enrofloxacin 50 mg/kg + SE. Chickens received Enrofloxacin in the drinking water from days 1 to 5 of age. On day 6, all groups received fresh water without any treatment. All chickens were orally gavaged with 10^7 cfu/chick of SE at 7 days of age and euthanized on 8 days of age. In Experiment 2, turkey poults were assigned to one of the three groups; control + SH; probiotic + SH; and Enrofloxacin 50 mg/kg + SH. Poults received probiotic or Enrofloxacin in the drinking water from days 1 to 5 of age. On day 6, poults received fresh water without any treatment. Poults were orally gavaged with 10^7 cfu/poult of SH at 7 days of age. Poults were weighed and humanely killed 24 h post-SH challenge to evaluate serum concentration of fluorescein isothiocyanate-dextran to evaluate intestinal permeability, metagenomics, and SH infection. In both trials of Experiment 1, chickens treated with Enrofloxacin were more susceptible to SE organ invasion and intestinal colonization when compared with control non-treated chickens ($P < 0.05$). In Experiment 2, poults treated with 50 mg/kg of Enrofloxacin showed an increase in body weight, however, this group also showed an increase in SH susceptibility, intestinal permeability, and lower proportion of Firmicutes and Bacteroidetes, but with control group had the highest proportion of Proteobacteria. By contrast, poults that received the probiotic had the highest proportion of Firmicutes and Bacteroidetes, but lowest Proteobacteria. The results of the present study suggest

that prophylactic utilization of Enrofloxacin at five times the recommended dose in poultry increases the susceptibility to salmonellae infections, and confirms that probiotics may be an effective tool in salmonellae infections.

Keywords: Enrofloxacin, *Salmonella*, poultry, susceptibility, metagenomics

INTRODUCTION

Fluoroquinolones are the third generation of quinolone development. Nalidixic acid and pipemidic acid are examples of the first generation and currently have limited activity against Gram-negative bacteria. Fluorinated 4-quinolones were introduced to the market in the 1980s and were the top of the line antibiotics, offering a broad spectrum of activity and high efficacy in a wide range of infections both orally and parenterally (1, 2). Nevertheless, history has demonstrated that the extensive use of new antibiotics is eventually shadowed by the appearance of resistance to those chemicals that have become a major global problem. This was demonstrated by the higher incidence of salmonellae and *Campylobacter* infections worldwide, and several reports of fluoroquinolone resistance in clinical isolates for these and other enteric pathogens (3–7). Hence, the World Health Organization (WHO) published a list of antibiotics that should be reserved for human use only (8), and fluoroquinolones were among them, due to the alarming evidence of quinolone-resistant zoonotic pathogens. Soon after the publication of the WHO report, several countries banned the use of fluoroquinolones in animal production (9–11). With growing consumer and scientific pressures, the European Union went one step further, creating new legislations banning the use of all antibiotics as growth promoters as of January 2006 (12). However, in many countries, the indiscriminate use and misuse of antibiotics, including fluoroquinolones, are still a sad reality. Especially in countries where there is no legislation regulating the use of fluoroquinolones in animal agriculture and where there is an abundance of generic fluoroquinolones at a low cost. Typical management practices in those countries are to treat or dose healthy neonatal chickens and turkey poults with five times the recommended dose of Enrofloxacin for five consecutive days in the drinking water. Interestingly, in those countries, the incidence of *Salmonella* spp. and *Campylobacter* spp. rates in both humans and agriculture are also high (1, 13–16). Therefore, the objectives of the present study were to evaluate and confirm the risks involved in the use of Enrofloxacin for *Salmonella* enterica serovars Enteritidis or Heidelberg in commercial poultry and to determine if poultry selected probiotics have a prophylactic effect when birds are challenged with SE and SH.

MATERIALS AND METHODS

Enrofloxacin

Baytril® (Bayer Health Care LLC, Mission, KS 66201, USA) Enrofloxacin 3.23% concentrate solution for use in chickens and turkeys drinking water only.

Probiotic Culture

FloraMax®-B11 (Pacific Vet Group USA Inc., Fayetteville, AR 72703, USA) is a defined probiotic culture derived from gastrointestinal poultry origin that contains proprietary strains of lactic acid bacteria (LAB), selected by their *in vitro* ability to inhibit enteropathogens (17).

Animal Source

Day-of-hatch, male broiler chickens were obtained from Cobb-Vantress (Siloam Springs, AR, USA) for Experiment 1 or male turkey poults from a local hatchery in Experiment 2 and were randomly housed in heated brooder batteries in a controlled age-appropriate environment. For each experiment, birds were provided *ad libitum* access to water, and unmedicated corn–soybean diet, meeting the nutritional requirements of poultry recommended by National Research Council (18), respectively. All animal handling procedures were in compliance with Institutional Animal Care and Use Committee at the University of Arkansas. In each experiment, a small number of chicks or poults ($n = 10$) were humanely euthanized upon arrival by CO_2 asphyxiation. Ceca-cecal tonsils (CCT), liver, and spleen were aseptically cultured in tetrathionate enrichment broth (Catalog no. 210420, Becton Dickinson, Sparks, MD, USA). Enriched samples were confirmed negative for *Salmonella* by streak plating the samples on Xylose Lysine Tergitol-4 (XLT-4, Catalog no. 223410, BD Difco™) selective media.

Bacterial Strains and Culture Conditions

The challenge organism used in Experiment 1 was a poultry isolate of *Salmonella enterica* serovar Enteritidis (SE), bacteriophage type 13A, obtained from the USDA National Veterinary Services Laboratory, Ames, IA, USA. In Experiment 2, a primary poultry isolate of *Salmonella enterica* serovar Heidelberg (SH) isolated in our laboratory was used. Antimicrobial susceptibility test revealed that both isolates were sensitive to Enrofloxacin. Furthermore, SE and SH are resistant to 25 μg/mL of novobiocin (NO, catalog no.N-1628, Sigma) and were selected for resistance to 20 μg/mL of nalidixic acid (NA, catalog no.N-4382, Sigma) in our laboratory. For both experiments, 100 μL of SE or SH from a frozen aliquot was added to 10 mL of tryptic soy broth (Catalog no. 22092, Sigma) and incubated at 37°C for 8 h, and passed three times every 8 h to ensure that all bacteria were in log phase. Post-incubation, bacterial cells were washed three times with sterile 0.9% saline by centrifugation at 1,864 × *g* for 10 min, reconstituted in saline, quantified by densitometry with a spectrophotometer (Spectronic 20D+, Spectronic Instruments Thermo Scientific), and diluted to an approximate concentration of 10^8 cfu/milliliter. Concentrations of SE or SH were further verified by serial dilution and plating on brilliant green agar (BGA,

Catalog no. 70134, Sigma) with NO and NA for enumeration of actual cfu used to challenge the chickens and turkeys.

Experimental Design in Chickens and Turkeys

Evaluation of Enrofloxacin in Neonatal Chickens Challenged with *Salmonella* Enteritidis. Experiment 1

Two independent trials were conducted. In each trial, 36 chickens were randomly assigned to one of three groups ($n = 12$): control SE challenged without Enrofloxacin; Enrofloxacin 25 mg/kg SE challenged; and Enrofloxacin 50 mg/kg SE challenged. Chickens received Enrofloxacin from days 1 to 5 of age in the drinking water. At day 6, treated groups received fresh water without any treatment. Fresh water without antibiotic was administered to control chickens throughout the experiment. All chickens were orally gavaged with 10^7 cfu/chick of SE at 7 days of age. Chickens were humanely euthanized for culture at 8 days of age as describe below.

Salmonella Recovery

At 8 days, broilers were humanely euthanized and liver and spleen were collected aseptically and enriched in 10 mL of tetrathionate broth (Becton Dickinson) overnight at 37°C. Following enrichment, each sample was streaked for isolation on BGA plates containing 25 μg/mL of NO and 20 μg/mL of NA. The plates were incubated at 37°C for 24 h and examined for the presence or absence of antibiotic-resistant SE. CCT were collected aseptically, homogenized within sterile sample bags (Nasco, Fort Atkinson, WI, USA) using a rubber mallet and diluted with saline (1:4 by wt/vol) and 10-fold dilutions were plated on BGA with NO and NA, incubated at 37°C for 24 h to enumerate total SE colony forming units. The CCT samples were enriched in 2× concentrated tetrathionate enrichment broth and further incubated at 37°C for 24 h to enrich. Following this, enrichment samples were plated on BGA with NO and NA and incubated at 37°C for 24 h to confirm presence/absence of typical lactose-negative colonies of *Salmonella*.

Evaluation of Prophylactic Administration of FloraMax-B11® Enrofloxacin in Neonatal Turkey Poults Challenged with *Salmonella* Heidelberg. Experiment 2

In Experiment 2, 72 day-of-hatch turkey poults were neck tagged, weighed, and randomly assigned to one of the three groups ($n = 24$/group): control SH challenged without treatment; probiotic SH challenged; and Enrofloxacin 50 mg/kg SH challenged. Poults received FloraMax-B11® or Enrofloxacin from days 1 to 5 of age in the drinking water. Control group received fresh water without any treatment throughout the duration of the experiment. At day 6, treated groups received water without any treatment. All poults were orally gavaged with 10^7 cfu/poult of SH at 7 days of age. Poults were weighed and humanely euthanized 24 h post-SH challenge (day 8 of age) to evaluate serum concentration of fluorescein isothiocyanate-dextran (FITC-D) and cecal bacterial community compositions as describe below, as well as *Salmonella* recovery and plating from CCT as was previously described. Samples from CCT were also plated in Man Rogosa Sharpe (Difco™ Lactobacilli MRS Agar VWR Cat. No. 90004-084 Suwanee, GA 30024) to evaluate total number of LAB.

Serum Determination of FITC-D Leakage

Intestinal leakage of FITC-D (MW 3–5 KDa; Sigma-Aldrich Co., St. Louis, MO, USA) and the measurement of its serum concentration were done in experiment 2 as a marker of paracellular transport and mucosal barrier dysfunction (19–22). At 24 h, post-SH challenge (day 8 of age), poults in all groups were given an oral gavage dose of FITC-D (4.16 mg/kg). Following 2.5 h, they were killed by CO_2 asphyxiation. Blood samples were collected from the femoral vein kept at room temperature for 3 h and centrifuged ($500 \times g$ for 15 min) to separate the serum from the red blood cells. FITC-D levels of diluted serum samples (1:5 PBS) were measured at excitation wavelength of 485 nm and an emission wavelength of 528 nm with a Synergy HT, Multi-mode microplate fluorescence reader (BioTek Instruments, Inc., Vermont, USA). Fluorescence measured was then compared to a standard curve with known FITC-D concentrations. Gut leakage for each bird was reported as microgram of FITC-D/mL of serum (20).

DNA Extraction and Illumina-Based Analysis of Microbial Community Diversity

Cecal content from six poults was obtained, homogenized thoroughly in four volumes diluent (0.85% NaCl, 0.1% peptone), centrifuged at $300 \times g$ for 2 min to remove large debris, and finally, 0.5 mL of aliquots (average 8 mg dry weight) were pelleted at $10{,}000 \times g$ for 5 min. Extraction of DNA was performed immediately using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany). Bacterial community compositions at Phylum and Class level were performed using Illumina dye sequencing (Era7 Bioinformatics Inc., Cambridge, MA 02142, USA). The analysis corresponded to 16S rRNA amplicons from V6 region sequenced with Illumina technology (23). Reads were assigned to a taxon based on sequence similarity to 16S rRNA genes extracted from the NCBI nt database. The 16S rRNA sequences were extracted from NCBI based on their presence in the set of sequences included in the Ribosomal Database Project (RDP) (24) and on the specificity of their taxonomical assignment based on the lowest common ancestor (LCA) approach adopted metagenomics analysis as the last version of Meta-Genome Analyzer (MEGAN). The algorithm was similar to the assignment algorithm adopted by MEGAN tool (25). Phylum distribution in all the samples is expressed in % on the total merged reads of each sample.

Data and Statistical Analysis

Log_{10} cfu/g of SE and SH in cecal contents, body weight (BW), body weight gain (BWG), serum FITC-D concentration, and proportion of bacterial composition were subjected to analysis of variance as a completely randomized design, using the General Linear Models procedure of SAS (26). Significant differences among the means were determined by Duncan's multiple-range test at $P < 0.05$. Enrichment data were expressed as positive/total chickens (%), and the percent recovery of SE and SH was compared using the chi-squared test of independence, testing all possible combinations to determine the significance ($P \leq 0.05$) for these studies (27).

RESULTS

The results from experiment 1, evaluating the effect of Enrofloxacin on neonatal chickens challenged with SE 24 h after antibiotic treatment on organ invasion and cecal colonization, are summarized in **Table 1**. In trial 1, there was a significant ($P < 0.05$) increase in the incidence of SE in liver and spleen in chickens treated with either 25 or 50 mg/kg of Enrofloxacin when compared with control chickens. Furthermore, chickens treated with 50 mg/kg of Enrofloxacin showed a 3.23 log increased in the incidence of SE in CCT as well as total cfu of SE/gram of ceca content when compared with control chickens and 0.45 log increase when compared with chickens treated with 25 mg/kg of Enrofloxacin. Similar results were observed in trial 2, where chickens treated with both doses of Enrofloxacin showed an increase in SE incidence in CCT as well as total numbers of SE in the cecal content when compared with control non-treated chickens (**Table 1**).

The results from experiment 2 evaluating the prophylactic administration of FloraMax-B11® or Enrofloxacin on organ invasion and cecal colonization of SH in neonatal turkey poults are summarized in **Table 2**. No significant differences were observed in the SH organ invasion between treated or control groups ($P > 0.05$), nevertheless, poults treated with the probiotic showed a significant reduction in both incidence of SH in CCT and total numbers of SH in ceca content when compared with poults treated with 50 mg/kg of Enrofloxacin or control non-treated poults ($P < 0.05$). Enrofloxacin poults also had a significant reduction in the total numbers of LAB (**Table 2**).

The results of the evaluation of prophylactic administration of FloraMax-B11® or Enrofloxacin on BW, BWG, and serum concentration of FITC-D in neonatal turkey poults in Experiment 2 are summarized in **Table 3**. Poults treated with 50 mg/kg of Enrofloxacin showed a significant increase in BW and BWG when compared with probiotic or control non-treated poults. Interestingly, poults in this group also showed a significant increase in gut permeability (**Table 3**).

Table 4 shows the results of the Phylum distribution (cumulative% LCA) and class direct assignment in % for all ceca samples of turkey poults following prophylactic administration of FloraMax-B11® or Enrofloxacin in Experiment 2. At the phylum level microbiome analysis, poults treated with the probiotic had the higher proportion of Firmicutes, followed by control poults and poults treated with Enrofloxacin. A significant reduction was observed in Bacteroidetes in poults treated with

TABLE 1 | Evaluation of Enrofloxacin in neonatal chickens challenged with *Salmonella* Enteritidis (SE)[a] 24 h after antibiotic treatment on organ invasion and cecal colonization.

	Liver and spleen[b]	Log $_{10}$ SE g/CCT[c]	Cecal tonsils[b]
Trial 1			
Control + SE	0/12 (0%)[e]	1.23 ± 0.45[e]	5/12 (41.7%)[e]
Enrofloxacin 25 mg/kg + SE	4/12 (33.3%)[d]	2.01 ± 0.66[e]	6/12 (50%)[e]
Enrofloxacin 50 mg/kg + SE	5/12 (41.7%)[d]	4.46 ± 0.37[d]	12/12 (100%)[d]
Trial 2			
Control + SE	0/12 (0%)[e]	1.23 ± 0.45[e]	5/12 (41.7%)[e]
Enrofloxacin 25 mg/kg + SE	4/12 (33.3%)[d]	2.01 ± 0.66[e]	6/12 (50%)[e]
Enrofloxacin 50 mg/kg + SE	5/12 (41.7%)[d]	4.46 ± 0.37[d]	12/12 (100%)[d]

Experiment 1.

[a]Chickens received Enrofloxacin from days 1 to 5 of age in the drinking water. At day 6, all groups received fresh water without any treatment. All chickens were orally gavaged with 10^7 cfu/chick of SE at 7 days of age. Chickens were humanely killed for culture at 8 days of age.

[b]Data of liver and spleen or ceca-cecal tonsils is expressed as positive/total chickens (%).

[c]Log $_{10}$ SE/g of ceca-ceca tonsils (CCT) data is expressed as mean ± SD.

[d,e]Superscripts within columns indicate significant difference at $P < 0.05$.

TABLE 2 | Evaluation of prophylactic administration of FloraMax-B11® or Enrofloxacin on organ invasion and cecal colonization of *Salmonella* Heidelberg (SH)[a] in neonatal turkey poults.

	Liver and spleen[b]	Cecal tonsils[b]	Log_{10} SH/g of CCT[c]	Log_{10} Lactic acid bacteria/g of CCT[c]
Control SH	2/24 (8.33%)[d]	5/24 (20.83%)[d]	0.66 ± 0.29[d]	6.67 ± 0.26[d]
FloraMax-B11® + SH	0/24 (0%)[d]	0/24 (0%)[e]	0.0 ± 0.0[e]	7.16 ± 0.24[d]
Enrofloxacin 50 mg/kg + SH	0/24 (0%)[d]	8/24 (33.33%)[d]	1.95 ± 0.28[d]	4.06 ± 0.22[e]

Experiment 2.

[a]Poults received FloraMax-B11® or Enrofloxacin from days 1 to 5 of age in the drinking water. At day 6, all groups received fresh water without any treatment. All poults were orally gavaged with 10^7 cfu/poult of SH at 7 days of age. Poults were humanely killed for culture at 8 days of age.

[b]Data of liver and spleen as well as cecal tonsils is expressed as positive/total poults (%).

[c]Log $_{10}$g of ceca-cecal tonsil (CCT) data is expressed as mean ± SD, n = 12.

[d,e]Superscripts within columns indicate significant difference at $P < 0.05$.

TABLE 3 | Evaluation of prophylactic administration of FloraMax-B11® or Enrofloxacin on body weight, body weight gain, and serum concentration of FITC-D[a] in neonatal turkey poults.

	Body weight (grams)	Body weight gain (grams)	Serum FITC-D (μg/mL)
Control SH	105.59 ± 2.31[c]	51.14 ± 2.45[c]	1.24 ± 0.08[c]
FloraMax-B11® + SH	106.54 ± 2.24[c]	52.15 ± 2.39[c]	0.23 ± 0.06[c]
Enrofloxacin 50 mg/kg + SH	120.57 ± 2.60[b]	63.87 ± 2.71[b]	7.28 ± 3.09[b]

Experiment 2.

[a]Poults received FloraMax-B11® or Enrofloxacin from days 1 to 5 of age in the drinking water. At day 6, all groups received fresh water without any treatment. All poults were orally gavaged with 10^7 cfu/poult of SH at 7 days of age. FITC-D was administered on 8 days of age.

[b,c]Superscripts within columns indicate significant difference at $P < 0.05$, n = 24.

TABLE 4 | Phylum distribution (cumulative% lowest common ancestor) and class direct assignment in % for all ceca samples of turkey poults following prophylactic administration of FloraMax-B11® or Enrofloxacin.

	Control + SH	FloraMax-B11® + SH	Enrofloxacin 50 mg/kg + SH
Phylum			
Firmicutes	42 ± 10[b]	55 ± 8[a]	9 ± 4[c]
Bacteroidetes	19 ± 6[a]	23 ± 4[a]	10 ± 2[b]
Proteobacteria	29 ± 4[a]	18 ± 5[b]	31 ± 3[a]
Class			
Gammaproteobacteria	15.07 ± 2.58[a]	6.16 ± 0.083[b]	24.95 ± 2.76[a]
Clostridia	5.01 ± 2.22[a]	4.25 ± 1.30[a]	2.40 ± 0.04[b]
Bacilli	3.05 ± 0.01[a]	4.21 ± 0.01[a]	1.11 ± 0.06[b]

Experiment 2.

[a,b]*Superscripts within rows indicate significant difference at $P < 0.05$, $n = 6$.*

the antibiotic. Furthermore, significant increases in the proportion of Proteobacteria were observed in poults that received Enrofloxacin or control poults when compared with poults that received FloraMax-B11®. At the class level, it was interesting to observe that both control and Enrofloxacin poults had an increase in Gammaproteobacteria, but Clostridia and Bacilli were decreased in Enrofloxacin birds when compared with control or poults treated with the probiotic (**Table 4**).

DISCUSSION

Considerable scientific evidence has shown that the use of certain antibiotics increases enteric colonization of antibiotic-resistant strains of enteric pathogens in domestic animals (28–32). Because some of these pathogens are extremely resistant to many antibiotics and are capable of rapidly developing resistance when exposed (7, 13, 14), antibiotic prophylaxis or treatment has been reported to actually increase the occurrence and severity of these infections in commercial poultry (33, 34). In addition, the lack of effect of these antibiotics in resistant enteropathogens, some researchers have shown that antibiotics can actually cause disruption in the microbiome (35), accompanied with reduction of short chain fatty acids (36, 37) and increased luminal pH in the distal gastrointestinal tract (38). In the present study, we evaluate the management practice in certain countries of using five times the recommended dose of Enrofloxacin in neonatal chickens and turkey poults for five consecutive days after placement, and look at their susceptibility to salmonellae infections 24 h after treatment. In trial 1 of Experiment 1, chickens treated with either 25 or 50 mg/kg of Enrofloxacin were more susceptible to SE organ invasion when compared with control non-treated chickens. In addition, chickens treated with 50 mg/kg of Enrofloxacin in trial 1 and both Enrofloxacin doses in trial 2 had a significant increase in total SE cfu in cecae when compared with control chickens, suggesting that this management practice performed in poor antimicrobial stewardship countries, increased susceptibility to SE infections in broiler chickens.

Salmonella Heidelberg is among the top three *Salmonella* serovars isolated from humans when poultry products were linked to the infection (39–42). Furthermore, SH resistant to various antimicrobial agents has been isolated from domestic animals (43–45). In Experiment 2, our results are in agreement with previous publications from our laboratory, showing not only the low invasiveness of SH for internal organs, but also effectiveness of FloraMax-B11® in reducing SH intestinal colonization in turkey poults (46). Published studies have also shown that FloraMax®-B11 increased colonization resistance to *Salmonella* spp. infections (47–51), reduces idiopathic diarrhea in commercial turkey brooding houses (52), as well as increased performance and reduced costs in poultry production (53, 54). In the present study, it was remarkable to observe that poults treated with 50 mg/kg of Enrofloxacin were more susceptible to SH colonization and that this effect was associated with a significant reduction in the total number of LAB. Poults treated with 50 mg/kg of Enrofloxacin showed a significant increase in BW and BWG, however, this group also showed a significant increase in gut permeability. Metagenomic analysis of cecal content using the MEGAN software can be used to interactively analyze and compare metagenomic and metatranscriptomic data, thereby providing a percent identity filter that can be used to enforce the following levels of percentage sequence identities for an assignment at a given taxonomic level (25). In Experiment 2, poults treated with Enrofloxacin had a lower proportion of Firmicutes and Bacteroidetes, suggesting that the broad spectrum of Enrofloxacin had a profound impact upon the microbiome. Interestingly, these poults had the highest proportion of Proteobacteria (similar to control). Such a high dose of antibiotic also had a significant increase in Gammaproteobacteria. Changes in the proportion of phylum and class were associated with higher SH intestinal colonization since *Salmonella* belongs to phylum Proteobacteria, class Gammaproteobacteria. Furthermore, poults treated with Enrofloxacin had lower proportions of Clostridia and Bacilli when compared with control or probiotic poults. Antibiotics administered in low doses have been widely used as growth promoters in poultry for over half a century. However, the exact mechanisms for this effect are elusive. Similarly, there are no reports that have described the impact of Enrofloxacin at low or high therapeutic dose on the microbiome or metabolomics in poultry. This is the first report that describes profound changes in microbiome of turkey poults that received a high dose of Enrofloxacin, shifting it and making them more susceptible to a SH experimental challenge.

By contrast, poults that received the probiotic had the highest proportion of Firmicutes and Bacteroidetes, but the lowest amount of Proteobacteria. These birds also showed significant reduction in Gammaproteobacteria, but similar to the control group, a higher proportion in Clostridia and Bacilli. The shift in these bacterial populations had a positive effect on reducing SH colonization following challenge and confirms our previous research (46).

The results of these experiments suggest that, five times the recommended dose of Enrofloxacin, a broad-spectrum antibiotic can have a negative effect on the microbiome that may be responsible for an enhancement of SH colonization, which has been previously demonstrated with other enteropathogens (4, 28, 29, 31, 32). The mechanism of antibiotic-altered resistance was not investigated in the present study. However, regardless

of the mechanism involved, increased susceptibility of turkey poults to *Salmonella* was observed in two experiments following Enrofloxacin treatment. Furthermore, based on the microbiota changes following fluoroquinolone administration, including the increase in Proteobacteria, these results suggest that this practice may predispose to other infectious diseases that will further require the use of additional antibiotics and broaden the selection of antimicrobial resistance. Acquisition of resistance to fluoroquinolones has been reported to be a multifaceted process, which includes spontaneous point mutations that result in amino acid substitutions within the topoisomerase subunits GyrA, GyrB, ParC, or ParE, reduced expression of outer membrane porins, overexpression of multidrug efflux pumps, and/or plasmid-mediated quinolone resistance (1, 2, 7, 13, 15, 55, 56). It is remarkable to contemplate that the alarming incidence of certain enteric pathogens is associated with the indiscriminate use of some antibiotics in animal agriculture in some countries (42, 45, 57–61). Since poultry products have been identified as important reservoirs of human infections, this is a growing public health concern. Given that fluoroquinolones and other antibiotics are over used in animal production, any effort to diminish the risk of resistance is crucial. The results of the present study and of previous investigations involving antibiotics and other enteropathogens suggest that prophylactic utilization of some antibiotics in poultry increase the susceptibility to salmonellae colonization and organ invasion. Therefore, antibiotics should be limited to infections of specific bacteria with known antibiotic sensitivity. In addition, our findings also confirm previous studies suggesting that the use of alternatives, such as probiotics, can be an effective tool in controlling salmonellae infections.

AUTHOR CONTRIBUTIONS

EM-B, NC, JL-T, and VL: conception and design, acquisition of data, and drafting of manuscript. XH-V, JL, BH, GT: drafting the article and revising it critically for important intellectual content. OP-R, RM-G, VP-G, AW, MB, BM, KT, and LG: acquisition of data. BH and GT: analysis and interpretation of data, drafting of manuscript, and approval of the version to be submitted and any revised version.

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

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 20 October 2016
doi: 10.3389/fvets.2016.00095

Evaluation and Selection of *Bacillus* Species Based on Enzyme Production, Antimicrobial Activity, and Biofilm Synthesis as Direct-Fed Microbial Candidates for Poultry

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OPEN ACCESS

Edited by:
Carl James Yeoman, Montana State University, USA

Reviewed by:
Christi Swaggerty, United States Department of Agriculture, USA
Kenneth M. Bischoff, Agricultural Research Service, USA

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *26 August 2016*
Accepted: *05 October 2016*
Published: *20 October 2016*

Citation:
Latorre JD, Hernandez-Velasco X, Wolfenden RE, Vicente JL, Wolfenden AD, Menconi A, Bielke LR, Hargis BM and Tellez G (2016) Evaluation and Selection of Bacillus Species Based on Enzyme Production, Antimicrobial Activity, and Biofilm Synthesis as Direct-Fed Microbial Candidates for Poultry. Front. Vet. Sci. 3:95. doi: 10.3389/fvets.2016.00095

Social concern about misuse of antibiotics as growth promoters (AGP) and generation of multidrug-resistant bacteria have restricted the dietary inclusion of antibiotics in livestock feed in several countries. Direct-fed microbials (DFM) are one of the multiple alternatives commonly evaluated as substitutes of AGP. Sporeformer bacteria from the genus *Bacillus* have been extensively investigated because of their extraordinary properties to form highly resistant endospores, produce antimicrobial compounds, and synthesize different exogenous enzymes. The purpose of the present study was to evaluate and select *Bacillus* spp. from environmental and poultry sources as DFM candidates, considering their enzyme production profile, biofilm synthesis capacity, and pathogen-inhibition activity. Thirty-one *Bacillus* isolates were screened for *in vitro* relative enzyme activity of amylase, protease, lipase, and phytase using a selective media for each enzyme, with 3/31 strains selected as superior enzyme producers. These three isolates were identified as *Bacillus subtilis* (1/3), and *Bacillus amyloliquefaciens* (2/3), based on biochemical tests and 16S rRNA sequence analysis. For evaluation of biofilm synthesis, the generation of an adherent crystal violet-stained ring was determined in polypropylene tubes, resulting in 11/31 strains showing a strong biofilm formation. Moreover, all *Bacillus* strains were evaluated for growth inhibition activity against *Salmonella enterica* serovar Enteritidis (26/31), *Escherichia coli* (28/31), and *Clostridioides difficile* (29/31). Additionally, in previous *in vitro* and *in vivo* studies, these selected *Bacillus* strains have shown to be resistant to different biochemical conditions of the gastrointestinal tract of poultry. Results of the present study suggest that the selection and consumption of *Bacillus*-DFM, producing a variable set of enzymes and antimicrobial compounds, may contribute to enhanced performance through improving nutrient digestibility, reducing intestinal viscosity, maintaining a beneficial gut microbiota, and promoting healthy intestinal integrity in poultry.

Keywords: *Bacillus*, direct-fed microbial, enzyme, antimicrobial, biofilm

INTRODUCTION

The continuous tendency to reduce the use of antibiotic growth promoters (AGP) in poultry production, due to social concern about generation of antibiotic-resistant bacteria, has resulted in the crucial necessity to find economically viable alternatives that can maintain optimal health and performance parameters under commercial conditions (1, 2). One possible substitute for AGP that has been extensively studied is the utilization of probiotics to prevent and treat gastrointestinal infections (3). The most common microorganisms used as probiotics are lactic acid bacteria (LAB) from the genus *Lactobacillus* and *Pediococcus*; however, these microorganisms required refrigeration or lyophilization to survive for long storage periods, and microencapsulation to withstand feed application, therefore adding cost to their industrial production (4). Among the microorganisms used as direct-fed microbials (DFM), *Bacillus* spores have been increasingly included as feed additives in poultry diets, due to their remarkable resistance to harsh environmental conditions, and also have a long shelf life (5, 6). Bacteria from the genus *Bacillus* are Gram-positive, rod shaped, and usual inhabitants of the soil. However, different studies have shown that *Bacillus* spores can also be present, germinate, and survive in the gastrointestinal tract (GIT) of different animal species, suggesting that these bacteria could be considered facultative anaerobes and part of the metabolically active host microbiota (7–10). Rate of survival and persistence of some *Bacillus* strains in the GIT may be related to their capacity to synthesize biofilms, thereby, protecting themselves against the harsh environmental conditions present in the gut (11). Moreover, one of the principal sources of enzymes and antibiotics from bacterial origin used by biotechnology companies are produced by different *Bacillus* strains, making this multifunctional microorganism useful inside or outside a host (12, 13).

On the other hand, the increasing consumption of poultry meat globally, along with utilization of grains such as corn for biofuel production, has led to the use of less digestible energy sources in poultry diets. Alternative cereals, such as wheat, barley, triticale, or rye, have been previously included in poultry feed (14–16). However, the incorporation of these raw materials in monogastric diets have a negative impact on growth performance due to an elevated concentration of antinutritional factors, such as the non-starch polysaccharides (NSP), in comparison to corn-based diets (17). Diets rich in NSP generate an increase in intestinal viscosity, affecting digestibility and absorption of nutrients by the intestinal surface (18). An alternative to reduce the negative effects generated by NSP is the inclusion of microbial enzymes, such as xylanase, which have been shown to reduce intestinal viscosity and *Clostridium*-associated enteritis (19). Additionally, utilization of other microbial enzymes, such as α-amylase, protease, lipase, and phytase, have demonstrated to increase degradation of low-quality proteins, improve bone quality, and enhance absorption of carbohydrates and fatty acids (20–22). In this regard, the exogenous enzymes produced by *Bacillus* spp. that may help to degrade complex antinutritional factors in poultry diets and improve nutrient absorption include cellulase (23), α-amylase (24), β-glucanase (25), α-galactosidase, β-mannanase (26), xylanase (27), protease (28), lipase (29), keratinase (30), and phytase (31). Nonetheless, it is important to mention that not all *Bacillus* bacteria synthesize the same type of enzymes, therefore require selection and characterization of adequate isolates according to the specific target substrates in the diet.

Besides the capacity of certain *Bacillus* spp. to produce enzymes and increase utilization of nutrients from different feedstuffs, spores from various *Bacillus* strains have also been included in poultry diets to control the incidence of different gastrointestinal diseases through the production of antimicrobial compounds or acting as competitive exclusion agents against *Salmonella* Typhimurium (32), *Clostridium perfringens* (33), *Escherichia coli* (34), and *Campylobacter jejuni* (35). Additionally, *Bacillus*-DFM have shown to enhance cellular and humoral immune responses by increasing the number of solitary lymphoid follicles in the intestinal mucosa, influencing the development of the gut-associated lymphoid tissue (GALT), enhancing antibody responses after vaccination, and augmenting macrophage function (36–38). Dietary supplementation with *Bacillus* spores may also have a positive effect on other beneficial bacteria populations, such as LAB, through production of subtilisin and catalase, as well as reducing pH and oxygen concentration in the gut to generate a more favorable environment (39, 40). In the case of intestinal epithelial integrity, it has been shown *in vitro* (Caco2 cells) and *ex vivo* that a *Bacillus subtilis* quorum-sensing signal molecule known as the competence and sporulation-stimulating factor (CSF), induces expression of the heat-shock protein, Hsp27, therefore enhancing protection of enterocytes against oxidative damage and preventing detrimental effects on the intestinal barrier (41). At the end, all the characteristics mentioned before support the utilization of selected *Bacillus* spp. spores as a feasible alternative to AGP, improving performance parameters through production of enzymes and maintaining an optimal health status by synthesis of antimicrobial compounds. Therefore, the purpose of the present study was to evaluate and select *Bacillus* isolates from environmental and poultry sources as candidate DFM based upon enzyme production profiles, pathogen-inhibition capacity, and biofilm synthesis, therefore, extending our understanding of the mechanism of action of *Bacillus*-DFM and its applicability in the poultry industry.

MATERIALS AND METHODS

Bacillus spp. Isolation

Previous research conducted in our laboratory focused on isolation of several *Bacillus* spp. from environmental and poultry sources as described by Wolfenden et al. (42). Briefly, samples from intestinal content, fecal material, and environmental sources were collected using sterile cotton swabs and placed into sterile borosilicate tubes for transport. All samples were pasteurized by heat treatment at 70°C for 15 min to eliminate the presence of vegetative cells and allow the isolation of spore-formers only. Swabs were then plate struck on tryptic soy agar (TSA, Becton Dickinson, Sparks, MD, USA) to be able to collect individual

colonies after 24 h incubation at 37°C. Additionally, all the strains used in the present study were previously selected as negative for alpha and beta hemolysis after being inoculated on TSA plates containing 50 mL/L of defibrinated sheep blood (Remel, Lenexa, KS, USA).

In Vitro Determination of Enzyme Activity

Thirty-one *Bacillus* spp. isolates obtained from the Poultry Health Laboratory at the University of Arkansas were screened for production of α-amylase, protease, lipase, and phytase. All *Bacillus* strains were grown in tryptic soy broth (TSB, Becton Dickinson, Sparks, MD, USA) at 37°C for 24 h. Then the isolates were washed with a saline solution (0.9%) and centrifuged three times at 1864 × *g* for 15 min to prepare a clean inoculum. Then, 10-fold dilutions of the inoculum from each strain were plated on TSA, followed by 24 h of incubation at 37°C, to determine the cfu/mL used for assessment of enzyme activity. During the screening process, 10 µl with 10^8 cfu/mL of each *Bacillus* strain were placed on the center of each selective media according to the enzyme under evaluation. After incubation, all plates were evaluated and the diameters of the zones of clearance were measured removing the diameter of the bacterial colony. The relative enzyme activity (REA) was determined by using the formula: REA = diameter of zone of clearance divided by the diameter of the bacterial colony in millimeters. Based on REA test organisms were categorized into excellent (REA > 5.0), good (REA > 2.0–5.0), or poor (REA < 2.0) (43). Each *Bacillus* strain was evaluated by triplicate, and values are presented in **Table 1**. More details about the composition of the selective media and incubation periods used to evaluate the capacity to produce each enzyme are described below.

Production of Amylase

To determine α-amylase enzyme activity, a starch agar media was used and consisted of 10 g of tryptone, 3 g of soluble starch, 5 g of KH_2PO_4, 10 g of yeast extract, 15 g of noble agar, and 1000 mL of distilled water. The starch media was autoclaved at 121°C for 15 min and poured in Petri dishes when the temperature reaches 50°C. Then each tested *Bacillus* strain was inoculated and incubated at 37°C for 48 h. For visualization of the zone of clearance, all Petri dishes were flooded with 5 mL of Gram's iodine solution (24).

Production of Protease

For evaluation of protease activity, a skim milk agar media was prepared containing 25 g of skim milk, 25 g of noble agar, and 1000 mL of distilled water. The mixture was stirred thoroughly and autoclaved at 121°C for 15 min. For plating, the skim milk agar solution was held in a water bath at 50°C, and then it was poured quickly into plates. Each *Bacillus* strain was inoculated on Petri dishes and incubated at 37°C for 24 h to observe if a zone of clearance was developed (44).

Production of Lipase

Lipase activity was assessed using the Spirit blue agar media (Difco Laboratories, Detroit, MI, USA) composed by 10 g of pancreatic digest of casein, 5 g of yeast extract, 20 g of noble agar, and 0.15 g of the die spirit blue. A total of 35 g spirit blue agar were used per 1000 mL of distilled water. The media was sterilized at 121°C for 15 min and cooled to 50°C in a water bath, before being mixed with 30 mL of a lipoidal solution prepared with 100 mL of olive oil, 1 mL of polysorbate 80, and 400 mL of warm water (60°C). Plates were inoculated and incubated at 37°C for 24 h, before the determination of a zone of clearance around each bacterial colony (45).

Production of Phytase

For determination of phytase activity *Bacillus* isolates were screened in a medium that contained: 10 g dextrose, 0.3 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4$, 0.1 g $CaCl_2$, 0.01 g $MnSO_4$, 0.01 g $FeSO_4$, 5 g Na-phytate, and 20 g of noble agar per 1000 mL of distilled water. The phytate media was autoclaved at 121°C for 15 min and poured into Petri dishes when the temperature reached 50°C. Isolates were inoculated and incubated at 37°C for a maximum of 120 h to evaluate if a zone of clearance was generated surrounding the tested bacterial strains (46, 47).

TABLE 1 | Relative enzyme activity (REA)[a] values produced by *Bacillus* spp. strains evaluated as enzyme producer candidates.

Bacillus isolates[b]	Amylase	Protease	Lipase	Phytase
AM0902	1.0 ± 0.00	1.0 ± 0.00	1.9 ± 0.15	1.0 ± 0.00
AM0904	5.3 ± 0.19	2.7 ± 0.08	2.3 ± 0.06	1.2 ± 0.07
AM0905	5.8 ± 0.44*	3.0 ± 0.26	2.7 ± 0.17	1.6 ± 0.24
AM0908	5.3 ± 0.06	2.1 ± 0.08	2.3 ± 0.07	1.4 ± 0.10
AM0923	5.7 ± 0.19	2.8 ± 0.04	2.2 ± 0.26	1.5 ± 0.02
AM0933	5.3 ± 0.21	2.3 ± 0.09	2.1 ± 0.07	1.3 ± 0.07
AM0934	4.5 ± 0.18	3.1 ± 0.34	2.4 ± 0.35	1.2 ± 0.08
AM0938	5.0 ± 0.50	3.4 ± 0.30*	2.7 ± 0.17	2.1 ± 0.08
AM0939	3.9 ± 0.12	2.9 ± 0.44	2.2 ± 0.12	1.4 ± 0.13
AM0940	5.9 ± 0.27	1.8 ± 0.19	2.4 ± 0.21	1.4 ± 0.12
AM0941	1.0 ± 0.00	1.7 ± 0.40	2.8 ± 0.27	2.0 ± 0.12
AM1002	6.3 ± 0.12*	2.8 ± 0.15	3.0 ± 0.35*	2.1 ± 0.11
AM1010	5.7 ± 0.16	2.1 ± 0.11	2.6 ± 0.21	1.5 ± 0.12
AM1011	4.4 ± 0.30	3.0 ± 0.13	2.5 ± 0.29	1.3 ± 0.10
AM1012	6.1 ± 0.18*	2.5 ± 0.15	2.3 ± 0.17	1.4 ± 0.02
AM1013	4.1 ± 0.08	2.3 ± 0.09	2.0 ± 0.09	1.3 ± 0.05
AM1109A	2.7 ± 0.27	1.8 ± 0.10	2.2 ± 0.11	1.4 ± 0.11
AM1109B	1.8 ± 0.42	1.0 ± 0.00	2.4 ± 0.21	1.4 ± 0.07
B2/53	4.0 ± 0.64	2.7 ± 0.16	2.5 ± 0.08	1.6 ± 0.05
BL	2.2 ± 0.13	1.0 ± 0.00	1.0 ± 0.00	1.0 ± 0.00
JD17	4.0 ± 0.29	2.9 ± 0.20	2.6 ± 0.11	2.3 ± 0.15*
JD19	3.4 ± 0.33	2.1 ± 0.17	2.2 ± 0.12	1.5 ± 0.01
NP001	4.3 ± 0.19	2.3 ± 0.14	1.9 ± 0.11	1.1 ± 0.04
NP002	3.0 ± 0.40	2.3 ± 0.29	2.1 ± 0.11	1.2 ± 0.12
NP117B	2.7 ± 0.48	3.0 ± 0.06	2.1 ± 0.14	1.3 ± 0.12
NP121	3.1 ± 0.46	2.2 ± 0.13	2.0 ± 0.09	1.5 ± 0.14
NP122	4.7 ± 0.36	2.8 ± 0.40	2.3 ± 0.15	1.3 ± 0.12
NP124	1.6 ± 0.40	2.1 ± 0.29	2.2 ± 0.12	1.1 ± 0.00
NP126	3.3 ± 0.23	2.5 ± 0.15	2.2 ± 0.12	1.2 ± 0.07
MM65	3.8 ± 0.31	1.0 ± 0.00	3.0 ± 0.22	2.5 ± 0.06*
RW41	4.2 ± 0.88	1.3 ± 0.11	2.0 ± 0.04	1.2 ± 0.04

**Identified bacterial strains as superior enzyme producers with a higher REA value, $P < 0.05$.*

[a]REA was calculated dividing the diameter of area of clearance by the diameter of the Bacillus colony. Organism were classified as excellent (REA > 0.5), good (REA > 2.0–5.0), or poor (REA < 2.0) enzyme producers. Data expressed as mean ± SE.

[b]All Bacillus spp. isolates were tested by triplicate.

In Vitro Assessment of Antimicrobial Activity against *Salmonella enterica* serovar Enteritidis and *Escherichia coli*

Thirty-one *Bacillus* spp. strains were screened by triplicate for *in vitro* antimicrobial activity against *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis), bacteriophage type 13A, obtained from the USDA National Veterinary Services Laboratory (Ames, IA, USA), and a wild-type poultry field strain *E. coli*, as reported previously by Wolfenden et al. (42). Briefly, 10 µl with 10^8 cfu/mL of each *Bacillus* isolate were placed on the center of TSA plates and incubated for 24 h at 37°C. Then, the Petri dishes with visible *Bacillus* colonies were overlaid with a TSA soft agar containing either 10^6 cfu/mL of *S.* Enteritidis or *E. coli*. After aerobic incubation for 24 h at 37°C, all plates were observed and the diameters of the zones of inhibition were measured removing the diameter of the bacterial colony.

In Vitro Assessment of Antimicrobial Activity against *Clostridioides difficile*

All tested *Bacillus* spp. isolates were cultured aerobically overnight on TSA plates and screened for *in vitro* antimicrobial activity against *Clostridioides difficile* (*C. difficile*) ATCC 9689D, formerly known as *Clostridium difficile* (48). Briefly, 10 µl with 10^8 cfu/mL of each *Bacillus* strain were placed in the center of TSA plates. After 24 h of incubation at 37°C, the plated samples were overlaid with TSA containing sodium thioglycolate (0.25 g/L) and 10^6 cfu/mL of *C. difficile*. Then, all plates were incubated anaerobically using a BD GasPak EZ container system (Becton Dickinson, Sparks, MD, USA). After 24 h of incubation at 37°C, plates were evaluated for the presence of zones of inhibition, and the diameter of the inhibition zone was measured as mentioned above for *S.* Enteritidis and *E. coli* antimicrobial activity evaluation.

Biofilm Assay

To determine biofilm synthesis a previously published crystal violet staining method was used with slight modifications (49). Briefly, *Bacillus* isolates were grown in TSB overnight at 37°C, and 10 µl of each strain were inoculated in 0.5 mL of Casein-Mannitol (CM) broth in 1.5 mL polypropylene tubes. The CM broth contained per liter: 10 g casein digest (Sigma-Aldrich Co., St. Louis, MO, USA) and 10 g D-mannitol. After 12 h of incubation of the CM broth at 37°C without shaking, the liquid supernatant was removed and the tubes were gently rinsed with distilled water. Then, 1 mL of a 1% w/v crystal violet solution was added to the tubes to stain the cells adhered to the walls forming a ring. After 25 min, the crystal violet solution was removed, and the tubes were washed with distilled water. The qualitative measurement of biofilm synthesis was based on color intensity and size of the adherent crystal violet ring with a score ranging from negative (−) to strong (++) biofilm formation described by Fall et al. (50). Additionally, all samples were scored by the same person to minimize variability and maintain results consistency.

Identification of *Bacillus*-DFM Candidates

Bacillus spp. strains laboratory identified as AM1002, AM0938, and JD17 were selected as superior enzyme producers based on their enzyme production profile. These candidates were identified and characterized based on biochemical evaluation tests using a bioMerieux API 50 CHB test kit (bioMerieux, Marcy l'Etoile, FRA). Selected candidates were also subjected to 16S rRNA sequence analysis in a specialized laboratory using Sherlock® DNA microbial analysis software and database (Midi labs, Newark, DE, USA). Briefly, the 16S rRNA gene was PCR amplified from genomic DNA isolated from pure bacterial colonies. Primers used are universal 16S primers that correspond to positions 0005F and 0531R for a 500 bp sequence and 0005F and 1513R for the 1500 bp sequence. Amplification products were purified from excess primers and dNTPs and checked for quality and quantity by running a portion of the products on an agarose gel. Cycle sequencing of the 16S rRNA amplification products was carried out using DNA polymerase and dye terminator chemistry. Excess dye-labeled terminators were then removed from the sequencing reactions. The samples were electrophoresed on either a 3130 or 3130xl Genetic Analyzer.

Statistical Analysis

Data from all measurements were subjected to one-way analysis of variance as a completely randomized design using the General Linear Models procedure of SAS (version 9.1, SAS Institute Inc., Cary, NC, USA) (51). Means were separated with Duncan's multiple-range test and considered significant at $P < 0.05$. Data were reported as mean ± SE.

RESULTS

Determination of *In Vitro* Enzyme Activity

Bacillus spores were isolated by heat treatment of intestinal, fecal, and environmental samples, eliminating the presence of vegetative cells. Although enzyme activity was detected for the majority of the strains, there were considerable differences in their REA values. Three of the 31 screened *Bacillus* spp. strains showed a significantly higher REA value for amylase production in comparison to other bacterial colonies. Isolates AM1002, AM1012, and AM0905 obtained REA values of 6.3, 6.1, and 5.8, respectively, all of them categorizing these *Bacillus* isolates as excellent amylase producers (REA > 5.0). In the case of protease activity, strain AM0938 showed a REA value of 3.4 which is considered good (REA > 2.0–5.0), surpassing the enzyme activity values of all other screened strains. Lipase synthesis was significantly superior in the isolate AM1002 (REA = 3.0), meanwhile, phytase production was classified as good for the strains JD17 (REA = 2.3) and MM65 (REA = 2.5) in comparison to the other screened *Bacillus* spp. isolates. A complete description of the enzyme activity profile of all the evaluated isolates and the appearance of each selective media are presented in **Table 1** and **Figure 1**, respectively.

In Vitro Evaluation of Antimicrobial Activity

An overlay method was used to assess the production of antimicrobial compounds by the 31 *Bacillus* strains against Gram-positive and Gram-negative enteropathogens (**Table 2**; **Figure 2**). Although antimicrobial activity was observed in a greater number

of isolates, individual differences were evident in the degree of inhibition and spectrum of activity. In the case of *S.* Enteritidis, isolate NP122 generated the largest diameter of the zone of inhibition with 13.7 mm, followed by the strain AM0904 with a inhibition diameter of 12.0 mm. Activity against *E. coli* was more evident in isolates AM1010 and AM1012, both with a diameter of clearance of 20 mm. Interestingly, *C. difficile* was the most susceptible microorganism in the presence of almost all *Bacillus* spp. strains, with an average zone of inhibition of 19 mm for the 31 isolates, where the strain AM1010 produced larger pathogen-inhibition activity with a diameter of clearance of 28 mm.

Biofilm Synthesis

Biofilm production was evaluated by generation of an adherent crystal violet-stained ring in polypropylene tubes. All the screened *Bacillus* spp. strains produced biofilms; however, isolates AM0905, AM0933, AM0940, AM0941, AM1002, AM1011, AM1012, AM1109A, AM1109B, NP122, and MM65

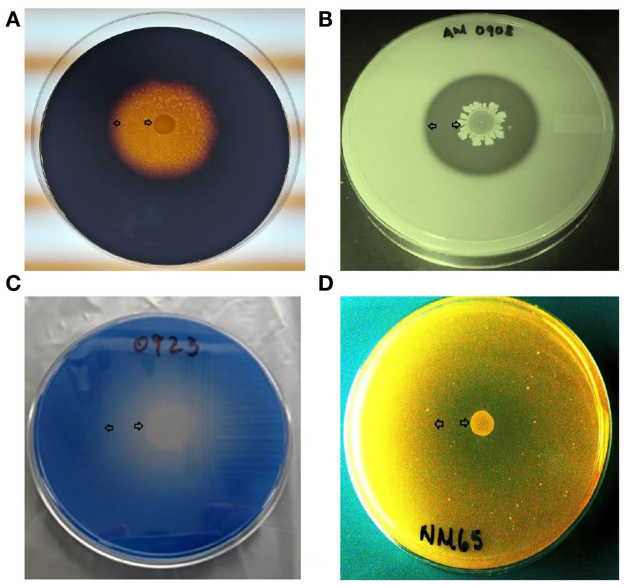

FIGURE 1 | Representative examples of microbial enzyme activity using a different selective media for each enzyme under evaluation. An area of clearance around a bacterial colony can be observed, representing enzyme production of **(A)** amylase, **(B)** protease, **(C)** lipase, and **(D)** phytase. All *Bacillus* spp. strains were screened by triplicate. Arrows indicate the bacterial colony and the outer limit of the zone of clearance.

TABLE 2 | Evaluation of antimicrobial activity[a] and biofilm synthesis[b] of different *Bacillus* spp. isolates.

Bacillus isolates	*S.* Enteritidis (mm)	*E. coli* (mm)	*C. difficile* (mm)	Biofilm formation
AM0902	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	+
AM0904	12.0 ± 0.38*	16.0 ± 2.31	26.0 ± 1.86	+
AM0905	6.7 ± 0.67	14.0 ± 1.15	20.3 ± 1.67	++
AM0908	6.0 ± 0.56	4.3 ± 0.33	22.0 ± 2.31	+
AM0923	7.7 ± 0.30	10.0 ± 3.06	24.0 ± 3.06	+
AM0933	1.3 ± 0.33	4.0 ± 0.58	10.0 ± 1.15	++
AM0934	6.3 ± 0.40	8.7 ± 1.76	22.7 ± 2.40	+
AM0938	8.0 ± 1.15	10.0 ± 2.00	22.0 ± 2.00	+
AM0939	6.3 ± 0.88	8.3 ± 1.33	26.0 ± 2.60	+
AM0940	8.0 ± 1.12	10.3 ± 1.67	21.0 ± 1.76	++
AM0941	0.7 ± 0.27	0.0 ± 0.00	0.0 ± 0.00	++
AM1002	5.7 ± 0.58	8.7 ± 1.76	16.0 ± 2.08	++
AM1010	8.0 ± 1.10	20.0 ± 1.45*	28.0 ± 2.67*	+
AM1011	8.5 ± 0.90	10.7 ± 1.76	20.3 ± 2.33	++
AM1012	8.7 ± 0.88	20.0 ± 2.19*	10.0 ± 1.75	++
AM1013	4.0 ± 1.15	10.0 ± 1.15	22.0 ± 1.15	+
AM1109A	10.3 ± 1.20	12.0 ± 1.50	24.0 ± 1.11	++
AM1109B	0.3 ± 0.33	0.0 ± 0.00	14.7 ± 1.62	++
B2/53	10.3 ± 1.20	12.0 ± 0.58	26.0 ± 3.08	+
BL	0.0 ± 0.00	4.0 ± 0.52	10.0 ± 2.00	+
JD17	6.3 ± 0.33	10.0 ± 1.15	20.6 ± 3.53	+
JD19	2.0 ± 0.58	2.7 ± 0.67	19.0 ± 1.72	+
NP001	8.0 ± 0.88	6.0 ± 0.58	12.0 ± 1.13	+
NP002	4.3 ± 1.33	6.0 ± 1.10	20.7 ± 2.40	+
NP117B	2.7 ± 0.67	6.0 ± 1.15	18.0 ± 3.46	+
NP121	2.3 ± 0.33	14.0 ± 3.06	16.0 ± 2.31	+
NP122	13.7 ± 1.86*	12.0 ± 2.00	26.0 ± 4.16	++
NP124	6.0 ± 1.73	12.0 ± 1.86	22.0 ± 2.03	+
NP126	0.3 ± 0.30	2.0 ± 1.89	21.7 ± 1.76	+
MM65	8.0 ± 0.55	10.0 ± 1.15	20.3 ± 1.45	++
RW41	5.7 ± 0.88	10.0 ± 2.00	22.0 ± 2.28	+

**Identified bacterial strains with the enhanced antimicrobial activity, $P < 0.05$.*
[a]Represents the diameter of the zone of inhibition observed at 24 h of incubation without the diameter of the bacterial colony. Data expressed as mean ± SE.
[b]The qualitative measurement of biofilm synthesis was based on color intensity and size of the adherent crystal violet ring with a score ranging from negative (–) to strong (++) biofilm formation. All Bacillus spp. isolates were tested by triplicate.

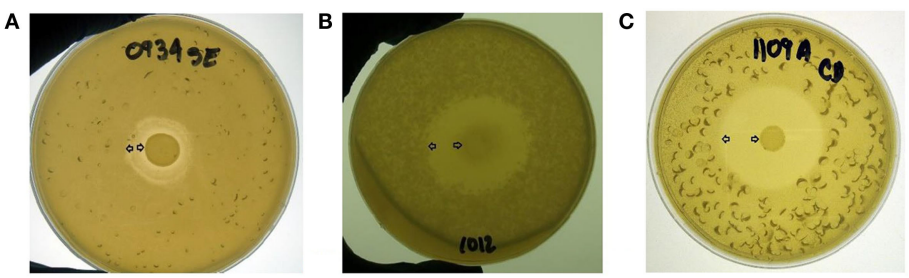

FIGURE 2 | Evaluation of antimicrobial activity from different *Bacillus* spp. isolates using an overlay method. A zone of inhibition is shown surrounding a tested bacterial colony located in the middle of the plate against **(A)** *S.* Enteritidis, **(B)** *E. coli*, and **(C)** *C. difficile*. All *Bacillus* spp. strains were screened by triplicate. Arrows indicate the bacterial colony and the outer limit of the zone of inhibition.

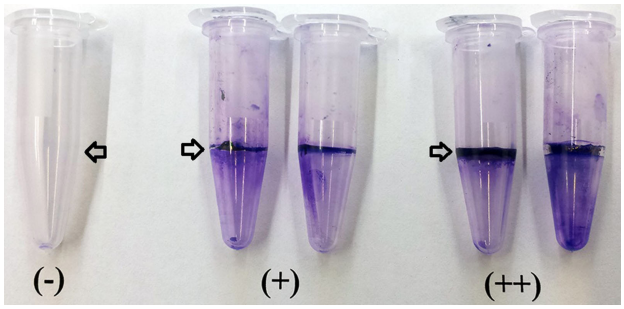

FIGURE 3 | Determination of biofilm synthesis was performed using a crystal violet staining method. Measurement of biofilm synthesis was based on color intensity and size of the adherent crystal violet ring with a score ranging from negative (–) to strong (++) biofilm formation. All *Bacillus* spp. strains were screened by triplicate. Arrows indicate the presence or absence of the biofilm ring.

were identified as strong biofilm formers with a wider and more colorful intense ring of adherence present on the wall of the test tubes (**Table 2**; **Figure 3**).

Characterization and Selection of *Bacillus*-DFM Candidates

Based on the REA results, three *Bacillus*-DFM candidates were selected with excellent to good REA values for each of the evaluated enzymes. These candidates were then identified and characterized using a bioMerieux API 50 CHB test kit (bioMerieux, Marcy l'Etoile, France). This set of biochemical tests classified *Bacillus* spp. strains based on their capacity to metabolize 49 different carbohydrates (**Table 3**). According to the fermentation profile, all isolates were categorized as *B. subtilis/Bacillus amyloliquefaciens* with an identification percentage of 99.0% or higher. To further assist in identification of the strains, each isolate was also subjected to 16S rRNA sequence analysis in a specialized laboratory (Midi labs, Newark, DE, USA). 16S rRNA sequence analysis identified isolate AM1002, as *B. subtilis* (GenBank Match: 100%, accession number AB201120); AM0938 as *B. amyloliquefaciens* (GenBank Match: 100%, accession number GU191912); and JD17 as *B. amyloliquefaciens* (GenBank Match: 100%, accession number GU191912). These three isolates have been deposited at the Agricultural Research Service Culture Collection (NRRL Peoria, IL, USA) by Pacific Vet Group USA, Inc., with the NRRL numbers: AM1002/B-67143; AM0938/B-67144; and JD17/B-67142.

TABLE 3 | Characterization and identification of selected *Bacillus*-DFM candidate strains based on biochemical carbohydrate metabolism tests.[a,b]

Item	AM1002	AM0938	JD17
Amidon (starch)	+	+	+
Amygdalin	+	+	+
Arbutin	+	+	+
D-Adonitol	–	–	–
D-Arabinose	–	–	–
D-Arabitol	–	–	–
D-Cellobiose	+	+	+
D-Fructose	+	+	+
D-Fucose	–	–	–
D-Galactose	–	–	–
D-Glucose	+	+	+
D-Lactose (bovine origin)	+	+	+
D-Lyxose	–	–	–
D-Maltose	+	+	+
D-Mannitol	+	+	+
D-Mannose	+	+	+
D-Melezitose	–	–	–
D-Melibiose	+	–	+
D-Raffinose	+	+	+
D-Ribose	+	+	+
D-Saccharose (sucrose)	+	+	+
D-Sorbitol	+	+	–
D-Tagatose	–	–	–
D-Trehalose	+	+	+
D-Turanose	–	–	–
Dulcitol	–	–	–
D-Xylose	+	+	+
Erythritol	–	–	–
Esculin (ferric citrate)	+	+	+
Gentibiose	+	+	–
Glycerol	+	+	+
Glycogen	+	+	+
Inositol	+	+	+
Inulin	+	–	–
L-Arabinose	+	+	+
L-Arabitol	–	–	–
L-Fucose	–	–	–
L-Rhamnose	–	–	–
L-Sorbose	–	–	–
L-Xylose	–	–	–
Methyl-αD-glucopyranoside	+	+	+
Methyl-αD-mannopyranoside	–	–	–
Methyl-βD-xylopyranoside	–	–	–
N-Acetylglucosamine	–	–	–
Potassium 2-ketogluconate	–	–	–
Potassium 5-ketogluconate	–	–	–
Potassium gluconate	–	–	–
Salicin	+	+	+
Xylitol	–	–	–

[a]*BioMerieux API50 CHB test kit (bioMerieux, Marcy l'Etoile, France).*
[b]*Different scores (+ or –) reflect the capacity of the tested Bacillus spp. isolate to ferment an specific carbohydrate or carbohydrate derivative.*

DISCUSSION

Nowadays, poultry diets include a variety of ingredients from different plant and animal sources. Due to an increasing demand of cereal grains for production of biofuels, rising corn prices have had a direct impact on diet costs (52). Consequently, the necessity to reduce costs of production has required the inclusion of less digestible and more available raw materials in poultry diets. Distillers' dried grains with solubles (DDGS) are usually available to be included in the ingredient matrix, as a result of the continuous development of the ethanol industry (53). However, the main concern with the inclusion of high percentages of DDGS in poultry diets is related to its variable nutritional content and nutrient digestibility. Moreover, it has been observed that high levels of DDGS in the diet could act as a predisposing factor for presentation of necrotic enteritis (54). On the other hand, alternative grains, such as wheat, barley, rye, and sorghum, conform

a different group of unconventional feed ingredients that have increased their participation in poultry diets as energy sources; nevertheless, it is important to mention that these feedstuffs often contain a higher concentration of antinutritional factors, such as NSP, in comparison to corn (55). An elevated concentration of arabinoxylans or β-glucans in the intestinal content has been related to reduced nutrient absorption and increased intestinal viscosity and microbial growth (56). Therefore, as an alternative to improve nutrient utilization and increase flexibility of the ingredient matrix used in poultry diets, multiple researchers have been evaluating the inclusion of different exogenous feed enzymes either alone or in diverse combinations (57). It has been well established that incorporation of carbohydrases (xylanase, β-glucanase, or amylase) and phytase can reduce the adverse impact of antinutritional factors in monogastric animals fed with different raw materials (58). Additionally, a growing interest on the reduction of environmental pollution generated by livestock production has been one of the principal targets supporting the inclusion of enzymes in animal feed (59). Nevertheless, research results have been variable due to the different sources of exogenous enzymes under evaluation. Some of these enzymes are denatured at acidic pH (proventriculus) or do not resist high temperatures commonly used during feed pelletization. One of the principal sources of microbial enzymes is produced by bacteria from the genus *Bacillus* (24, 27). For this reason in the present study, 31 *Bacillus* spp. were screened for production of amylase, protease, lipase, and phytase (**Table 1**). Three strains were selected based on superior REA values on at least one of the enzymes under evaluation. These results demonstrate that not all *Bacillus* spp. synthesize the same type of enzymes over time, suggesting that this capacity is a strain-specific characteristic (**Figure 1**). The combination and feed inclusion of these superior enzyme producer isolates as a *Bacillus*-DFM cocktail has been previously evaluated during *in vivo* experiments with broiler chickens and turkeys (9, 60). In these experiments, results showed that consumption of the DFM significantly improved performance parameters, intestinal viscosity, bacterial translocation, and bone quality in poultry fed with a rye-based diet containing high amounts of NSP.

On the other hand, despite of the success showed by the development of the LAB probiotics for use in commercial poultry, there is still an urgent necessity for commercial DFM that are shelf-stable, cost-effective, and feed-applicable to increase widespread utilization of viable substitutes of AGP in the poultry industry. In this regard, *Bacillus* spp. spores have been isolated from the GIT of multiple animal species, including poultry and pigs suggesting that this microorganism could be an active member of the host microbiota (11, 61). Moreover, some *Bacillus* spp. endospores have been extensively studied as DFM, showing to be a safe and reliable prophylactic tool to diminish the presentation of gastrointestinal diseases in livestock and humans (62–64). In the present study, the majority of the tested *Bacillus* spp. strains showed antimicrobial activity against different food-borne pathogens, including *S.* Enteritidis (25/31) and *E. coli* (27/31). This could be the result of the capacity of some *Bacillus* to synthesize antimicrobial compounds, compete for nutrients, and/or change the environmental conditions of the media (**Figure 2**). Furthermore, it was remarkable to observe that the most susceptible enteropathogen to the presence of almost all *Bacillus* isolates was *C. difficile* (28/31). This anaerobic spore-former bacteria is the principal etiological agent of nosocomial diarrhea in patients under antibiotic therapy, and it has also been isolated from animals and retail meat (65, 66). Therefore, these results suggest that utilization of selected *Bacillus*-DFM may be a suitable alternative to reduce the incidence of bacterial gastrointestinal diseases in humans and animals, including cases of *C. difficile* infection. However, as observed in the enzyme-production profile, the ability to produce antimicrobial compounds appears to be a specific feature for each *Bacillus* spp. isolate (**Table 2**).

In the case of biofilm formation, it is possible that this polysaccharide structure served as a mechanism of survival for some *Bacillus* isolates to resist the harsh environmental conditions of the GIT. Additionally, generation of biofilms could help *Bacillus* cells to be attached to the gut epithelia, therefore, increasing their persistence in the intestinal mucosa, as well as, preventing adherence of enteropathogens as suggested by Barbosa et al. (11). Results of the biofilm assay showed that 11 of 31 *Bacillus* spp. synthesized a thicker and stronger adherent layer, therefore classifying this isolates as superior biofilm formers. Previous studies from our laboratories has evaluated germination, distribution, and persistence of *B. subtilis* spores in the GIT of poultry, and it was observed that spores from the isolate NP122 which synthesized biofilms, persisted for 120 h after a single gavage dose, that is longer than the estimated half-life, based on gut-passage time of the digesta in poultry (10). This finding could be an important strain-specific characteristic influencing the viability of different *Bacillus* candidates in the GIT; however, more studies need to be conducted to confirm this hypothesis.

In summary, our results confirm that *Bacillus* spp. isolates differ in their capacity to produce enzymes, antimicrobial compounds, and biofilms even if they are from the same species. Therefore, an exhaustive selection process must be performed according to the purpose the DFM is going to be used for. *Bacillus* strains selected as superior enzyme producers were different from the isolates showing the highest antimicrobial activity; however, all *Bacillus* isolates showed certain pathogen-inhibition activity. As observed in previous *in vivo* experiments in poultry consuming rye-based diets, it is expected that the consumption of the *Bacillus*-DFM candidate selected in this study, based on enzyme activity, may contribute to enhanced performance parameters by improving nutrient digestibility, maintaining a balanced microbiota, and promoting healthy intestinal integrity in poultry consuming conventional corn-based diets and/or diets containing alternative feed ingredients with a higher content of NSP.

AUTHOR CONTRIBUTIONS

JL: conception and design, acquisition of data, and drafting of manuscript. XH-V: drafting the article or revising it critically for important intellectual content. RW: acquisition of data and drafting the article. JV: acquisition of data. AW: acquisition of data. AM: acquisition of data. LB: acquisition of data. BH: approval of the version to be submitted and any revised version. GT: conception and design, acquisition of data, analysis and interpretation of data, and drafting of manuscript.

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

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 16 September 2016
doi: 10.3389/fvets.2016.00083

Reduction of *Salmonella* Typhimurium by Fermentation Metabolites of Diamond V Original XPC in an *In Vitro* Anaerobic Mixed Chicken Cecal Culture

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OPEN ACCESS

Edited by:
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Reviewed by:
Lisa Bielke, Ohio State University, USA
Michael J. Rothrock, United States Department of Agriculture, USA

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *22 July 2016*
Accepted: *02 September 2016*
Published: *16 September 2016*

Citation:
Rubinelli P, Roto S, Kim SA, Park SH, Pavlidis HO, McIntyre D and Ricke SC (2016) Reduction of Salmonella Typhimurium by Fermentation Metabolites of Diamond V Original XPC in an In Vitro Anaerobic Mixed Chicken Cecal Culture. Front. Vet. Sci. 3:83. doi: 10.3389/fvets.2016.00083

Fermentation metabolites of Diamond V Original XPC™ (XPC), a biological product derived from yeast fermentation, were evaluated for their ability to reduce the *Salmonella* Typhimurium population using an *in vitro* mixed anaerobic culture system containing cecal microbiota to simulate chicken hindgut conditions. Four different samples were prepared: anaerobic mixed culture containing (1) feed only, (2) cecal only (ceca were harvested from 42 days old broiler chickens), (3) feed and cecal contents, and (4) feed, cecal contents, and 1% XPC. Two experimental conditions were investigated: Group 1, in which the cecal content was added at the same time as a *S.* Typhimurium marker strain and Group 2, in which the cecal content was preincubated for 24 h prior to the inoculation with the *S.* Typhimurium marker strain. The mixed cultures were incubated anaerobically at 37°C, and the *S.* Typhimurium marker strain was enumerated at 0, 24, and 48 h. Analysis of short chain fatty acids was also conducted for 24 h. In the Group 1 experiment, adding XPC did not exhibit significant reduction of *S.* Typhimurium. However, the presence of XPC resulted in rapid reduction of *S.* Typhimurium in Group 2. *S.* Typhimurium was reduced from 6.81 $\log_{10}$ CFU/ml (0 h) to 3.73 $\log_{10}$ CFU/ml and 1.19 $\log_{10}$ CFU/ml after 24 and 48 h, respectively. These levels were also 2.47 $\log_{10}$ and 2.72 $\log_{10}$ lower than the *S.* Typhimurium level recovered from the control culture with feed and cecal contents, but without XPC. Based on these results, it appears that the ability of XPC to reduce *S.* Typhimurium requires the presence of the cecal microbiota. Short chain fatty acid analysis indicated that acetate and butyrate concentrations of cultures containing XPC were twofold greater than the control cultures by 24 h of anaerobic growth. Results from the present study suggest that dietary inclusion of XPC may influence cecal microbiota fermentation and has the potential to reduce *Salmonella* in the cecum. Implications of these findings suggest that XPC may decrease preharvest levels of *Salmonella* in broilers and layers.

Keywords: *Salmonella* Typhimurium, Diamond V Original XPC, mixed anaerobic culture, *in vitro*, reduction, short chain fatty acids

INTRODUCTION

Food-borne disease continues to be one of the primary public health concerns throughout the world. Infections by *Salmonella* are one of the leading causes of food-borne gastroenteritis to systemic infections in humans. Annually, it is estimated that over one million Americans contract *Salmonella* (1), and yearly costs for *Salmonella* control efforts are estimated to be up to $14.6 billion (2, 3). Salmonellosis usually occurs by consumption of foods or water contaminated with *Salmonella*, and common sources are poultry and poultry products (4), thus it is essential to control pathogenic *Salmonella* in poultry products.

Because the use of antibiotic growth promoters provoke a negative reaction from many consumers due to public health concerns such as the appearance of antibiotic resistance, the food industry has been searching for effective alternatives to replace antibiotics (5–7). Prebiotics can be defined as non-digestible food ingredients that selectively simulate the growth of beneficial bacteria and/or minimize pathogen growth in the colon, and they are occasionally used in the poultry industries to improve poultry health as a replacement of antibiotic growth promoters (8–11).

However, there are several ingredients that do not stringently fit the definition of prebiotics, but nevertheless provide similar and beneficial effects on host health with different modes of action compared to prebiotics. These ingredients are referred as "prebiotic-like compounds" (12). Fermentation metabolites of Diamond V Original XPC™ (XPC; Diamond V, Cedar Rapids, IA, USA) is a common prebiotic-like compound, which includes post-fermentation growth medium residues, residual yeast cells, and yeast cell wall fragments (mannan-oligosaccharides and β-glucans) (13). To date, several studies of XPC have focused on its effects on the host including feed uptake, growth performance, reproductive performance, and immunomodulatory functions with different animal model systems (13–17); however, few studies have examined inhibitory/bactericidal effects against pathogenic *Salmonella* (18, 19).

Because the environment of the chicken gut is anaerobic, the *in vitro* methodology using an anaerobic mixed culture can provide more empirical data since it can mimic the chicken cecal environment effectively while minimizing confounding host variables and is considered cost-effective (20). The gut microbiota ferment non-digestible ingredients to produce various compounds including short chain fatty acids (SCFA), methane, hydrogen, and ammonia (21). Among these, SCFA are potential metabolites that can be inhibitory to pathogens such as *Salmonella* (22, 23). In the present study, the ability of XPC in feed to reduce *S.* Typhimurium was investigated using a mixed anaerobic culture system to mimic conditions within the chicken hindgut. Additionally, the requirement for cecal microbiota on the reduction of *S.* Typhimurium by XPC was established. Finally, SCFA analysis was performed on the anaerobic cultures with or without XPC to further characterize the effect of XPC on cecal fermentation.

MATERIALS AND METHODS

Preparation of Anaerobic Dilution Solution

Our *in vitro* anaerobic mixed culture experiment was based on the method of Donalson et al. The mixed cultures were grown in anaerobic dilution solution (ADS), consisting of 0.45 g/l K_2HPO_4, 0.45 g/l KH_2PO_4, 0.45 g/l $(NH_4)_2SO_4$, 0.9 g/l NaCl, 0.1875 g/l $MgSO_4$-$7H_2O$, 0.12 g/l $CaCl_2$-$2H_2O$, 1 ml/l 0.1% resazurin, 0.05% cysteine-HCl, and 0.4% CO_2-saturated sodium carbonate, with the sodium carbonate added last as described previously (24–29). ADS was sparged with an anaerobic gas mixture (90% nitrogen/5% carbon dioxide/5% hydrogen) for 30 min in an anaerobic chamber using an aquarium air pump and airstone prior to autoclaving. Autoclaved ADS was cooled to room temperature and allowed to equilibrate overnight in an anaerobic chamber (Coy Laboratories, Grass Lake, MI, USA) with the same atmosphere described above to remove all traces of oxygen.

Bacterial Culture

Salmonella Typhimurium marker strain ST97, a nalidixic acid-resistant (NA^R) isolate (gift of Dr. Billy Hargis, Poultry Health Laboratory, University of Arkansas) was used in the present study. This isolate was grown in sterile glass culture tubes with agitation for 16 h in Luria–Bertani (LB) medium containing 20 μg/ml nalidixic acid, 37°C at 250 rpm. The bacterial suspension was washed three times in phosphate-buffered saline (PBS).

Cecal Sample Preparation

Ceca from three different CO_2-euthanized 42-day-old Cobb male broiler chickens (Cobb-Vantress, Siloam Springs, AR, USA) were collected separately using alcohol-dipped, flame-sterilized tools. A University of Arkansas Institutional Animal Care and Use Committee (IACUC)-approved protocol was used to ensure humane treatment of the chickens (IACUC # 15052). Ceca were placed in sterile sample bags (VWR, Radnor, PA, USA). The bags were then placed in a portable anaerobic box (Mitsubishi Gas Chemical Co., Japan) containing oxygen-scrubbing sachets. Immediately after harvest, ceca were transferred to an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA). Two palladium catalyst scrubbers running continuously maintained an anaerobic environment inside the chamber.

Anaerobic *In Vitro* Mixed Cultures

A portion of the cecal contents from three individual chickens were each removed aseptically within the chamber, weighed, and diluted 1:3000 by addition of 0.1 g of cecal content to 300 ml ADS for each chicken. A total of 20 ml of this diluted cecal content was transferred to each serum bottle with or without ground chicken feed (40 mesh) and XPC as indicated below. An additional culture received sterile ADS, but no cecal content. An initial inoculum of approximately 1×10^7 CFU/ml of *S.* Typhimurium was added to each 20 ml culture. Cultures were stoppered with airtight rubber stoppers and aluminum crimps, removed from the anaerobic chamber, and incubated at 37°C with 150 rpm shaking for 48 h.

Two different experimental designs were employed, referred to as Group 1 (unadapted) and Group 2 (adapted), respectively. The experimental designs are illustrated in **Figure 1**. In Group 1, the *Salmonella* NA^R marker strain was added at the beginning of the culture incubation along with cecal bacteria, and/or chicken feed, and/or XPC. In Group 2, *S.* Typhimurium was added after a 24 h preincubation of the cecal bacteria with the chicken feed and/or XPC. Three control cultures were run in parallel as indicated in **Figure 1**.

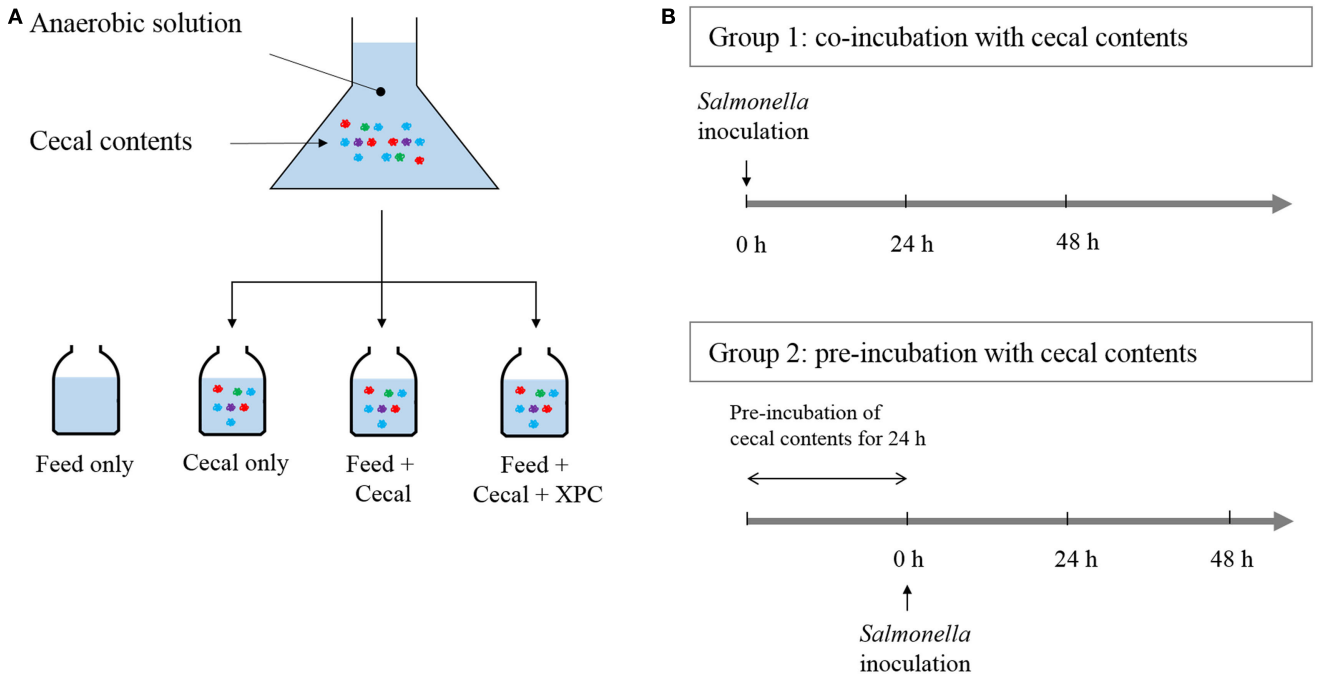

FIGURE 1 | **Anaerobic experimental strategy**. **(A)** Cecal content is added to anaerobic solution with chicken feed and fermentation metabolites of Original XPC. Controls contain (1) chicken feed, but no cecal content; (2) cecal content, but no chicken feed and; (3) feed + cecal content, respectively. Experimental treatments contain feed, cecal contents, and XPC. **(B)** Group 1 cultures receive *Salmonella* at the same time as cecal content (at 0 time). Group 2 cultures receive *Salmonella* after a 24 h incubation of cecal content under anaerobic conditions. Each culture is then incubated with *Salmonella* for 48 h.

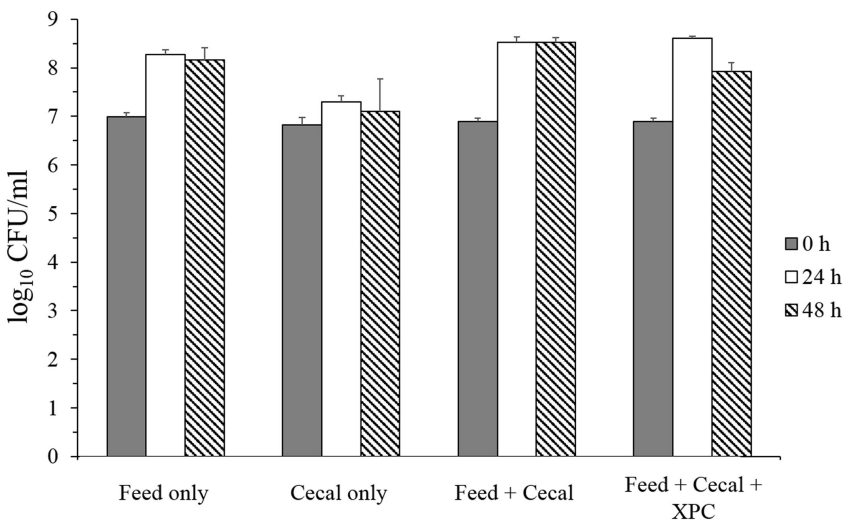

FIGURE 2 | ***Salmonella*** **Typhimurium survival in unadapted anaerobic cultures (Group 1) with and without fermentation metabolites of Original XPC**. Bars and brackets represent the mean and SE of three biological replicates.

Salmonella Enumeration

At 0, 24, and 48 h, an aliquot of each culture was removed, diluted, and spread on Brilliant Green Agar medium (BG, BD Biosciences, Franklin Lakes, NJ, USA) supplemented with 20 ug/ml nalidixic acid for quantitation of colony forming units (CFU) of marker strain *S.* Typhimurium per milliter of culture. The diluted cecal contents were also tested for NA^R bacteria prior to addition of marker strain *S.* Typhimurium by inoculation into tetrathionate (TT) enrichment broth (BD Biosciences, Franklin Lakes, NJ, USA), and none were detected. If no *Salmonella* were

detected at a particular time point in undiluted culture, that culture was inoculated into TT enrichment broth to confirm that no *S.* Typhimurium survived.

Short Chain Fatty Acid Analysis

Anaerobic culture supernatants were stored at −20°C until they could be analyzed by gas chromatography. A 1 ml portion of culture supernatant was centrifuged at 14,000 × *g* to remove solids. An aliquot of the supernatant (450 µl) was then mixed with 50 µl of GC reagent (50 mM 4-methyl-valeric acid, 5% meta-phosphoric acid, 1.6 mg/ml copper sulfate). This mixture was allowed to incubate at 25°C for 10 min and subsequently centrifuged at 14,000 × *g*. The supernatant was transferred to a fresh tube and 1 µl was loaded into a Shimadzu 2010 gas chromatograph (Kyoto, Japan) fitted with a 30 m × 0.25 mm BP21 glass capillary column with 0.25 mm film thickness (SGE, Austin, TX, USA) operated at 100 kPa He carrier gas pressure, with 170 kPa H_2, Ar, and air pressure, at 100°C for 3 min, followed by a temperature gradient of 4°C/min to 120°C, holding at 120°C for 1 min, followed by a further gradient of 3°C/min to 150°C. The SPL was maintained at 220°C with split ratio = 30. FID was maintained at 230°C. Carrier gas flow rate was set to 30 ml/min. A 1:100 mixture of acetic, propionic, and butyric acids was serially diluted, mixed with GC reagent, and used as standards. Peak areas were normalized for loading differences using the valeric acid internal control from the GC reagent.

Statistical Analysis

Means were determined to be significantly different if $P < 0.05$ by two-tailed paired Student *t*-test using Microsoft Excel.

RESULTS AND DISCUSSION

The main objective of this study was to investigate the inhibitory effect of XPC on *S.* Typhimurium when combined *in vitro* with cecal microbiota. The ceca are the main site where pathogens including *Salmonella* colonize (30). Since poultry have a relatively slow digestion transit time, poultry ceca have a large number of bacteria, and the majority of these are strictly anaerobic (27, 31, 32). Cecal bacteria in poultry become more diverse as the host matures, and they can maximize metabolic fermentation in an anaerobic environment (12). Cecal contents used in this study were obtained from mature chickens (42-day-old chickens), thus it should serve as a source of a fairly diverse microbiota containing a wide range of anaerobic bacteria. Also, using an anaerobic mixed culture in this study could help to understand the actual fate of *Salmonella* in ceca by various feeding conditions.

Two conditions were investigated in the present study: Group 1 (unadapted), in which the cecal microbiota was added at the same time as the *S.* Typhimurium and Group 2 (adapted), in which the cecal microbiota was allowed to metabolize anaerobically for 24 h prior to the inoculation of *S.* Typhimurium (see **Figure 1** for design). Results on *S.* Typhimurium reduction by XPC were different between groups. In the unadapted condition (Group 1), the population of *S.* Typhimurium was slightly increased or maintained during 48 h incubation in all controls (feed only, cecal only, and feed + cecal) and treatment (feed + ceca + XPC); the population after incubation was not significantly different from the initial population (**Figure 2**). In the feed + cecal sample, *S.* Typhimurium populations were increased from 6.89 $\log_{10}$ CFU/ml to 8.52 and 8.53 $\log_{10}$ CFU/ml after 24 and 48 h, respectively

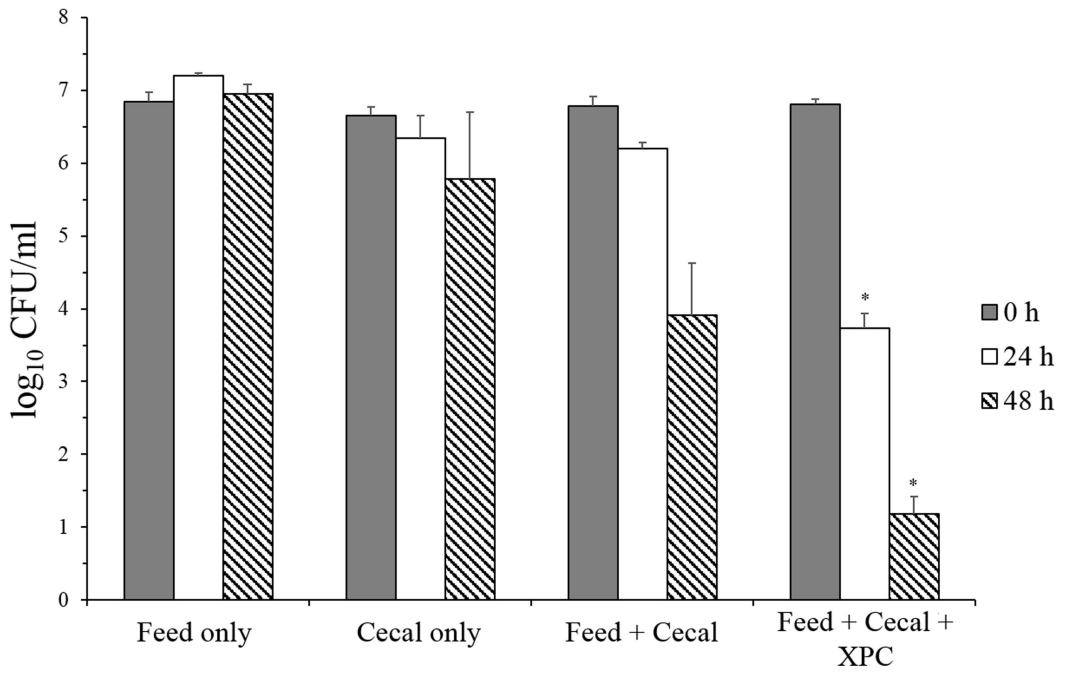

FIGURE 3 | *Salmonella* Typhimurium survival in adapted anaerobic cultures (Group 2) with and without fermentation metabolites of Original XPC. Bars and brackets represent the mean and SE of three biological replicates. Asterisks indicate significant difference ($P < 0.05$) from the "feed + cecal" control.

($P < 0.05$). When XPC was added to the feed + cecal sample, *S.* Typhimurium populations were increased from 6.89 $\log_{10}$ CFU/ml to 8.60 and 7.92 $\log_{10}$ CFU/ml after 24 and 48 h ($P < 0.05$), respectively, indicating that XPC had little or no effect on *Salmonella* survival when *S.* Typhimurium was added at the same time as cecal contents.

In contrast, XPC-containing cultures exhibited a significant reduction in *Salmonella* survival under adapted conditions (Group 2) (**Figure 3**). There was no reduction in *S.* Typhimurium in the feed-only control sample, and only a 0.87 $\log_{10}$ reduction of populations of *S.* Typhimurium was achieved after 48 h incubation in the cecal-only control sample. In addition, the *S.* Typhimurium population was decreased in both the feed + cecal control and the feed + cecal + XPC treatments. However, the presence of XPC resulted in a greater reduction of *S.* Typhimurium compared with the feed + cecal control. When *S.* Typhimurium was inoculated to the feed + cecal control, a 2.87 $\log_{10}$ reduction in the bacterial population was observed after 48 h. With XPC, the $\log_{10}$ reductions achieved after 24 and 48 h incubation were 3.08 and 5.62 $\log_{10}$ reduction, respectively. These levels are 2.47 $\log_{10}$ (24 h) and 2.72 $\log_{10}$ (48 h) lower than the *Salmonella* level recovered from the feed + cecal control.

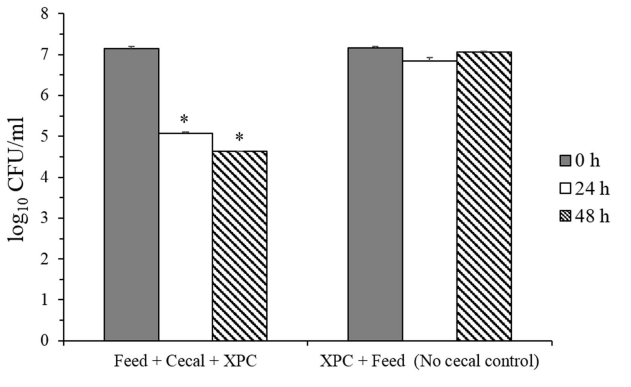

FIGURE 4 | *Salmonella* Typhimurium survival in feed + cecal + fermentation metabolites of Original XPC and in control anaerobic cultures lacking cecal contents. Asterisks indicate significant difference ($P < 0.005$) from the corresponding control.

These results suggested that adaptation of the cecal microbiome in the *in vitro* mixture to XPC prior to inoculation of *S.* Typhimurium appears to generate a more inhibitory environment for *Salmonella* than XPC unadapted cecal cultures. To evaluate the role of the microbiota on reduction of *S.* Typhimurium, survival of *S.* Typhimurium in "Feed + cecal + XPC" and "Feed + XPC without cecal contents" were compared (**Figure 4**). When *S.* Typhimurium was exposed to XPC in the absence of broiler cecal content, no reduction in *S.* Typhimurium was observed, suggesting that XPC acts in concert with cecal microbiota to inhibit *S.* Typhimurium (**Figure 4**). This is a further indication that cecal microbiota are essential to the reduction of *Salmonella* by XPC. These results are in accordance with a previous study reporting higher inhibitory activities of fructooligosaccharide in samples preincubated with cecal microbiota prior to inoculation of bacteria (25). Furthermore, the results from both *in vitro* studies suggest that dietary inclusion of XPC may influence cecal microbiota fermentation and has the potential to reduce *Salmonella* colonization in the cecum.

The SCFA analysis of supernatants from the mixed cultures indicated that acetate and butyrate concentrations of cultures containing XPC + cecum and XPC + feces were twofold greater than the control cultures after 24 h of anaerobic growth (**Figure 5**). This suggests one or more microorganisms have potentially increased acetate and/or butyrate production as a result of being exposed

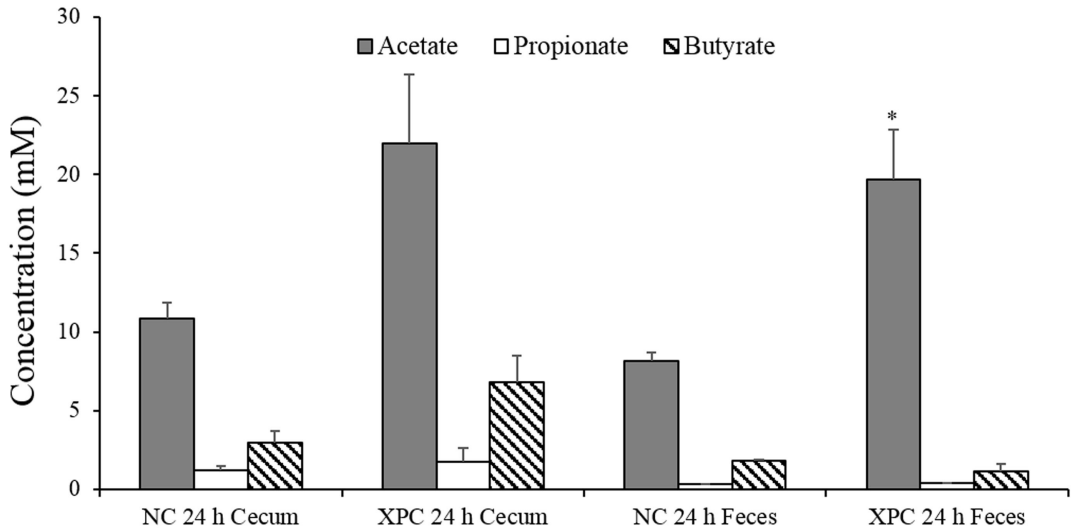

FIGURE 5 | Short chain fatty acid analysis of 24 h anaerobic cultures containing 6-week-old broiler cecal contents or feces with and without 1% fermentation metabolites of Original XPC. Bars and brackets represent the mean and SE of three biological replicates (chickens). Asterisk indicates significant difference ($P < 0.05$) from corresponding negative control (NC).

to components of XPC. This additional acetate and butyrate may be contributing to the inhibition of *Salmonella* due to the direct toxic effect of intracellular anion accumulation when these acids dissociate in the cytosol of sensitive bacteria such as *Salmonella* (22, 23, 33). Interestingly, butyrate has been found to inhibit *Salmonella* invasion of host cells by downregulating *Salmonella* pathogenicity island 1 (SPI-1) gene expression (22, 34). Along these lines, Feye et al. has shown that XPC fed to broilers reduces the virulence regulatory gene *hilA* in the intestine (19).

In conclusion, XPC can effectively reduce *S.* Typhimurium survival (5.62 $\log_{10}$ reduction) in an *in vitro* anaerobic mixed cecal culture, and XPC and cecal microbiota are both required for the reduction of *S.* Typhimurium survival. Incubation of cecal microbiota with XPC increased SCFA levels (particularly acetate) in anaerobic cultures. The use of XPC as a prebiotic-like compound has a number of advantages for use in poultry: (1) there are no concerns over usage of antibiotics or growth promoters or the appearance of antibiotic-resistant bacteria, (2) the use of XPC is acceptable to the both industries and consumer since it is a naturally derived yeast product (also an environmentally friendly product), (3) its use by the poultry industry is also feasible because XPC was classified as generally recognized as safe (GRAS) by US FDA (13). To the best of our knowledge, this is the first study to examine the inhibitory effects of XPC in feed with an anaerobic mixed cecal inocula culture to mimic the chicken cecal environment. The implication of these findings is that XPC may decrease preharvest levels of *Salmonella* in the ceca of broilers and layers, thus it could be a suitable alternative to antibiotics currently used in poultry industries.

AUTHOR CONTRIBUTIONS

PR and SR performed experiments, drafted the manuscript, collected test data, and analyzed the data. SK, HP, and DM revised the manuscript. PR, SP, and SR designed the study and revised the manuscript.

ACKNOWLEDGMENTS

We would like to thank the University of Arkansas, Fayetteville, FDSC program for supporting a graduate student assistantship to SR. We thank the Michael G. Johnson Endowed Scholarship for Excellence in Food Microbiology Research, Teaching, and Peer Mentoring for providing financial assistance to SR. We would like to thank Diamond V Mills (Cedar Rapids, IA, USA) for support of this study. Author SK is supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2015R1A6A3A03016811).

FUNDING

Funding is provided by Diamond V (Cedar Rapids, IA, USA).

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

frontiers in Veterinary Science

GENERAL COMMENTARY
published: 31 August 2016
doi: 10.3389/fvets.2016.00074

General Commentary on: Alternatives to Antibiotic Growth Promoters in Animals

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Keywords: antibiotics growth promoters, phytogenic feed additives, microbiota, antibacterial activity, probiotics

A commentary on

Development and Evaluation of a Herbal Formulation with Antipathogenic Activities and Probiotics Stimulatory Effects
by Qian Z, Si-Si W, Guang Y, Wen Z, Hui-Ling L. J Integr Agric (2016) 15:1103–11. doi:10.1016/S2095-3119(15)61146-7

Phytogenic Feed Additives as an Alternative to Antibiotic Growth Promoters in Broiler Chickens
by Murugesan GR, Syed B, Haldar S, Pender C. Front Vet Sci (2015) 2:21. doi:10.3389/fvets.2015.00021

OPEN ACCESS

Edited by:
Guillermo Tellez, University of Arkansas, USA

Reviewed by:
Kenneth James Genovese, United States Department of Agriculture, USA
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *04 June 2016*
Accepted: *23 August 2016*
Published: *31 August 2016*

Citation:
Anonye BO (2016) General Commentary on: Alternatives to Antibiotic Growth Promoters in Animals. Front. Vet. Sci. 3:74. doi: 10.3389/fvets.2016.00074

Recent studies have evaluated the impact of alternatives to antibiotic growth promoters (AGPs) such as phytogenic feed additives (PFAs) *in vitro* and *in vivo*. Zhou and colleagues studied the antibacterial properties of 30 herbs on pathogenic Gram-negative and positive bacteria (1). Thirteen of the 30 herbs exerted a significant effect against *Escherichia coli* ATCC 25922 ($p < 0.01$, $n = 11$ and $p < 0.05$, $n = 2$). More than 30% of the herbs exhibited activity against *Salmonella enteritidis* ATCC 13076 and *Salmonella typhimurium* ATCC 14028. These pathogenic organisms commonly infect humans and animals especially poultry such as chickens leading to loss and decrease in their market value. Additionally, Zhou reported that more than 50% of the herbal extracts ($n = 16$) possessed antibacterial activity against *Staphylococcus aureus* ATCC 25923.

Furthermore, based on the results of the individual herbs on the pathogenic bacteria, two formulations were performed with five of the herbs. For formulation 1, the herb, *Fructus mume* was the main ingredient (35%) and *Galla chinensis* (30%) for formulation 2 with varying proportions of four other herbs. Both formulations 1 and 2 had significant antibacterial activity against the pathogenic bacteria ($p < 0.05$) with no significant difference in activity when compared to the AGPs, aureomycin, and flavomycin (1). Formulation 1 also led to increased counts of *Lactobacillus acidophilus* ATCC 4356 and *Bifidobacterium longum* ATCC 15707 relative to the control, indicating the possibility that these herbs could have a synergistic effect on beneficial bacteria in the intestinal microbiota. This raises the potential of using herbs as an alternative to antibiotics to increase growth in animals and modulate the microbiota. However, these herbs must be carefully chosen as formulation 2 did not produce the same probiotic effects as formulation 1 but led to reduced amounts of the *L. acidophilus* and *B. longum* compared to the control.

Similarly, Murugesan et al. compared the effects of Digestarom® Poultry, a commercial PFA produced by BIOMIN, to the AGP, bacitracin methylene disalicylate in broiler chickens (2). Chicks were randomly assigned to receive either a corn–soybean meal only or supplemented with the PFA or AGP, respectively, over a 39-day period. This period was divided into pre-starter (days 1–7), starter (days 8–21), and grower (days 22–39) phases. The authors noted differences based on the period of growth. For example, in the starter phase, AGP-fed birds gained more body weight relative to control, while PFA-fed birds had increased body weight in the grower phase. Also, increase in the villus height

across the small intestine was observed in birds fed with AGP or PFA relative to control (2). As the villi help to increase the surface area of the intestinal walls, an increase in digestion and absorption of nutrients is likely to be observed (3). Coliforms were significantly decreased ($p < 0.01$), and *Lactobacillus* spp. was significantly increased ($p < 0.01$) through plating of the cecal microbiota when compared to the control or AGP-fed birds. Similar results have been obtained with respect to increased *Lactobacillus* spp. in PFA-fed birds using similar PFA as Murugesan et al. (4) or a phytoncide (5). However, next-generation sequencing could provide a better picture of the changes taking place in the cecal microbiota with respect to the bacterial groups.

The results from these and other studies suggest the ability of the PFAs to modulate the intestinal microbiota. These could occur through various mechanisms by influencing the digestibility of nutrients and thereby enhancing the growth performance of the animals (1, 2, 5). PFAs can potentially stimulate the secretion of digestive enzymes, thereby promoting gut functions. Moreover, the bioactive compounds produced by the PFAs have been shown to possess antibacterial properties *in vivo* against chickens challenged with *S. enteritidis*, *E. coli*, and *Clostridium perfringens* (4, 6). PFAs, such as *F. mume*, may exert their antibacterial effect through the production of organic acids, leading to increased acidity as revealed by HPLC (7). Another possible mechanism by which PFAs exert their beneficial effects is by acting as antioxidant against oxidative stress in animals.

However, the search for PFAs with these desirable properties is not trivial. Single and different combinations of PFAs need to be tested against different strains of pathogenic bacteria *in vitro* and *in vivo* to determine their antimicrobial activity. A desirable PFA ideally should be able to stimulate the gut microbiota in a number of ways. This could be through increasing colonization resistance without having any adverse effect on beneficial bacteria and creating a favorable environment for increased nutrient intake leading to weight gain. Both studies described above have shown to some extent these desirable characteristics, but more studies will be needed to determine the exact mode of action of these PFAs.

It would also be worth looking at other alternatives to antibiotics such as prebiotics, probiotics, and bacteriocins to determine their effects on animals (8, 9). The development of PFAs and other substances that can give similar or more beneficial outcomes, as the AGPs will go a long way in reducing the increase of antibiotic-resistant bacteria.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 29 June 2017
doi: 10.3389/fvets.2017.00103

Growth Characterization of Single and Double *Salmonella* Methionine Auxotroph Strains for Potential Vaccine Use in Poultry

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OPEN ACCESS

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Reviewed by:
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *05 April 2017*
Accepted: *13 June 2017*
Published: *29 June 2017*

Citation:
Rubinelli P, Kim SA, Park SH, Baker CA and Ricke SC (2017) Growth Characterization of Single and Double Salmonella Methionine Auxotroph Strains for Potential Vaccine Use in Poultry. Front. Vet. Sci. 4:103. doi: 10.3389/fvets.2017.00103

Poultry meat is an important source of zoonotic *Salmonella* infection. Oral vaccination of chickens with live attenuated *Salmonella* during grow-out is an attractive approach to control *Salmonella* colonization in the chicken gastrointestinal tract. In this study, we report the construction of methionine-dependent and growth of *Salmonella* Typhimurium mutant strains with methionine auxotrophy (Δ*metR* and ΔΔ*metRmetD*) and survival in chicken feed and fecal matrices. The methionine auxotroph mutant ΔΔ*metRmetD* grew slowly on L-methionine but failed to grow on D-methionine, as expected, and exhibited lower affinity for methionine compared with the isogenic parent strain (Δ*metR* single mutant) in whole-cell affinity experiments. Preliminary data conducted as part of a previously published bird challenge study indicated that the methionine auxotroph was less effective at protection in chickens to a challenge with virulent wild-type parent strain but generated greater *Salmonella*-specific serum IgG. Although the auxotroph could not sustain itself in minimal media it was able to survive when incubated in the presence of chicken and fecal material. The immune response appears promising but further work may be needed to alter low-affinity methionine transporters and methionine biosynthesis genes in combination with the knock-out of the high affinity transporter *metD* reported here to ensure timely clearance of the candidate vaccine strain.

Keywords: *Salmonella* Typhimurium, vaccine, methionine auxotrophy, poultry, Δ*metR*, ΔΔ*metRmetD*

INTRODUCTION

As an essential amino acid for animals, methionine is an important component of animal feeds, including poultry feed (1). Methionine is one of the nutritionally limiting components of animal feeds and is limited in plant proteins. Methionine is essential for protein synthesis and serves as a source of methyl groups for the biosynthesis of lipids, biotin, nucleic acids, and polyamines (2, 3).

Methionine synthesis and uptake have been extensively investigated in *Escherichia coli* and *Salmonella* Typhimurium (4–7). Transport of methionine into the bacterial cell is mediated by both a high-affinity transporter (Km approximately 0.1 mM) and one or more low-affinity transporters (Km approximately 20–40 mM) (6). The high-affinity transporter is referred to as metD in both *E. coli* and *S.* Typhimurium, and mutants in this transporter are unable to transport D-methionine. More recently, the metD transporter gene has been sequenced and shown to consist of an operon comprised of three genes, recently named *metNIQ* (8). We have tested the hypothesis that a methionine auxotrophic strain of *S.* Typhimurium with limited uptake and synthesis of

methionine can serve as an effective vaccine for the pre-harvest control of *Salmonella* in poultry. Poultry is a significant source of food-borne *Salmonella* illness in humans (9). Thus, pre-harvest control measures such as vaccination are desirable.

One approach to poultry vaccination with live attenuated *Salmonella* has focused on auxotrophy by deletion of genes encoding essential regulators of metabolism. One of these is the regulation of synthesis and uptake of methionine. The *metR* gene encodes a transcription factor of the LysR family that regulates several genes of the methionine biosynthesis pathway. The *metR* controls primarily genes involved with the last steps of methionine biosynthesis: *metF*, *metE*, and *metH*. The *metF* gene product produces a methyl donor, 5-methyltetrahydrofolate, which provides the terminal methyl group for methionine. Both *metE* and *metH* encode cobalamin-independent and cobalamin-dependent enzymes, respectively, that add the terminal methyl group to homocysteine to form methionine (6, 10). The *metD* deletion eliminates the high-affinity methionine transporter (7). We hypothesized that use of this mutant in combination with the *metR* deletion might further reduce the ability of a *Salmonella* vaccine strain to survive in the host by limiting methionine uptake to that of the remaining (low-affinity) methionine transporters.

In this study, single (Δ*metR*) and double (ΔΔ*metRmetD*) *S.* Typhimurium UK-1 mutants were constructed and characterized as potential vaccine strains for control of *Salmonella* colonization. Here we present preliminary data on the ΔΔ*metRmetD* unpublished part of the bird challenge study (11) and compare it with the previously published responses to the wild-type parent strain of *S.* Typhimurium, UK-1 (positive control) and a P_{BAD}-*mviN* vaccine strain from our past research (11). The P_{BAD}-*mviN* strain is a genetically attenuated strain that has the native promoter of the *mviN* gene (a gene required for cell wall synthesis) (12) removed and replaced with an arabinose-inducible promoter (P_{BAD}) and the gene encoding the upstream activator, *araC*. By growing this strain in arabinose, but then washing away this medium and inoculating the washed cells orally to the chicken, the bacterium undergoes delayed lysis as cell wall synthesis shuts down (11). To assess the environmental characteristics of the methionine auxotrophs, growth kinetics in minimal medium with L-methionine as well as growth curves in D-methionine, and survival of the auxotroph strains in chicken feed and feces are presented in the current study.

MATERIALS AND METHODS

Bacterial Strains

A wild-type *S.* Typhimurium UK-1 strain was utilized to construct potential vaccine strains. A nalidixic acid (NA) resistant *S.* Typhimurium UK-1 derived from the wild type was used as the challenge strain. The P_{BAD}-*mviN* vaccine strain discussed in the current study for comparative purposes was generated from UK-1 as described in our previous report (11).

Construction of ΔΔ*metRmetD* *S.* Typhimurium UK-1

Single and double deletion mutants affecting methionine metabolism were produced in *S.* Typhimurium strain UK-1. This strain and the plasmids for the Red recombinase system were obtained from Dr. Young Min Kwon, Department of Poultry Science, University of Arkansas (Fayetteville, AR, USA). The Red recombinase system was used for the targeted gene deletions as previously described (13). Briefly, disruption of the targeted genes was accomplished by first transforming strain UK-1 with plasmid pKD46. This plasmid confers ampicillin resistance and harbors the genes for phage lambda Red recombinase, which mediates the exchange of DNA between the gene of *Salmonella* to be deleted and the gene disruption construct. This plasmid also contains a temperature-sensitive origin of replication, which facilitates its removal following recombination. Gene disruption constructs were synthesized by amplifying a region of plasmid pKD4 (13) by polymerase chain reaction (PCR), from the P1 site (nucleotides 31–50 of pKD4) to the P2 site (nt. 1488–1507). This region consists of a central kanamycin (Kan) resistance gene (encoding aminoglycoside 3′-phosphotransferase), flanked by two FLP recognition target (FRT) sites. Genomic DNA sequences corresponding to upstream and downstream regions surrounding the appropriate target gene of strain UK-1 were subsequently introduced on either side of the FRT-Kan^R-FRT region by overlap extension PCR (14), using the primers indicated in **Table 1**.

The method for deletion of the metD transporter sequence, which is comprised of the met*NIQ* operon is shown in **Figure 1**. The resulting PCR products were gel purified, treated with restriction endonuclease Dpn1 to degrade any trace amount of template pKD4, gel purified again, and then electroporated into electroporation-competent UK-1:pKD4 cells that had been grown at 30°C in Luria-Bertani (LB) broth with 1 mM arabinose to induce the Red recombinase. After a 1 h incubation of the electroporated cells at 37°C in SOC medium, the electroporated cells were spread on LB/Kan agar plates and grown at 37°C overnight. Four tranformants were then streaked for isolation on LB/Kan, and tested on LB/ampicillin plates to confirm curing of plasmid pKD46.

The Kan resistance marker was subsequently removed from the genomic insertion sites, leaving a gene deletion, by introducing a second plasmid, pCP20, which expresses a second recombinase, the *S. cerevisiae* FLP recombinase and confers ampicillin resistance (15). The FLP mediates the removal of the antibiotic resistance marker by recombining the flanking FRT sites. The FLP was induced and pCP20 removed by shifting the temperature

TABLE 1 | Polymerase chain reaction primers used in the present study.

Primer	Sequence (5'–3')
metR-F	TCTAAATAGTTCGGCTTGCAG
metR-R	GTATAAACGTCTGATGGAGACC
metR-Up-F	AGGTACTGTATATTCCTCAAGCG
metR-Up-R	CAGCTCCAGCCTACACGATGAGACAGAGCGGATTG
metR-Dn-F	GAGGATATTCATATGGCGATCATCTGCCGTTTGTG
metR-Dn-R	GAACTATGGCGCTACCCAG
metNIQ-F1	CGACTAAGTCTTCAGCATTGG
metNIQ-F2	GATCTGCTTAGCATGGAACAAC
metNIQ-Up-R	CAGCTCCAGCCTACACGTGTACGAAGCCGCAAATAAAG
metNIQ-Dn-F	GAGGATATTCATATGGCCCCTGCTGGAACACTTTG
metNIQ-R1	TCATGTACGTAGCCGTGATCC
metNIQ-R2	CCACCTTTTATAGCTCCTGAGTAAAG

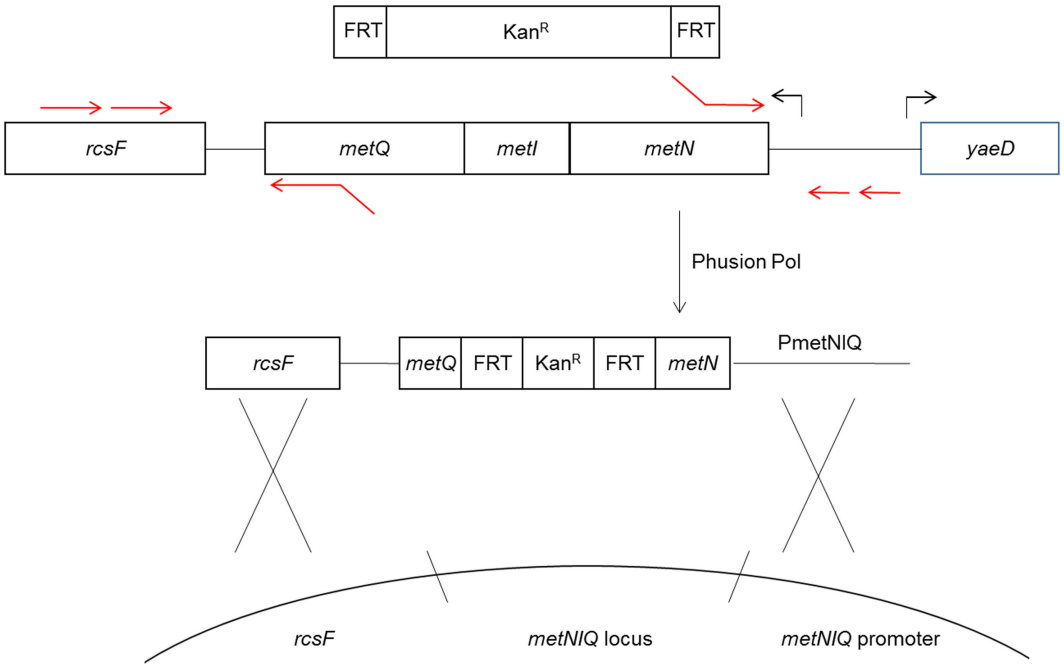

FIGURE 1 | Diagram of *metD* mutant construction. The *metR* mutant was constructed in a similar fashion. The *metD* transporter consists of three subunits, encoded by the *metNIQ* operon. Primer sequences fusing the *metN* and *metQ* sequences to FRT-Kan expression cassette sequences (13) were used to delete the entire metI gene and part of the *metN* and *metQ* genes, replacing these with the FRT-Kan cassette. The resulting construct was introduced into the *metD* locus by electroporation and homologous recombination using the Red recombinase system (13).

from 30 to 42°C as described previously (13). Removal of the Kan^R genomic insertion and pCP20 were confirmed by failure to grow on LB/Kan and LB/ampicillin, respectively, with appropriate growth on LB in parallel.

Whole-Cell Affinity Measurements

Whole-cell affinity measurements were conducted as described previously (16). Briefly, cultures of the Δ*metR* single mutant and ΔΔ*metRmetD* double mutant were grown in M9 minimal medium + 10 μM L-methionine at 37°C for 16 h and then diluted to an OD_{600} of 0.05 in M9 minimal medium + L-methionine at 3, 7, 10, 13, 15, and 17 μM in culture tubes containing 4 ml each of minimal medium at the L-methionine concentrations indicated, and three technical replicate culture tubes were prepared at each methionine concentration. Cultures were grown at 37°C in a shaking water bath at 220 RPM, and the OD_{600} was measured every 15 min for a total of 6 h. The replicate data were then averaged and transformed for Lineweaver–Burk plots using Microsoft Excel software.

Vaccination

Details of the vaccination challenge trial have been described elsewhere (11). Briefly male Cobb 500 broiler chicks (Siloam Springs, AR, USA) were obtained on day of hatch and randomly assigned to four pre-sterilized Horsfall units. A University of Arkansas Institutional Animal Care and Use Committee-approved protocol was used to ensure humane treatment of the chickens. Chicks vaccinated with Δ*metR*Δ*metD* double mutant *Salmonella* was one treatment group (designated Group 2) of the four treatment groups (Group 1: unvaccinated, challenged; Group 3: vaccinated with the P_{BAD}-*mviN* vaccine strain *Salmonella*, challenged), and Group 4: vaccinated with the wild-type parent strain UK-1, challenged. The vaccine and control inocula were grown for 16 h in LB broth at 37°C, followed by three washes in phosphate-buffered saline (PBS) and adjustment of the cell density by dilution in PBS to 5×10^8 cells/ml. Chicks were orally inoculated with 1×10^8 CFU *Salmonella* cells via sterile gavage needle on Day 2 post-hatch and again on Day 7 post-hatch. The unvaccinated chicks received an equal volume (0.2 ml) of sterile PBS via sterile gavage needle on Days 2 and 7 post-hatch. The challenge strain, which had been passaged repeatedly through chicks to increase its virulence followed by cryopreservation, was grown for 16 h in LB + 20 μg/ml NA. The challenge strain was then passaged twice for 8 h each passage to ensure a log phase culture. The resulting cells were then diluted to 5×10^8 cells/ml with PBS and orally inoculated via sterile gavage needle to chickens at 2 weeks post-hatch with 1×10^8 cells (0.2 ml).

At the time of chicken necropsy reported previously (11), ceca and ilea organs were collected aseptically and transferred to sterile sample bags, subsequently removed and transferred to 10 ml tetrathionate (TT) broth for enrichment. The TT broth was incubated for 24 h at 37°C, followed by streaking of a 10 μl loopful of the TT broth for isolation on Brilliant Green (BG) agar supplemented with 20 μg/ml NA and another 10 μl loopful on BG agar supplemented with 50 μg/ml Kan + 1 mM arabinose.

Agar plates were incubated for 24 h at 37°C, examined for *Salmonella* colony appearance to enumerate the NA—resistant *S.* Typhimurium challenge strain per gram cecal content, and live vaccine strains (Kan—resistant *Salmonella* per gram of cecal content). A 0.1 g portion of cecal content was aseptically added to a sterile microtube, weighed, and then combined with nine volumes of sterile PBS to obtain a 1:10 dilution, followed by vortexing. After serial dilution in sterile PBS, mixed cecal contents were spread-plated aseptically onto selective media (BG + 20 μg/ml NA) for enumeration of the respective treatment groups. Since *S.* Typhimurium is naturally resistant to novobiocin (NO), the wild-type control NA-sensitive strain was distinguished from the NA-resistant challenge strain by direct plating of cecal contents from the wild-type-inoculated chickens on both BG + NA and BG + NO plates, and subtracting the number of colonies on BG + NA (challenge strain) from the total colonies on BG + NO (challenge strain + wild-type control strain).

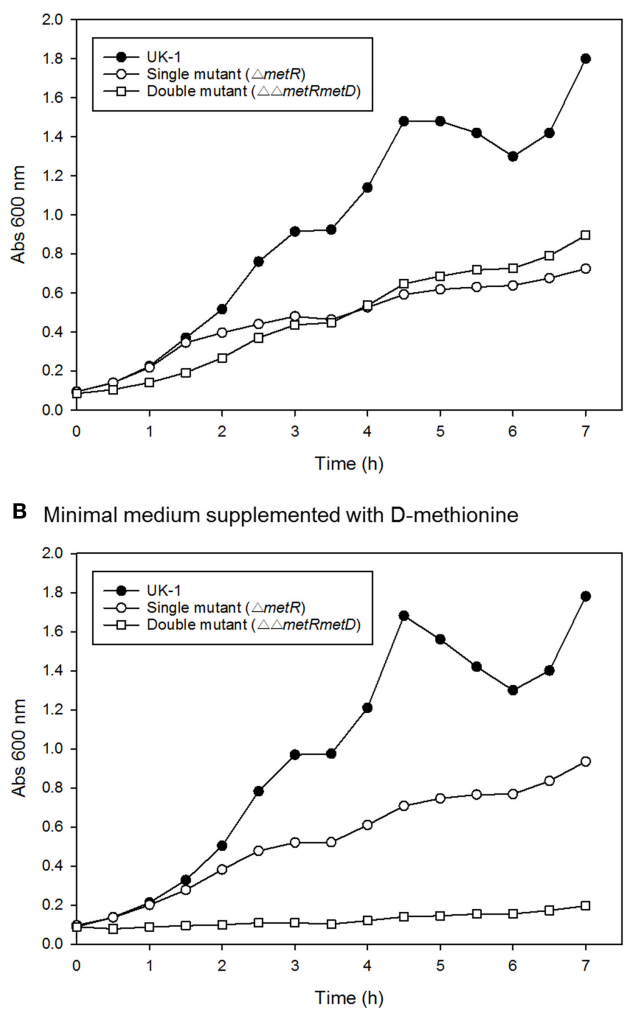

FIGURE 2 | Growth curves of *S.* Typhimurium wild-type (UK-1), single (Δ*metR*), and double mutant (ΔΔ*metRmetD*) mutant in minimal medium supplemented with **(A)** L-methionine or **(B)** D-methionine.

Enzyme-Linked Immunosorbent Assay (ELISA)

As described previously (11), indirect ELISA reactions were performed by placing 1 μg of *Salmonella* protein from sonicated UK-1 cells in each well of 96-well microlon medium binding microtiter plates (Grainer, Frickenhausen, Germany) after diluting to 100 μl in carbonate buffer, pH 9.6. After allowing proteins to bind for 2 h 37°C, the plates were allowed to air-dry overnight at 23°C then blocked with Superblock (Thermo Scientific, Rockford, IL, USA) 2 h, 37°C. Chicken sera from unvaccinated and vaccinated chickens from all treatment groups were serially diluted in Superblock and the plates incubated at 37°C, 2 h followed by washing four times with ELISA plate wash buffer (50 mM Tris pH 8, 140 mM NaCl, 0.05% Tween-20). Anti-chicken IgG-HRP conjugate was diluted 1:20,000 with Superblock and plates incubated 37°C, 1 h, followed by washing four times in wash buffer. The TMB substrate (KPL, Gaithersburg, MD, USA) was incubated 10 min in each well, stopped with 1 N HCl and absorbance measured in a Tecan Infinite M200 plate reader at 450 nm.

Growth and Survival of Mutants in M9, Feed, and Fecal Broth

Wild-type parent strain and mutants Δ*metR* and Δ*metR*Δ*metD* were grown in M9 minimal medium supplemented with 10 μM L- or D-methionine as indicated for 16 h and subsequently washed three times in PBS. Five grams of chicken feed were blended with 100 ml dionized water at high speed for 3 min. The same was done for 5 g of chicken feces, separately. These blends

TABLE 2 | Growth rates and doubling times of the single and double mutants.

Exp. #	Mutant	Met conc. (μM)	Growth rate (OD/h)	Doubling time (min)
1	*metR*	3	0.0091	76.17
		7	0.014	49.51
		10	0.0148	46.83
		13	0.0149	46.52
		15	0.0157	44.15
1	*metRmetD*	3	0.0016	433.21
		7	0.0022	315.07
		10	0.0067	103.45
		13	0.0109	63.59
		15	0.0124	55.90
2	*metR*	3	0.0105	66.01
		5	0.0122	56.82
		7	0.0127	54.58
		9	0.0136	50.97
		11	0.0141	49.16
2	*metRmetD*	9	0.0057	105.02
		11	0.0073	81.55
		13	0.0093	63.59
		15	0.0097	53.73
		17	0.0098	56.82

were then filtered through cheese cloth and autoclaved. These were subsequently aliquoted into sterile culture tubes and the washed *Salmonella* was added to a final density of 1×10^6 cells/ml. The cultures were incubated at 37°C, 200 RPM and growth of the cultures was monitored by spread-plating and colony counting of appropriate dilutions on LB medium at 0, 2, 5, 24, 48, 96, 264, and 504 h.

Statistical Analysis

The enumeration of the challenge (NA-resistant marker) strain in ceca of unvaccinated and vaccinated chickens was compared by a two-tailed Student's *t*-test, using the Microsoft Excel program.

RESULTS AND DISCUSSION

Growth curves of single Δ*metR* and ΔΔ*metRmetD* in M9 minimal medium supplemented with L- or D-methionine are shown in **Figure 2**. Both mutants exhibited reduced growth when compared with the control strain (*S.* Typhimurium UK-1) in both media with either L- or D-methionine. The double mutant exhibited little or no growth in D-methionine, as expected. The growth rates and doubling times of the mutants in M9 minimal medium are shown in **Table 2**. The growth rates were 10–20-fold lower than that reported by Froelich et al. for an *E. coli* methionine auxotroph (16). The whole-cell affinities of the single and

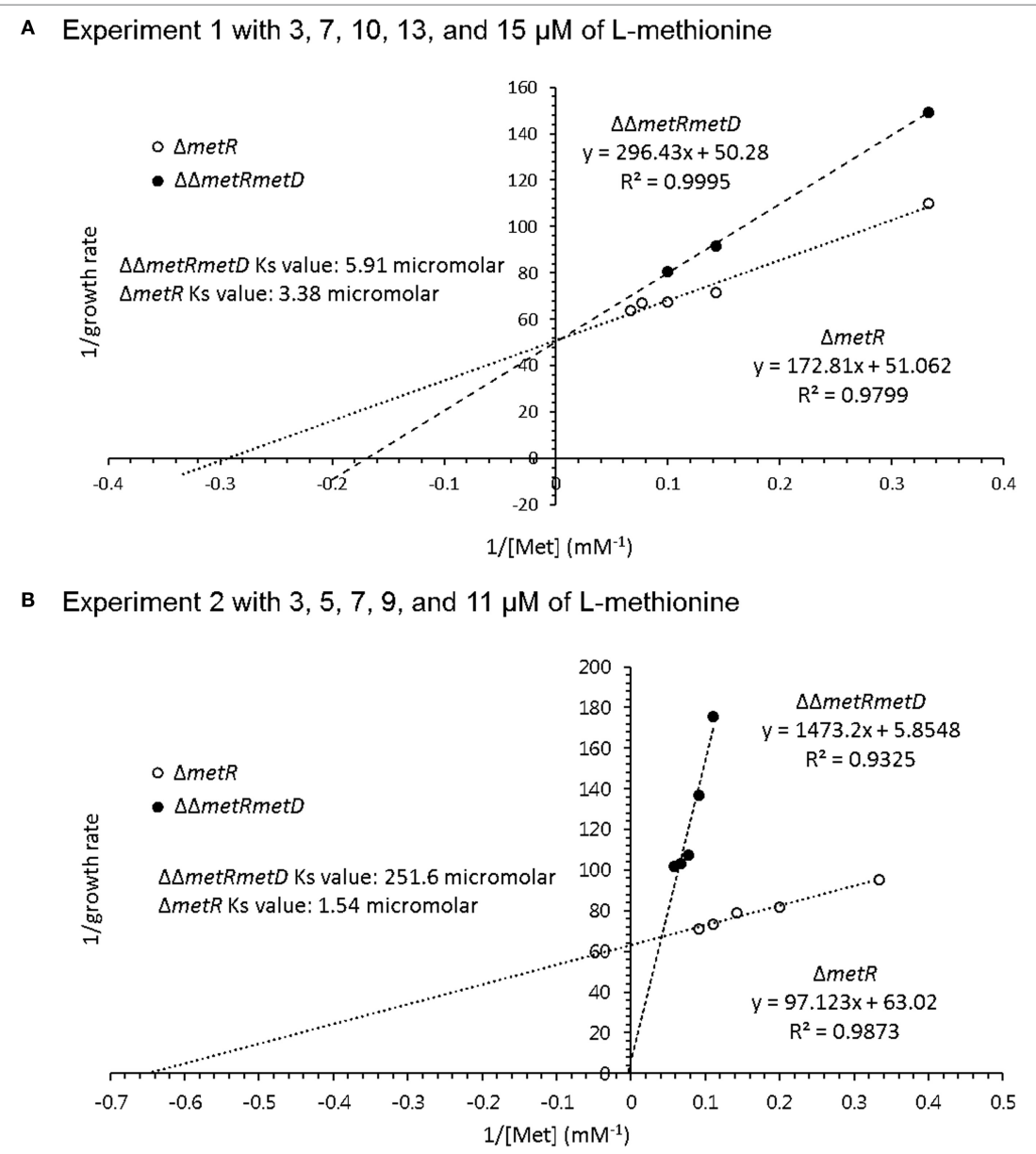

FIGURE 3 | Whole-cell affinity comparing growth of Δ*metR* and ΔΔ*metRmetD* mutant in minimal medium with added L-methionine. **(A)** Experiment 1 with 3, 7, 10, 13, and 15 μM of L-methionine; **(B)** Experiment 2 with 3, 5, 7, 9, and 11 μM of L-methionine.

double mutants for methionine are shown in **Figure 3**. Whole-cell affinity measurements indicated that the ΔΔ*metRmetD* double mutant had a consistently lower affinity (Ks) for L-methionine compared with the Δ*metR* single mutant (**Figure 3**). The Ks of the *metRmetD* double mutant was 5.90 and 251.6 μM in Experiments 1 and 2, respectively, while the corresponding values for the *metR* single mutant were 3.38 and 1.54 μM. Except for the outlier value of 251.6 μM, these values are similar to the Ks values of 6.41 and 7.00 reported by Froelich et al. for an *E. coli* methionine auxotroph, ATCC 23798 (16). The discrepancy between the much lower growth rate of the *Salmonella* auxotroph described in the present paper compared with the *E. coli* ATCC 23798 could be due to genetic differences. The genotype of ATCC 23798 is not known, but the parent strain is described as having been mutagenized with *N*-methyl-*N*-nitrosoguanidine (17).

Cecal prevalence of the *Salmonella* challenge strain was evaluated, and 100% were positive for the challenge strain in the unvaccinated and *metRmetD* vaccinated groups at the end of the trial, whereas 75 and 40% were positive for the challenge strain in the P_{BAD}-*mviN* and wild-type vaccinated groups as reported previously, respectively (11). Colonization of ceca was also measured by enumeration of challenge strain colonies on selective agar plates containing NA. Both the *metRmetD* and P_{BAD}-*mviN* vaccine strains significantly reduced ($P < 0.01$) the number of challenge strain *Salmonella* in the cecal contents when compared with the unvaccinated control group (the means ± SD were 4.71 ± 1.41 log CFU/g for *metRmetD*, 2.62 ± 0.8 log CFU/g for P_{BAD}-*mviN*, and 6.49 ± 0.61 log CFU/g for the unvaccinated group, partially reported in our previous study of the P_{BAD}-*mviN* vaccine) (11).

This suggests the vaccine strains partially protected against challenge strain colonization but based on the greater level of prevalence, the *metRmetD* vaccine candidate strain was not as well cleared by the birds. This is supported by two independent lines of evidence. For one, the survival curves of the methionine mutants in chicken feed and fecal material indicated a high degree of survival in these matrices versus incubation in minimal M9 medium (**Figure 4**). This may also be reflective of the fact that chickens vaccinated orally with the ΔΔ*metRmetD* mutant exhibited elevated levels of serum IgG binding specifically to *Salmonella* proteins in ELISA relative to an attenuated mutant strain, P_{BAD}-*mviN* and the unvaccinated group. The *metRmetD* mutant had a mean titer of 7840 ± 1711, while the P_{BAD}-*mviN* strain had a mean titer of 4520 ± 1544, and the unvaccinated group mean titer was 1700 ± 352.5 (11).

Given the superior immune response of the *metRmetD* mutant, this strain may warrant further research as a vaccine strain. However, this mutant does not appear to be easily cleared out by the inoculated birds and this could be problematic from an environmental contamination standpoint. There are possible remedies for this. To eliminate this problem and further reduce intracellular survival, further investigations may be required that eliminate some of the low-affinity methionine transporters in combination with the knock-out of the high affinity transporter *metD* reported here. Finally, different genes involved in methionine biosynthesis could also be targeted in addition to the transport genes. For example, *S.* Gallinarum *met*C mutants have been shown to possess diminished virulence capabilities, lowered levels in reticuloendothelial organs and competitiveness defects in challenged birds (18).

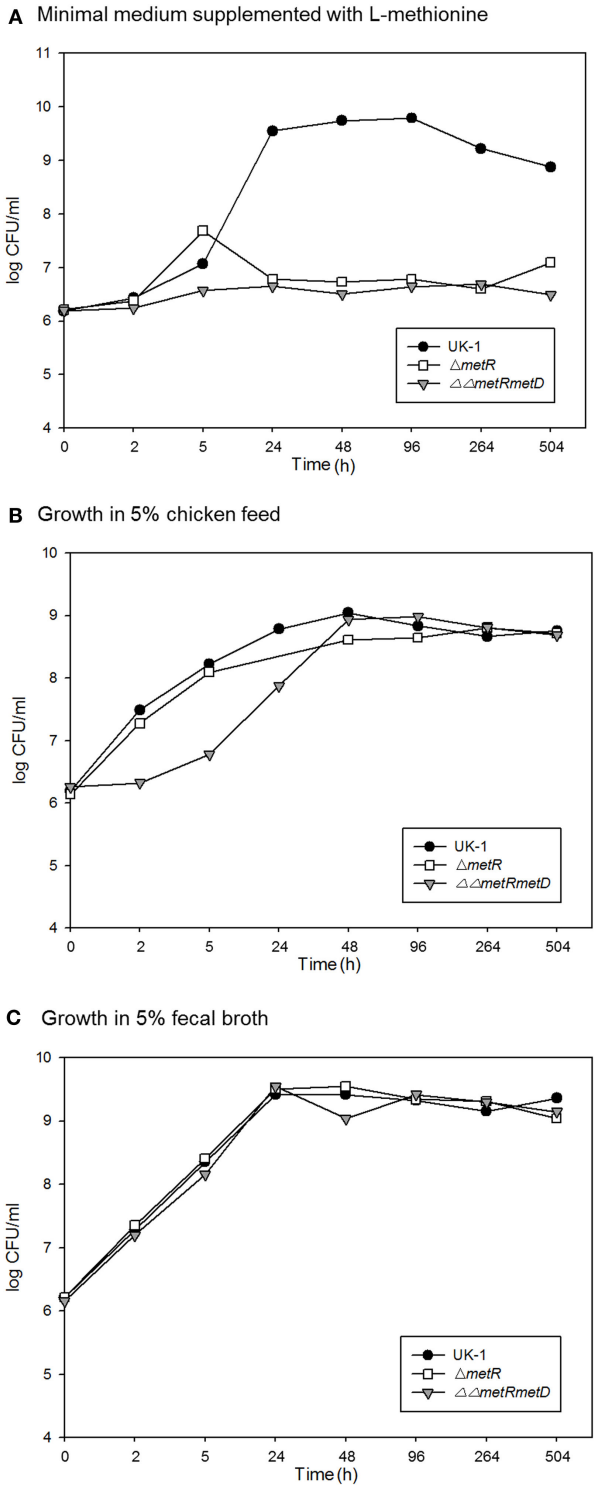

FIGURE 4 | Survival over time of the wild-type parent strain (*S.* Typhimurium UK-1) and methionine auxotrophic mutants in **(A)** M9 minimal medium; **(B)** 5% chicken feed; **(C)** 5% chicken feces.

In conclusion, new vaccine strains (Δ*metR* single mutant and ΔΔ*metRmetD*) were constructed in this study. The methionine auxotroph ΔΔ*metRmetD* generated a greater *Salmonella*-specific serum IgG level and reduced the level of *Salmonella* in cecal contents of approximately 100-fold relative to the unvaccinated control group. Particular combinations of methionine biosynthesis and transport mutants could result in optimal vaccine candidates that can be retained sufficiently to stimulate an optimal immune response but yet easily cleared via dietary manipulation.

AUTHOR CONTRIBUTIONS

PR performed experiments, drafted the manuscript, collected test data, and analyzed the data. SK drafted and revised the manuscript. PR, SP and SR designed the study and revised the manuscript.

FUNDING

This work was funded by USDA-SBIR grant award 2012-33610-19529.

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

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 25 August 2016
doi: 10.3389/fvets.2016.00066

Prophylactic Administration of Vector-Encoded Porcine Granulocyte-Colony Stimulating Factor Reduces *Salmonella* Shedding, Tonsil Colonization, and Microbiota Alterations of the Gastrointestinal Tract in *Salmonella*-Challenged Swine

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OPEN ACCESS

Edited by:
Guillermo Tellez, University of Arkansas, USA

Reviewed by:
Jason Kindrachuk, National Institutes of Health, USA
Sherry Layton, Vetanco S.A., Argentina

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *30 June 2016*
Accepted: *08 August 2016*
Published: *25 August 2016*

Citation:
Bearson SMD, Bearson BL, Loving CL, Allen HK, Lee I, Madson D and Kehrli ME Jr. (2016) Prophylactic Administration of Vector-Encoded Porcine Granulocyte-Colony Stimulating Factor Reduces Salmonella Shedding, Tonsil Colonization, and Microbiota Alterations of the Gastrointestinal Tract in Salmonella-Challenged Swine. Front. Vet. Sci. 3:66. doi: 10.3389/fvets.2016.00066

Salmonella colonization of food animals is a concern for animal health and public health as a food safety risk. Various obstacles impede the effort to reduce asymptomatic *Salmonella* carriage in food animals, including the existence of numerous serovars and the ubiquitous nature of *Salmonella.* To develop an intervention strategy that is non-specific yet effective against diverse *Salmonella* serovars, we explored the prophylactic use of a cytokine to decrease *Salmonella* in swine by boosting the host's innate immune system. Granulocyte-colony stimulating factor (G-CSF) is the major cytokine regulating the production, differentiation, function, and survival of neutrophils. Neutrophils play a critical role in the response to *Salmonella;* therefore, we evaluated the vectored-delivery of porcine G-CSF as a prophylactic to reduce *Salmonella* in pigs. Crossbred pigs, 5 weeks of age, were intramuscularly injected with a replication-defective human adenovirus (Ad5) engineered to express porcine G-CSF (Ad5-G-CSF, $n = 9$). Control pigs received the same Ad5 vector lacking the gene encoding G-CSF (Ad5-empty, $n = 7$). Four days later, all pigs ($n = 16$) were intranasally inoculated with 1×10^7 colony forming unit (CFU) of *Salmonella enterica* serovar Typhimurium UK1. At 2 and 3 days post-challenge with *Salmonella*, Ad5-G-CSF-treated pigs shed significantly less *Salmonella* (~10^3 CFU/g) in their feces than Ad5-empty-treated pigs (~10^4–10^5 CFU/g; $P < 0.05$). A significant 4-log reduction in tonsil colonization was also observed in the Ad5-G-CSF-treated pigs at 7 days post-challenge ($P < 0.05$). In the gastrointestinal tract, the Peyer's patch region of the ileum exhibited a significant 0.5-log reduction in colonization in the Ad5-G-CSF-treated pigs ($P < 0.05$). The microbiota of all challenged pigs was assessed

by sequencing and analyzing the V1–V3 region of the 16S rRNA gene from fecal DNA samples. The microbial community structure of *Salmonella*-challenged pigs was less disturbed post-challenge in the Ad5-G-CSF-treated pigs than the Ad5-empty-treated pigs. This suggests that Ad5-G-CSF administration mitigated changes in the microbial community structure caused by *Salmonella* challenge. Collectively, these data suggest that delivery of a targeted immunostimulant to enhance neutropoiesis may be a strategy to reduce *Salmonella* colonization, potentially during periods of immunological stress.

Keywords: *Salmonella*, granulocyte-colony stimulating factor, swine, immune stimulation, alternatives to antibiotics

INTRODUCTION

Asymptomatically colonized food animals are a major reservoir of the human foodborne pathogen *Salmonella* (1–3). Intervention strategies are needed to not only decrease the overall prevalence of *Salmonella* in food animals but also reduce an animal's susceptibility to *Salmonella* during times of production stress, such as farrowing, weaning, mixing, and transportation. Controlling *Salmonella* is challenging due to the broad host range, ubiquitous distribution, and number of *Salmonella* serovars (>2,500). To overcome the complexity of *Salmonella*, management strategies that target innate immune mechanisms warrant exploration to control the commensal-like state of this human foodborne pathogen in the gastrointestinal tract of animals contributing to our food (animal) supply.

An animal's innate immune system offers multiple pathways that can be modulated to fight disease-causing agents without activation of the adaptive immune system, which is the primary target of vaccination strategies. Instead, bolstering an innate immune response during stressful events in animal production or periods of immune dysfunction could reduce pathogen recrudescence and infection susceptibility. One possible intervention to address this vulnerability is the prophylactic use of biotherapeutic proteins, such as cytokines [reviewed in Ref. (4)]. Granulocyte-colony stimulating factor (G-CSF) is a cytokine involved in the production, differentiation, and function of granulocytes (especially neutrophils) from bone marrow (5–7). Neutrophils are phagocytic cells of the innate immune system, and their killing mechanism provides a critical first line of defense against bacterial and viral infections (8). Recombinant human G-CSF (Neulasta, Amgen Inc.) is FDA-approved for use in humans to decrease the incidence of infection in neutropenic patients receiving myelosuppressive anti-cancer drugs (9). Recombinant bovine G-CSF (Imrestor, Elanco) has also been approved by the FDA for use in dairy cattle to restore neutrophil function and neutrophil numbers during periparturient immune suppression (10–13). Previous work by our group established that the delivery of a replication-defective human adenovirus 5 encoding porcine G-CSF increased the number of functional neutrophils in circulation (14), thus demonstrating the potential for modulating the swine immune system by targeting the G-CSF pathway.

Rapid neutrophil influx into the intestines is the hallmark of a *Salmonella* infection (15, 16). In our previous work, cytokines involved in neutrophil production and recruitment were upregulated in swine following *Salmonella* challenge (17, 18). Furthermore, van Diemen et al. demonstrated higher numbers of circulating neutrophils with greater polymorphonuclear neutrophil (PMN) function in pigs bred for resistance to *Salmonella enterica* serovar Choleraesuis (19). Thus, we hypothesized that elevating the abundance of circulating neutrophils in pigs prior to *Salmonella* exposure may assist in controlling *Salmonella* colonization and shedding. The results demonstrate the beneficial effects of Ad5-G-CSF-induced neutrophilia on the reduction of *S. enterica* serovar Typhimurium (*S.* Typhimurium) colonization and shedding in swine, as well as decreased *Salmonella*-induced disturbance of the gastrointestinal microbiota, suggesting prophylactic use of porcine Ad5-G-CSF may serve as a biotherapeutic approach to reduce *Salmonella* in pigs.

MATERIALS AND METHODS

Swine Experiment

Sixteen crossbred, conventionally reared piglets from three *Salmonella*-fecal-negative sows were weaned at 12 days of age and shipped to the National Animal Disease Center, Ames, IA, USA. Siblings from each litter were divided and raised in two isolation rooms. Piglets tested fecal-negative for *Salmonella spp*. twice over a 2-week period using bacteriological culture with selective enrichment (20). At 5 weeks of age, piglets received an intramuscular injection of 10^{10} $TCID_{50}$/pig of a replication-defective human adenovirus (Ad5) engineered to express porcine G-CSF (Ad5-G-CSF, $n = 9$) (14). As previously described, Ad5-G-CSF was derived by directionally cloning G-CSF cDNA into the AdEasyTM XL System (Stratagene, La Jolla, CA, USA) and propagated in specialized AD-HEK-293 cells. Control pigs received the same Ad5 vector lacking the gene encoding G-CSF (Ad5-empty, $n = 7$). Four days later, all pigs ($n = 16$) were intranasally inoculated with 1×10^7 colony forming unit (CFU) of a nalidixic acid-resistant derivative of *S. enterica* serovar Typhimurium UK1 (21) that had been passaged in swine and isolated from the ileocecal lymph node of a pig (strain name: SB 377). Fecal samples were collected at 0, 1, 2, 3, and 7 days post-inoculation (d.p.i.) for microbiota analysis as well as quantitative and qualitative *Salmonella* culture analyses (see below). Blood samples were collected from the jugular vein at −4, −2, 0, 1, 2, 3, and 7 d.p.i. for enumeration of circulating blood cells by flow cytometry (see below). At 7 d.p.i., all pigs were euthanized and necropsied to obtain tissue samples from the tonsil and the intestinal tract (ileal Peyer's patches, ileocecal lymph nodes, and cecum) for quantitative and qualitative *Salmonella* culture analysis (see below). Procedures involving

animals followed humane protocols as approved by the USDA, ARS, NADC Animal Care and Use Committee in strict accordance with the recommendations in the Guide for the Care, and Use of Laboratory Animals of the National Institutes of Health.

Bacteriology

For quantitative bacteriology, 1 g of pig feces was combined with 5 ml PBS, vortexed, and 0.1 ml directly plated to XLT-4 medium (Beckton, Dickinson and Co., Sparks, MD, USA) containing 30 μg/ml of nalidixic acid. For tissue samples, 1 g of each tissue was combined with 2 ml of PBS in a whirlpak bag, pounded with a mallet, and homogenized in a Stomacher (Seward, Westbury, NY, USA) for 1 min. One hundred microliters of the resulting solution was aliquoted onto XLT-4 medium containing nalidixic acid. One hundred microliters of a 10-fold dilution of each fecal and tissue sample was also plated, and additional dilutions were performed when CFU reached >300/plate. Following 48 h of incubation at 37°C, black colonies were enumerated and a single colony from each plate was confirmed to be *Salmonella* by serogroup antiserum agglutination (Beckton, Dickinson and Co., Sparks, MD, USA). The total number of CFU for each quantitative tissue or fecal sample was calculated per gram of sample by obtaining the number of *Salmonella* per plate and multiplying by the dilution factor.

Qualitative bacteriology of *Salmonella* was performed as follows: 1 g (fecal) or 0.1 ml (homogenized tissue) samples were inoculated in 10 ml tetrathionate broth (TET; VWR, Rutherford, NJ, USA) for 48 h of growth at 37°C. Following incubation, 0.1 ml of each culture was transferred to 10 ml Rappaport–Vassiliadis medium (RV; Difco) and incubated at 37°C for 18–20 h. Cultures were streaked on XLT-4 medium containing nalidixic acid. Colonies suspicious for *Salmonella* were confirmed by serogroup antiserum agglutination.

Statistical analysis for *Salmonella* shedding in feces (CFU/g) was Log10-transformed and analyzed using a mixed linear model for repeated measures (Proc Mixed in SAS for Windows, version 9.2; SAS Institute Inc., Cary, NC, USA). Covariance structures within pigs across time were tested and modeled using the REPEATED statement to determine the optimal covariance structure. Linear combinations of the least-squares mean estimates were used in *a priori* contrasts after testing for a significant ($P < 0.05$) treatment group effect. Comparisons were made between each group at each time point, using a 5% level of significance ($P < 0.05$) to assess statistical differences. The endpoint data for bacterial colonization (CFU/g) of tissues collected at necropsy were Log10-transformed and analyzed by analysis of variance using a general linear model for unbalanced data. A 5% level of significance ($P < 0.05$) was used to assess statistical differences.

Whole Blood Cell Differential

White blood cell counts were performed via flow cytometry as previously described (14). Briefly, a 50-μl aliquot of anticoagulated (EDTA) whole blood was added to a tube containing monoclonal antibody to porcine granulocytes (6D10, Serotech, USA) with appropriate secondary fluorochrome-labeled antibody. After a 20-min incubation, cells were fixed and red blood cells lysed with the addition of 1 ml FACS lyse (BD Biosciences, USA). Microbeads (Spherotech, USA) were added to the tube immediately prior to data acquisition on a flow cytometer (BD LSR II, Becton Dickinson, USA). A gate was drawn around the beads and events were collected on each parameter (neutrophil gate was based on forward and side scatter properties and antibody labeling) until the bead event number was 500. A ratio of total counts to bead counts was used to determine the number of neutrophils per microliter of blood. Calculation of statistical significance of neutrophils per microliter of blood by treatment group was performed using a one-way ANOVA with a Dunnett's Multiple Comparison Test. A 5% level of significance ($P < 0.05$) was used to assess statistical differences.

16S rRNA Gene Sequencing and Analysis

Amplicon libraries of the 16S rRNA gene were generated and sequenced according to Kozich et al. (22), with our primers and procedures described previously (23). Briefly, PCRs contained the following: 17 μl AccuPrime Pfx SuperMix (Life Technologies, Grand Island, NY, USA), 5.0 μM each of the primers i5 + V3 and i7 + V1, and 25 ng of fecal DNA. The following PCR conditions were used: 2 min at 95°C, 22 cycles of (20 s at 95°C, 15 s at 55°C, 5 min at 72°C), 72°C for 10 min. Libraries were normalized using the SequalPrep Normalization Plate Kit (LifeTechnologies) and quantified using both Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and Kapa SYBR Fast qPCR (Kapa Biosystems, Wilmington, MA, USA). Normalized pools were sequenced using version 3 (300 × 2) chemistry on the MiSeq instrument (Illumina, San Diego, CA, USA) according to manufacturer's instructions.

Contig assembly, sequence alignment, chimera removal, and non-bacterial sequence removal were performed in the program mothur (version 1.33.3) (24). Sequences that only occurred once or twice across all samples were removed as potentially spurious. Sequences were rarified to 3,000 sequences, clustered into operational taxonomic units (OTUs) at 97% similarity, and analyzed for community metrics, including richness (25), evenness, and diversity. Analysis of similarity (ANOSIM) and non-metric multidimensional scaling (NMDS) analyses were conducted in PAST (26). Additionally, the OTUs were assigned to bacterial taxonomy using mothur's implementation of the SILVA database (27). One sample from a pig in the Ad5-G-CSF group at day 7 yielded insufficient sequences to be analyzed. The 16S rRNA gene sequences associated with this study were deposited in Genbank under Bioproject PRJNA339155.

RESULTS

Both *Salmonella* Typhimurium Challenge and Porcine Ad5-G-CSF Administration Increased Circulating Neutrophils in Pigs

The effects of Ad5-G-CSF administration and *S.* Typhimurium challenge on circulating neutrophils were determined by enumerating neutrophils in the blood at various days after Ad5-G-CSF administration and *S.* Typhimurium challenge. *S.* Typhimurium challenge alone induced a significant approximately threefold increase in circulating neutrophil counts, as values post-challenge were greater when compared to values on the day of challenge

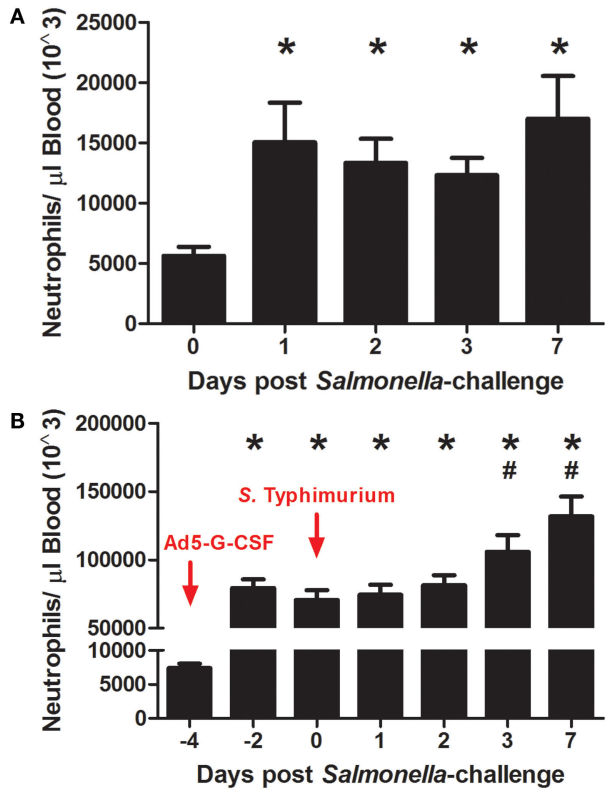

FIGURE 1 | The number of circulating neutrophils following *S.* Typhimurium challenge and Ad5-G-CSF administration. Blood samples were collected at the noted day relative to *Salmonella* challenge for neutrophil enumeration by flow cytometry as described in Section "Materials and Methods." Data are reported as the mean + SEM. **(A)** Pigs were intranasally challenged with 1×10^7 CFU of virulent *S.* Typhimurium UK1 strain ($n = 7$). Significant difference ($P < 0.05$) in circulating neutrophils relative to day 0 (*). **(B)** Pigs received a single intramuscular injection of Ad5-G-CSF followed by S. Typhimurium challenge with 1×10^7 CFU of *S.* Typhimurium UK1 ($n = 9$). Significant difference ($P < 0.05$) in circulating neutrophils relative to day −4 (*) or day 0 (#).

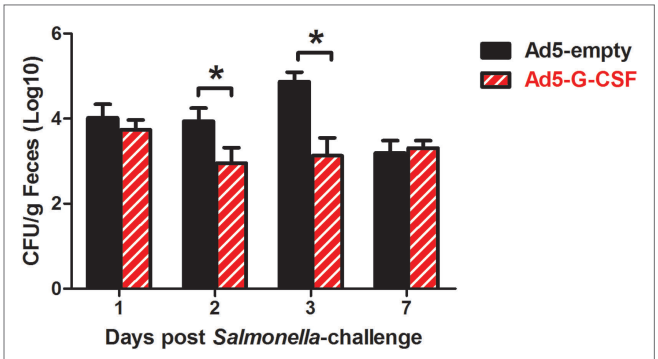

FIGURE 2 | Fecal shedding from *Salmonella*-challenged pigs, with or without prior Ad5-G-CSF administration. On day 4 following Ad5-G-CSF or Ad5-empty administration, all 16 pigs were challenged with 1×10^7 CFU of *S.* Typhimurium UK1. *Salmonella* fecal shedding was monitored via bacteriological analysis of fecal samples collected at 1, 2, 3, and 7 d.p.i. *At each timepoint, significant difference ($P < 0.05$) in *Salmonella* CFU/g feces comparing pigs administered Ad5-G-CSF versus Ad5-empty.

(day 0) (**Figure 1A**). Circulating neutrophils were also enumerated in pigs that were intramuscularly injected with 10^{10} $TCID_{50}$ Ad5-G-CSF prior to *Salmonella* exposure. As expected on the day of *S.* Typhimurium challenge (day 0), which was 4 days after Ad5-G-CSF administration, a significant neutrophilia occurred compared to pre-Ad5-G-CSF numbers (day −4, **Figure 1B**) or compared to Ad5-empty-treated controls on day 0 (day 0, **Figure 1A**). Following *Salmonella* challenge of the Ad5-G-CSF group, an additional significant increase in circulating neutrophils was observed at 3 and 7 d.p.i. compared to day 0 (day of *Salmonella* challenge). Collectively, Ad5-G-CSF administration induced a significant and sustained ~10-fold increase in the number of circulating neutrophils, and *Salmonella* challenge also induced significant increases in circulating neutrophils.

Ad5-G-CSF Treatment Reduced *Salmonella* Fecal Shedding and Tissue Colonization

Salmonella shedding and tissue colonization were compared between Ad5-G-CSF and Ad5-empty-treated pigs. Ad5-G-CSF-treated pigs shed significantly less *Salmonella* (10^3 CFU/g feces) when compared to the Ad5-empy-treated pigs at 2 and 3 d.p.i. (10^{4-5} CFU/g feces) (**Figure 2**). This 1- to 2-log difference between the treatment groups dissipated by 7 d.p.i. as *Salmonella* shedding in the feces of Ad5-empty-treated pigs declined to the level of the Ad5-G-CSF-treated pigs. Typical for swine, a transient fever was observed in the *S.* Typhimurium-challenged pigs, peaking at 2 days post-challenge; no significant difference was observed in the elevated body temperatures between treatment groups (data not shown). Gastrointestinal tissues (ileocecal lymph nodes, Peyer's patch region of the ileum, and cecum) were analyzed at 7 d.p.i., and all tissues were *Salmonella* positive in both Ad5-G-CSF-treated and Ad5-empty-treated pigs. Of these three tissues, the Peyer's patch region of the ileum exhibited a significant 0.5-log reduction in *Salmonella* colonization in the Ad5-G-CSF-treated pigs compared to the Ad5-empty-treated group (**Figure 3A**). A striking difference in tonsil colonization was observed between treatment groups (**Figure 3B**). Eight of the nine Ad5-G-CSF-treated pigs harbored no detectable *Salmonella* in the tonsil, with only one pig being qualitatively positive for *Salmonella* in the tonsils (i.e., by enrichment). By contrast, all seven Ad5-empty-treated pigs harbored *Salmonella* in the tonsils at an average of ~10,000 CFU/g. These data suggest that prophylactic administration of Ad5-G-CSF can reduce *Salmonella* colonization and subsequent fecal shedding, including the tonsils that have been implicated in the carrier-status of *Salmonella* (28–31).

The Gastrointestinal Microbiota of *Salmonella*-Challenged Pigs Was More Stable in the Ad5-G-CSF-Treated Pigs

Fecal 16S rRNA gene sequence data were used to compare the gastrointestinal bacterial communities of the Ad5-G-CSF and Ad5-empty treatment groups following *Salmonella* challenge. No significant differences in indices for diversity, evenness, or richness were detected among treatments or timepoints. OTU-based analysis of bacterial community structure showed that at

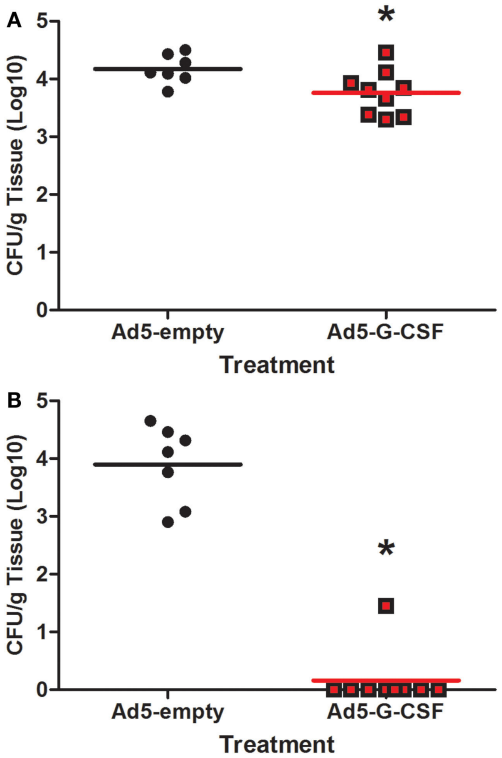

FIGURE 3 | Tissue colonization from *Salmonella*-challenged pigs, with or without prior Ad5-G-CSF administration. At 7 d.p.i., *Salmonella* bacteriological analysis (CFU/g) of the **(A)** Peyer's patch region of the ileum and **(B)** tonsils obtained during necropsy. *Significant difference ($P < 0.05$) comparing Ad5-G-CSF-treated to Ad5-empty-treated pigs at the same time point.

7 days post-*Salmonella* challenge, the microbiota of pigs that had received Ad5-G-CSF was not significantly different from that of day 2 or 3 (ANOSIM, $p > 0.05$; $R < 0.1$), but the microbiota of pigs that received Ad5-empty treatment was significantly different at day 7 compared to all previous time points (ANOSIM, $p < 0.05$; $R > 0.25$). However, the difference between the Ad5-G-CSF-treated and Ad5-empty-treated groups at day 7 was insignificant. The dissimilarity of the microbiotas between days 3 and 7 was visualized via an NMDS plot, which showed the disturbed microbiota at day 7 in the Ad5-empty-treated animals compared to Ad5-G-CSF-treated animals (**Figure 4**). These results demonstrate that Ad5-G-CSF administration slightly decreases the beta-diversity changes in the microbiota that are caused by *Salmonella* challenge, suggesting that Ad5-G-CSF mitigated the disturbance to the gut microbiota that was caused by *Salmonella*.

DISCUSSION

Granulocyte-colony stimulating factor is a cytokine that influences the proliferation, differentiation, maturation, function, and survival of neutrophils (32). Neutrophils are a critical mediator of antimicrobial defense during the initial stages of infection and have effects on a number of microbial targets (8). Circulating neutrophil numbers in swine have been correlated with resistance to salmonellosis, with pigs most resistant to *Salmonella* exhibiting higher numbers of circulating neutrophils and enhanced neutrophil function (19). In the current study, treatment of swine with Ad5-G-CSF increased the number of circulating neutrophils by 10-fold, decreased *Salmonella*-induced disturbance of the gastrointestinal microbiota, and reduced *Salmonella* fecal shedding 1–2 logs during the acute stage of infection. Thus, prophylactic use of G-CSF as an immunostimulant may be an effective strategy to reduce *Salmonella* in swine herds. A farm-to-consumption quantitative microbiological risk assessment (QMRA) for *Salmonella* in pigs in the European Union concluded that interventions should focus on decreasing the level of *Salmonella* in the feces of infected swine because the vast majority of human risk is derived from a subset of pigs with a high concentration of *Salmonella* in their feces ($\geq 10^4$ CFU/g) (33). In our study, the Ad5-empty-treated pigs shed *Salmonella* at 10^{4-5} CFU/g, and Ad5-G-CSF treatment reduced the level of *Salmonella* fecal shedding to 10^3 CFU/g, further supporting G-CSF administration as a possible risk mitigation strategy.

The dramatic reduction in *Salmonella* colonization of the tonsils in the Ad5-G-CSF-treated pigs also highlights prophylactic treatment with G-CSF as a potential control strategy for persistently infected pigs. *Salmonella* can reside in lymph nodes and especially the tonsils (28–31). In this carrier-state, a stressful event (farrowing, weaning, or transport) can trigger *Salmonella* to re-emerge and reseed the gastrointestinal tract, resulting in shedding recrudescence (34, 35). Reduction of tonsil colonization, as observed in the Ad5-G-CSF-treated pigs, may reduce the reseeding process during stress. An intriguing follow-up study would be to evaluate the recrudescence of *Salmonella* in colonized pigs that are given Ad5-G-CSF prior to an applied stress (mixing, transportation, etc.). While additional investigations of the efficacy and safety of Ad5-G-CSF administration in swine are warranted, our data suggest that increasing the number of circulating neutrophils via Ad5-G-CSF administration may offer a non-specific yet effective method for reducing *Salmonella* colonization in swine.

Inflammation-associated intestinal dysbiosis can result in pathogen expansion, especially for microorganisms, such as *Salmonella*, that are capable of taking advantage of an inflamed environment (36). We have previously shown that *Salmonella* colonization of the porcine gastrointestinal tract causes a disturbance within the gut microbial community (37) and triggers an inflammatory response from the host (17, 18). Intervention strategies that target *Salmonella* during the initial stages of colonization could reduce overall gut inflammation and subsequently prevent the development of a "nutrient-niche" that can be selectively used by *Salmonella* (38). In the current study, prophylactic Ad5-G-CSF administration was beneficial in reducing the *Salmonella*-induced microbiota disturbance. Nevertheless, neutrophils are a primary player in the inflammatory response, and their contribution to the inflammatory response that provides an optimal environment for *Salmonella* expansion needs to be considered with an intervention strategy that increases neutrophils in circulation. It may be important to establish an optimal neutrophilia for the greatest *Salmonella* reduction with minimal neutrophil-stimulated tissue damage

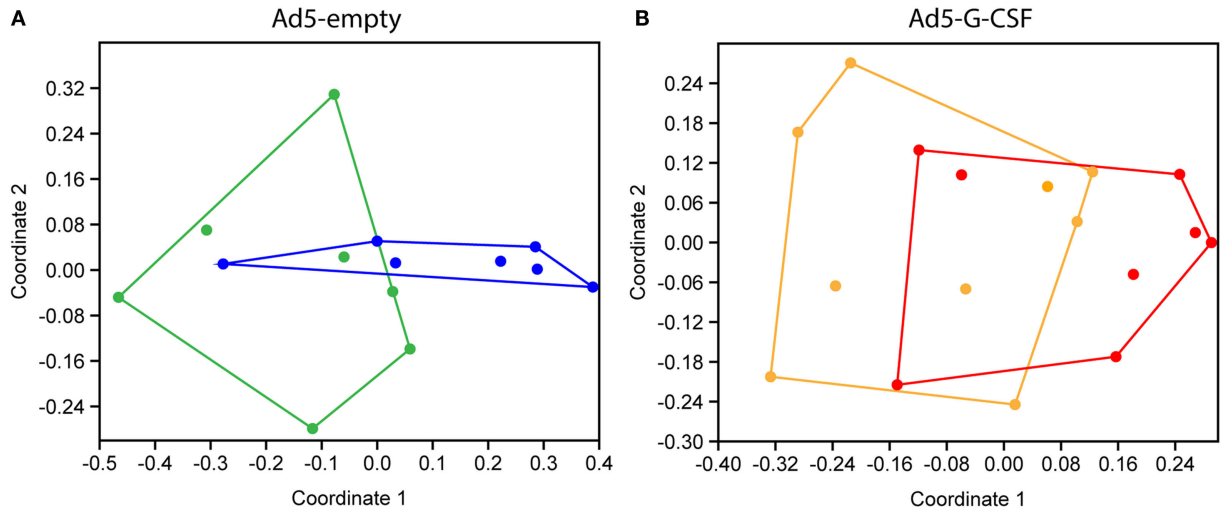

FIGURE 4 | Non-metric multidimensional scaling (NMDS) analyses of 16S rRNA gene OTUs from *Salmonella*-challenged pigs with prior administration of Ad5-empty (A) or Ad5-G-CSF (B). Shown are the fecal microbiotas from samples taken at 3 days [**(A)**, green; **(B)**, orange] and 7 days [**(A)**, blue; **(B)**, red] post-*Salmonella* challenge. OTU cutoff of 97% similarity was used. Stress = **(A)**, 0.1445; **(B)**, 0.1453.

that, in itself, could encourage *S.* Typhimurium virulence factor-induced inflammation (36).

As regulatory and public scrutiny necessitates the judicious use of antibiotics in food animals (39, 40), the need for antibiotic alternatives in animal production intensifies. Naturally occurring biotherapeutics engineered for pharmaceutical application offer an alternative to antibiotic usage, especially for prophylactic or possibly metaphylactic administration during periods of anticipated stress and host susceptibility. Through the general activation of innate immune defenses, immunostimulants may provide effective pathogen reduction or elimination with broad application against bacteria and viruses that pose a food safety threat or that negatively impact animal health. Our results suggest that prophylactic use of Ad5-G-CSF in swine could decrease subclinical or clinical disease by microorganisms that are targeted by neutrophils.

AUTHOR CONTRIBUTIONS

SB, BB, CL, HA, DM, and MK conceived and designed experiments. SB, BB, CL, HA, IL, and DM performed the experiments. SB, BB, CL, HA, and MK wrote and edited the manuscript.

ACKNOWLEDGMENTS

We are grateful of the superior technical support of Kellie Winter, Jennifer Jones, David Michael, Stephanie Jones, and Zahra Olson. This research was supported by USDA, ARS CRIS funds.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 19 April 2017
doi: 10.3389/fvets.2017.00056

Optimizing Fluorescein Isothiocyanate Dextran Measurement As a Biomarker in a 24-h Feed Restriction Model to Induce Gut Permeability in Broiler Chickens

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Edited by:
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Reviewed by:
Geetha Samak,
DVS College of Arts and Science, India
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 13 February 2017
Accepted: 03 April 2017
Published: 19 April 2017

Citation:
Baxter MFA, Merino-Guzman R, Latorre JD, Mahaffey BD, Yang Y, Teague KD, Graham LE, Wolfenden AD, Hernandez-Velasco X, Bielke LR, Hargis BM and Tellez G (2017) Optimizing Fluorescein Isothiocyanate Dextran Measurement As a Biomarker in a 24-h Feed Restriction Model to Induce Gut Permeability in Broiler Chickens. Front. Vet. Sci. 4:56. doi: 10.3389/fvets.2017.00056

Fluorescein isothiocyanate dextran (FITC-d) is a 3–5 kDa marker used to measure tight junction permeability. We have previously shown that intestinal barrier function can be adversely affected by stress, poorly digested diets, or feed restriction (FR), resulting in increased intestinal inflammation-associated permeability. However, further optimization adjustments of the current FITC-d methodology are possible to enhance precision and efficacy of results in future. The objective of the present study was to optimize our current model to obtain a larger difference between control and treated groups, by optimizing the FITC-d measurement as a biomarker in a 24-h FR model to induce gut permeability in broiler chickens. One *in vitro* and four *in vivo* independent experiments were conducted. The results of the present study suggest that by increasing the dose of FITC-d (8.32 versus 4.16 mg/kg); shortening the collection time of blood samples (1 versus 2.5 h); using a pool of non-FITC-d serum as a blank, compared to previously used PBS; adding a standard curve to set a limit of detection and modifying the software's optimal sensitivity value, it was possible to obtain more consistent and reliable results.

Keywords: broiler chickens, enteric inflammation, fluorescein isothiocyanate dextran, feed restriction, gut permeability

INTRODUCTION

Intestinal epithelial cells are not only responsible for digestion, secretion, and absorption but also act as a physical barrier separating external environmental agents from the internal host environment. In addition to preventing the entry of harmful intraluminal microorganisms, antigens, and toxins, this barrier increases the bodies' tolerance to nutrients, water, and electrolytes (1–3). Microbes that live inside and/or on animals outnumber the animals' actual somatic and germ cells by an estimated 10-fold (4). Hence, any alterations in gut permeability are associated with bacterial translocation to the portal and/or systemic circulation leading to systemic bacterial infections (5, 6). Consequently, our laboratory has recently developed several models to induce intestinal inflammation in poultry.

Those models include high non-starch polysaccharides diets (7, 8); dexamethasone (9); dextran sodium sulfate (DSS) (10, 11); and 24-h feed restriction (FR) (12, 13). In the above models, inflammation causes disruption of the epithelial tight junctions (TJs) increasing bacterial translocation and leakage of serum fluorescein isothiocyanate dextran (FITC-d). FITC-d is a large molecule (3–5 kDa) which under normal conditions is not able to cross the epithelial barrier (14). However, during intestinal inflammation, the TJs are disrupted allowing the FITC-d molecule to enter circulation. Previous results from our laboratory have demonstrated that in poultry, chemically induced disruption of TJs with DSS (10) increases transmucosal permeability as seen by elevated serum levels of FITC-d (11). On the other hand, recently, we have shown that dietary inclusion of a *Bacillus*-based direct-fed microbial ameliorated the adverse gut permeability inflammatory effects related to utilization of rye-based diets in turkeys and in broiler chickens (15, 16). We have previously shown that FITC-d can be used as a biomarker for intestinal barrier function (7–12). However, further optimization adjustments of the current FITC-d methodology are possible to enhance precision and efficacy of results in future studies as can be observed in **Figure 1** and **Table 5**. The objective of the present study was to optimize our current FITC-d model to obtain a larger difference between control and treated groups, using our 24-h FR model to induce gut permeability in broiler chickens.

MATERIALS AND METHODS

Fluorescein Isothiocyanate Dextran

Fluorescein isothiocyanate dextran (MW 3–5 KDa; Sigma Aldrich Co., St. Louis, MO, USA) was used as a marker of paracellular transport and mucosal barrier dysfunction.

In Vitro Evaluation of Different Fluorescence Gain Using Blank Chicken Sera from Chickens without FITC-d

Unlike absorbance assays where the gain on the plate reader is fixed and not user changeable, fluorescence assays have varying concentration ranges and require the gain on the photomultiplier to be adjusted. In this *in vitro* experiment, the following formula was used to predict the relative fluorescence units when changing the gain:

$$\text{Estimate of RFU at new gain setting} = (\text{new PMT gain}/\text{old PMT gain})^{7.3} \times \text{RFU at old PMT gain.}$$

To determine if fluorescence changes with varying gain, blank chicken sera and 0.9% saline were compared. Non-FITC-d chicken sera was diluted 1:5 in 0.9% saline onto black 96-well fluorescent plates and measured from gain 40 to gain 80 with continuous increments of 10. Non-FITC-d sera were also used to develop a standard curve adapted for every plate using six two-fold serial dilutions from the highest value 6,400 ng/mL until it reach 0 ng/mL (**Table 1**).

Experimental Animals

Four *in vivo* experiments were conducted to determine the optimal procedure for using FITC-d as a biomarker for intestinal permeability. In all trials, broiler chickens were obtained from a primary breeder company and all experiments were conducted in battery cages in a controlled age-appropriate environment.

FR Model

In all experiments, intestinal permeability was induced using FR as previously published (12, 13). Chickens were randomly

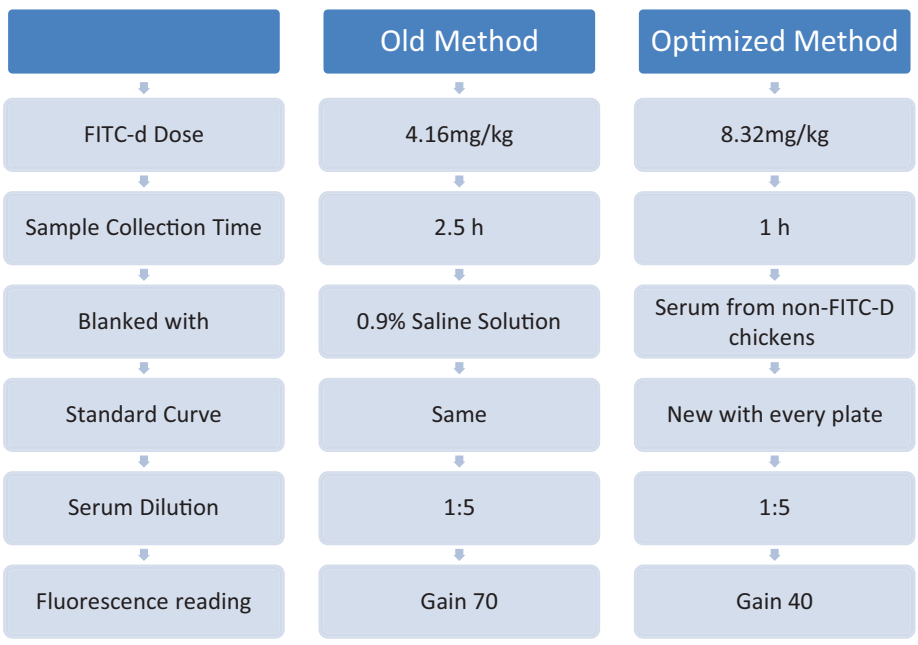

FIGURE 1 | **Comparing old versus optimized methodology**.

assigned to each experimental group and had unrestricted access to feed and water from 1 to 10 days of age. Beginning at 10 days, chickens in control FITC-d groups were allowed to continue with *ad libitum* access to feed, while chickens in FR FITC-d groups were subjected to 24 h of FR. Concentration of FITC-d was given based on group body weight; therefore, groups were weighed the day before FR began. At 11 days of age, chickens in all groups were given an appropriate dose of FITC-d by oral gavage for each experiment. After 1 h, or 2.5 h respectively, chickens were euthanized with CO_2 asphyxiation. Blood samples were collected from the femoral vein to quantify levels of FITC-d.

Serum Determination of FITC-d

In all experiments, blood was centrifuged (1,000 × *g* for 15 min) to separate the serum from the red blood cells. FITC-d levels of diluted sera were measured at excitation wavelength of 485 nm and emission wavelength of 528 nm (Synergy HT, Multi-mode microplate reader, BioTek Instruments, Inc., VT, USA). Fluorescence measurements were then compared to a standard curve with known FITC-d concentrations (old method) or non-FITC-d sera obtain from each independent experiment, respectively, to develop a standard curve as described in the *in vitro* methods.

TABLE 1 | Evaluation of different fluorescence gain using blank chicken sera, from chickens without fluorescein isothiocyanate dextran (FITC-d), versus 0.9% saline solution.

	Gain 40	Gain 50	Gain 60	Gain 70	Gain 80
0.9% saline solution	1.0 ± 0.27^{b}	0.75 ± 0.31^{b}	12.6 ± 0.18^{b}	37.9 ± 0.74^{b}	93.1 ± 1.3^{b}
Blank sera	1.6 ± 0.11^{a}	1.1 ± 0.10^{a}	20.6 ± 0.38^{a}	59.8 ± 1.1^{a}	255.6 ± 6.6^{a}

Non-FITC-d sera was diluted 1:5 in 0.9% saline onto black 96-well fluorescent plates and measured from gain 40 to 80.
^{a,b}Superscripts within columns indicate significant difference at P < 0.05.
Data are expressed as mean ± SE. n = 20 birds/treatment.

TABLE 2 | Comparing two serum dilution methods on serum fluorescein isothiocyanate dextran (FITC-d) (4.16 mg/kg) read at gain 70 in a 24-h feed restriction (FR) model to induce gut permeability in broiler chickens (Experiment 1).

Experimental group	Serum FITC-d (ng/mL) Diluted 1:5	Serum FITC-d (ng/mL) Diluted 1:10
Control no FITC-d	7.7 ± 2.7^{b,y}	1.0 ± 0.9^{b,z}
FR no FITC-d	11.4 ± 3.4^{b,y}	2.5 ± 1.6^{b,z}
Control FITC-d	9.1 ± 2.8^{b,y}	2.5 ± 1.2^{b,z}
FR FITC-d	23.1 ± 4.3^{a,y}	16.8 ± 3.1^{a,z}

^{a,b}Superscripts within columns indicate significant difference at P < 0.05.
^{y,z}Superscripts within rows indicate significant difference at P < 0.05.
Data are expressed as mean ± SE. n = 20 birds/treatment. In both comparisons, blanked serum was used to make a standard curve with every plate.

Experimental Designs

Experiment 1: Comparing Two Dilution Methods on Serum FITC-d Read at Gain 70 in a 24-h FR Model

Eighty chickens were randomly assigned to one of four groups ($n = 20$/group): (1) control no FITC-d; (2) FR no FITC-d; (3) control FITC-d 4.16 mg/kg; and (4) FR FITC-d 4.16 mg/kg. Control groups had *ad libitum* access to feed, meanwhile FR groups were feed restricted for 24 h before sampling. Serum was diluted at 1:5 or 1:10 to determine if a higher dilution factor would eliminate some of the background fluorescence. Readings were performed with a gain 70.

Experiment 2: Comparing Two Sampling Collection Times and Different Gain Readings of Serum FITC-d in a 24-h FR Model

In this experiment, all chickens received FITC-d (4.16 mg/kg) and samples were collected at 1 or 2.5 h post FITC-d administration. Eighty chickens were randomly assigned to one of four groups ($n = 20$/group): (1) control FITC-d collected 1 h post gavage; (2) FR FITC-d collected 1 h post-gavage; (3) control FITC-d collected 2.5 h post-gavage; and (4) FR FITC-d collected 2.5 h post-gavage. Control groups had *ad libitum* access to feed, meanwhile FR groups were feed restricted for 24 h before sampling. Serum was diluted at 1:5 and readings were done with gains 30, 35, 40, and 45.

Experiment 3: Comparing Collection Time of Serum FITC-d Diluted 1:5 and Read at Gain 40 in a 24-h FR Model

In this experiment, all chickens received FITC-d (8.32 mg/kg) and samples were collected at 1 or 2.5 h post FITC-d administration. Eighty chickens were randomly assigned to one of four groups ($n = 20$/group): (1) control FITC-d collected 1 h post-gavage; (2) FR FITC-d collected 1 h post-gavage; (3) control FITC-d collected 2.5 h post-gavage; and (4) FR FITC-d collected 2.5 h post-gavage. Control groups had *ad libitum* access to feed,

TABLE 3 | Comparing two sampling collection times and different gain readings of serum fluorescein isothiocyanate dextran (FITC-d) (4.16 mg/kg) in a 24-h feed restriction (FR) model to induce gut permeability in broiler chickens (Experiment 2).

Experimental group	Serum FITC-d (ng/mL) Gain 30	Serum FITC-d (ng/mL) Gain 35	Serum FITC-d (ng/mL) Gain 40	Serum FITC-d(ng/mL) Gain 45
Control FITC-d 1 h	0.00 ± 0.0.00^{c,z}	61.0 ± 21.4^{c,y}	49.6 ± 16.2^{c,y}	56.3 ± 16.2^{c,y}
FR FITC-d 1 h	207.5 ± 69.9^{b,y}	284.4 ± 37.7^{b,x}	185.6 ± 14.5^{a,z}	177.1 ± 13.9^{a,z}
Control FITC-d 2.5 h	390.8 ± 84.4^{a,x}	257.0 ± 25.0^{b,y}	118.9 ± 11.0^{b,z}	95.4 ± 8.8^{b,z}
FR FITC-d 2.5 h	468.1 ± 75.7^{a,x}	336.5 ± 41.9^{a,y}	191.3 ± 24.0^{a,z}	153.3 ± 19.4^{a,z}

^{a,b,c}Superscripts within columns indicate significant difference at P < 0.05.
^{x,y,z}Superscripts within rows indicate significant difference at P < 0.05.
Data are expressed as mean ± SE. n = 20 birds/treatment. Serum was diluted 1:5. Blanked serum was used to make a standard curve with every plate.

TABLE 4 | Comparing collection times of serum fluorescein isothiocyanate dextran (FITC-d) (8.32 mg/kg) diluted 1:5 and read at gain 40 in a 24-h feed restriction (FR) model to induce gut permeability in broiler chickens (Experiment 3).

Experimental group	Serum FITC-d (ng/mL)
Control 1 h	78.7 ± 9.4[c]
FR 1 h	136. 5 ± 7.3[a]
Control 2.5 h	67.1 ± 7.9[c]
FR 2.5 h	112.4 ± 6.5[b]

[a,b,c]*Superscripts within columns indicate significant difference at P < 0.05.*
Data are expressed as mean ± SE. n = 20 birds/treatment.

TABLE 5 | Comparing old method versus optimized method of serum fluorescein isothiocyanate dextran (FITC-d) in a 24-h feed restriction (FR) model to induce gut permeability in broiler chickens (Experiment 4).

Experimental group	Serum FITC-d (ng/mL) Old method	Serum FITC-d (ng/mL) Optimized method
Control	306.0 ± 41.1[a,y]	101.8 ± 36.0[b,z]
FR	388.0 ± 28.0[a,z]	397.3 ± 22.1[a,z]
FITC-d dose (mg/kg)	4.16	8.32
Sample collection time (h)	2.5	1
Blanked with	0.9% saline solution	Serum from non-FITC-d chickens
Standard curve	Same	New with every plate
Serum dilution	1:5	1:5
Fluorescence reading	Gain 70	Gain 40

[a,b]*Superscripts within columns indicate significant difference at P < 0.05.*
[y,z]*Superscripts within rows indicate significant difference at P < 0.05.*
Data are expressed as mean ± SE. n = 20 birds/treatment.

meanwhile FR groups were feed restricted for 24 h before sampling. Serum was diluted at 1:5 and readings were done using gain 40.

Experiment 4: Comparing the Old Method versus Optimized Method of Serum FITC-d in a 24-h FR Model

The objective of this experiment was to compare our previous FITC-d method to the new optimized FITC-d method. Eighty chickens were randomly assigned to one of four groups ($n = 20$/group): (1) control FITC-d (4.16 mg/kg) collected 2.5 h post-gavage; (2) FR FITC-d (4.16 mg/kg) collected 2.5 h post-gavage; (3) control FITC-d (8.32 mg/kg) collected 1 h post-gavage; and (4) FR FITC-d (8.32 mg/kg) collected 1 h post-gavage. In the old method, serum was diluted 1:5, fluorescence measurements were quantified using an equation from a previously determined standard curve with known FITC-d concentrations using 0.9% saline solution as a blank and measuring samples at gain 70. In the optimized method, serum from non-FITC-d chickens was obtained, to be used as a blank. Additionally, for each plate, a standard curve was adapted diluting known concentrations of FITC-d in the 1:5 diluted blank sera as described above in the *in vitro* method. All serum samples were also diluted 1:5 for fluorescence reading at gain 40 (**Table 5**).

Statistical Analysis

All data were subjected to Analysis of Variance as a completely randomized design using the General Linear Models procedure of SAS (17). In all trials, data are expressed as mean ± standard error. Significant differences among the means were determined by using Tukey's multiple-range test at $P < 0.05$.

RESULTS

The results of the *in vitro* evaluation of different fluorescence gains using blank chicken sera, from chickens without FITC-d, versus 0.9% saline solution are summarized in **Table 1**. There was a significant difference between blank sera and 0.9% saline solution at each of the gains measured (40, 50, 60, 70, 80) (**Table 1**). This indicates that blank sera has a higher amount of fluorescence activity than 0.9% saline and is affected by the gain. **Table 2** illustrates the results from Experiment 1 comparing two serum dilution methods on serum FITC-d (4.16 mg/kg) read at gain 70 in a 24-h FR model. In this study, using the same sera, samples were diluted 1:5 and 1:10 to determine if a higher dilution factor would eliminate some of the background fluorescence. A significant reduction in the background fluorescence was observed in all samples diluted at 1:10 ($P < 0.05$). Interestingly, serum samples from FR chickens treated with FITC-d diluted at 1:5 or 1:10 showed significantly higher amounts of serum FITC-d concentration when compared with control chickens.

Results from Experiment 2 comparing two sampling collection times and different gain readings of serum FITC-d (4.16 mg/kg) in a 24-h FR model are summarized in **Table 3**. Collecting the blood samples 1 h post FITC-d gavage not only showed significant increases in serum FITC-d concentration in chickens that received FR when compared with control chickens at all four gain readings but also the window of differences between feed restricted and control broilers were more evident when compared with serum collected at 2.5 h (**Table 3**).

Table 4 displays the results from Experiment 3, comparing collection time of serum FITC-d doubling the dose of FITC-d (8.32 mg/kg). Serum was diluted at 1:5 and read at gain 40 in a 24-h FR model. These results confirmed and extended the results of Experiment 2. Sample collection time gives a stronger reading of serum FITC-d in FR chickens when is performed 1 h after FITC-d oral administration when compared with 2.5 h (**Table 4**).

The results from Experiment 4, comparing old method versus optimized method of serum FITC-d in a 24-h FR model are summarized in **Figure 1** and **Table 5**. In the old method, chickens received 4.16 mg/kg FITC-d, serum samples were collected 2.5 h post gavage, samples were diluted 1:5 and fluorescence was measured using a previously determined standard curve, 0.9% saline solution was used as a blank and measured at gain 70. No significant differences were observed between control and FR chickens. In contrast, in the optimized method, chickens receiving 8.32 mg/kg FITC-d, serum samples were collected 1 h post gavage, were diluted 1:5, non-FITC-d serum was used as a blank, a standard curve was developed for each plate and a reading of gain 40, showed significant differences between control and FR chickens (**Figure 1**; **Table 5**).

DISCUSSION

Stress is known to affect gastrointestinal tract (GIT) homeostasis by altering gut motility, permeability, as well as alterations in ion, fluid, and mucus secretion and absorption (18–21). Several

investigators have reported that acute or chronic stress modifies gut permeability associated with a temporary redistribution of TJ proteins (22–25). Some of these alterations are linked to mast cells in the brain–gut axis which secrete several neurotransmitters and pro inflammatory cytokines, with profound effects on GIT physiology (26–28). Another hormone that increases during acute or chronic stress is corticotrophin-releasing factor, which increases intestinal paracellular permeability *via* mast cell-dependent release of TNF-α and proteases (29–31). Moreover, excessive cortisol may lead to GIT disturbances, opportunistic infections, and impaired wound healing (32–34). Due to intensive selection, modern chickens are the most efficient meat-producing animals because of their fast growth, supported by a virtually unlimited voluntary feed intake. However, these features also cause many problems in breeder hens because of the negative correlation between muscle growth and reproduction effectiveness. Hence, commercial restricted feeding programs in broiler breeders have been implemented, with negative effects on welfare and health, as birds are continuously hungry (35). Previous research in poultry has shown that FR increases plasma levels of corticosterone causing disruption of gut barrier integrity, systemic, and local inflammation (36–39). Similarly, we have previously shown in poultry that intestinal barrier function can be adversely affected by stress, poorly digested diets (7, 8), or FR (12, 13), resulting in increased intestinal inflammation-associated permeability. In those studies, we have described a correlation of liver bacterial translocation and serum concentrations of FITC-d as markers used to measure TJ permeability. FITC-d is a 3–5 kDa marker used to measure TJ permeability in chickens using enteric inflammation models. However, inconsistent results obtained from unpublished data suggested that current FITC-d methodology required further optimization. FITC-d has also been reported to be a viable method to measure enteric leakage in the murine model (40). However, they used a different methodology. Therefore, FITC-d methodology may vary with the animal model and this should be taken into consideration when using it to measure gut permeability. The results of the present study suggest that by increasing the dose of FITC-d (8.32 versus 4.16 mg/kg); shortening the collection time of the blood (1 versus 2.5 h); using a pool of non-FITC-d serum as a blank, compared to previously used 0.9% saline; generating a standard curve with every plate to set a limit of detection and modifying the software's optimal sensitivity value, it is possible to obtain more consistent and reliable results when measuring gut leakage in poultry.

ETHICS STATEMENT

All animal handling procedures were in compliance with Institutional Animal Care and Use Committee at the University of Arkansas.

AUTHOR CONTRIBUTIONS

MB, RM-G, JL, and GT contributed to the overall study design and supervised all research. BM, YY, KT, LG, and AW carried out the experiments and acquisition of data. MB and GT drafted and revised the first version of the manuscript. JL and GT analyzed the data. LB, XH-V, BH, and GT drafted the article and revised it critically for important intellectual content. XH-V and GT were responsible for the final editing of the manuscript. All the authors reviewed and finally approved the manuscript.

ACKNOWLEDGMENTS

This research was supported by the Arkansas Bioscience Institute under the project: development of an avian model for evaluation early enteric microbial colonization on the gastrointestinal tract and immune function.

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

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 14 February 2017
doi: 10.3389/fvets.2017.00015

Quantitative Tracking of *Salmonella* Enteritidis Transmission Routes Using Barcode-Tagged Isogenic Strains in Chickens: Proof-of-Concept Study

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OPEN ACCESS

Edited by:
Ryan Arsenault, University of Delaware, USA

Reviewed by:
Christi Swaggerty, United States Department of Agriculture, USA
Kenneth James Genovese, United States Department of Agriculture, USA

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *17 October 2016*
Accepted: *30 January 2017*
Published: *14 February 2017*

Citation:
Yang Y, Ricke SC, Tellez G and Kwon YM (2017) Quantitative Tracking of Salmonella Enteritidis Transmission Routes Using Barcode-Tagged Isogenic Strains in Chickens: Proof-of-Concept Study. Front. Vet. Sci. 4:15. doi: 10.3389/fvets.2017.00015

Salmonella is an important foodborne bacterial pathogen, however, a fundamental understanding on *Salmonella* transmission routes within a poultry flock remains unclear. In this study, a series of barcode-tagged strains were constructed by inserting six random nucleotides into a functionally neutral region on the chromosome of *S.* Enteritidis as a tool for quantitative tracking of *Salmonella* transmission in chickens. Six distinct barcode-tagged strains were used for infection or contamination at either low dose (10^3 CFUs; three strains) or high dose (10^5 CFUs; three strains) in three independent experiments (Experiment 1 oral gavage; Experiment 2 contaminated feed; Experiment 3 contaminated water). For all chick experiments, cecal and foot-wash samples were collected from a subset of the chickens at days 7 or/and 14, from which genomic DNA was extracted and used to amplify the barcode regions. After the resulting PCR amplicons were pooled and analyzed by MiSeq sequencing, a total of approximately 1.5 million reads containing the barcode sequences were analyzed to determine the relative frequency of every barcode-tagged strain in each sample. In Experiment 1, the high dose of oral infection was correlated with greater dominance of the strains in the ceca of the respective seeder chickens and also in the contact chickens yet at lesser degrees. When chicks were exposed to contaminated feed (Experiment 2) or water (Experiment 3), there were no clear patterns of the barcode-tagged strains in relation to the dosage, except that the strains introduced at low dose required a longer time to colonize the ceca with contaminated feed. Most foot-wash samples contained only one to three strains for the majority of the samples, suggesting potential existence of an unknown mechanism(s) for strain exclusion. These results demonstrated the proof of concept of using barcode tagged to investigate transmission dynamics of *Salmonella* in chickens in a quantitative manner.

Keywords: *Salmonella* Enteritidis, transmission, chickens, barcode-tagged isogenic strains, quantitative tracking

INTRODUCTION

Salmonella species induce bacterial illness and are also one of the leading causes of hospitalization among all the foodborne bacterial pathogens (1, 2). According to the Centers for Disease Control and Prevention, there are approximately 1.2–4 million human *Salmonella* infections in the United States each year (3–5). There are multiple sources of *Salmonella* infection in humans such as consumption of contaminated food and water or contact with infected animals (6). Among others, poultry products are a prominent source of human salmonellosis, and the contamination can originate from a multitude of sources during poultry production (7–9). *S.* Enteritidis is considered as one of the most commonly identified serovars in association with human infection in the United States (10). The number of human infections by *S.* Enteritidis continued to increase from the 1980s and had reached the point where *S.* Enteritidis became the predominant serovar in the 1990s and currently still remains a prominent foodborne disease-causing serovar (11, 12). Therefore, it is critical not only to understand the transmission modes of *S.* Enteritidis in chicken flocks but also to be able to quantitate their relative contribution of each route to contamination during poultry production. Knowing the quantitative contribution of various transmission routes would be very helpful in designing optimal strategies to minimize the spread of *Salmonella* within a chicken flock *via* interventions such as vaccines and antimicrobials administered in the feed or drinking water (13, 14).

The transmission of *Salmonella* in a chicken flock involves an initial infection with single or multiple *Salmonella* strains from different sources through oral or tracheal routes (15–19). While the oral route is believed to be the primary infection route of *Salmonella* based on experimental evidence (1, 2), there are indications that airborne transmission is also a possible route (20–23). Once infection occurs, the *Salmonella* population disseminates in the host from the entry site and may colonize the intestinal tract or systemically invade the host tissues (24). Once a host becomes infected locally in the intestinal tract or systemically, *Salmonella* can, in turn, be disseminated to other susceptible hosts (25).

Salmonella, as an enteric pathogen, can be disseminated to poultry flocks through several sources. Drinking water, feed, wildlife or pets, transportation mode, manure, or litter can be vehicles contributing to dissemination of *Salmonella* into poultry (26). Water is an important vehicle and can serve as a reservoir for *Salmonella* dissemination. *Salmonella* possesses the capacity to not only survive in the water for a long period of time but the expression of key virulence factors can also be increased when *Salmonella* is exposed to stressors in a water environment (27). *Salmonella* appears to possess the mechanisms to retain viability and successfully survive in river environments as well. The relationship between the contaminated feed and the occurrence of *Salmonella* in poultry has been substantiated by several studies (28, 29). For the reason of labor and technical simplification, most chicken feed is produced in the farm as milled and blended mash, most of which are not heat treated or pelleted. The vertical integration nature of the commercial poultry production cycle could impact the risk of introducing pathogens such as *Salmonella* to poultry production as a result of contaminated feed (30, 31).

Quantitative resolution of critical routes for *Salmonella* establishment in chickens requires the ability to track the strains introduced to the flock distinctively using some sort of recoverable signature. Traditionally, *Salmonella* monitoring has been based on techniques such as introducing foreign elements into the candidate strain to construct marker strains that are antibiotic resistant or express genes for fluorescence proteins (32–34). However, in these methods, the risk in introducing phenotypic features into the resulting marker strains is that it could alter the pathogenicity and physiological status such that the resulting strains no longer behave in exactly same fashion as the corresponding wild type. For example, green fluorescence proteins have been shown to alter growth physiology, while exposure to nalidixic acid can influence gene expression (35, 36). It is well established that acquisition of antibiotic resistance often entails fitness cost or enhanced fitness of the pathogenic strains in the absence of selection pressure (37).

The objective of the present study was to evaluate the proof of concept of barcode-tagged isogenic strains of *Salmonella* Enteritidis in broiler chickens using different routes of infection. A series of isogenic *S.* Enteritidis strains in which distinct DNA barcodes were inserted in a functionally neutral locus in the genome were constructed and the resulting strains employed to quantitatively track the transmission routes of the respective strains by profiling the barcode regions using high-throughput sequencing. The advantages of these barcode-tagged strains over previously used marker strains are that each strain can be tracked quantitatively as a distinguishable part of the entire population at high accuracy, allowing for differentiation among multiple barcode-tagged strains as well as discrimination from the environmental *Salmonella* without altering phenotypes or behaviors during infection, colonization, and dissemination.

MATERIALS AND METHODS

Bacterial Strains and Culture Condition

Salmonella enterica serovar Enteritidis phage type 13A strain, which is a primary poultry isolate, was originally obtained from the USDA National Veterinary Services Laboratory (Ames, IA, USA). The plasmid pKD4 was used as a template to amplify the kanamycin resistance gene for construction of the barcode-tagged strains. The *Escherichia coli* strain BW25141 carrying pKD4 was inoculated in Luria-Bertani (LB) broth overnight, and plasmid pKD4 was extracted with the illustra plasmidPrep Mini Spin Kit (GE Healthcare Life Sciences). The *Salmonella* Enteritidis strain (SE) containing pKD46 that encodes Red recombinase system was used for construction of barcode-tagged strains *via* electroporation (38, 39). The plasmid pKD46 contains an ampicillin resistance gene and is also a temperature-sensitive replicon requiring 30°C for replication of the plasmid in the cell. LB broth was used for cultivation of barcode-tagged strains. Super optimal broth with catabolite repression (SOC) media (Invitrogen, Carlsbad, CA, USA) was used for phenotypic expression of the transformed cells immediately after the electroporation. Appropriate antibiotics were used at the following concentrations when necessary: kanamycin (Km) at 50 μg/ml and ampicillin (Amp) at 100 μg/ml.

Rationale for the Genomic Location Selection

Ideally, the barcode along with the kanamycin resistance gene should be inserted into a functionally neutral genomic locus. Based on Chaudhuri et al. (40), we first searched for two adjacent genes that are not required for intestinal colonization in chickens and are also transcribed toward each other. We manually searched for the target locus for barcode insertion in the genome based on the result of Chaudhuri et al. (40) and found that SEN1521 and SEN1522 met these two conditions, and therefore, the intergenic region (141 bp) between these two genes was selected for insertion of a barcode plus the kanamycin resistance gene among other candidate loci (**Figure 1**). When foreign sequences are inserted in the middle of this intergenic region without removing any original genomic sequences, it can be ensured that the insertion would not cause any polar effect on the downstream genes that would minimize, if any, phenotypic change due to the barcode insertion.

Construction of Barcode-Tagged Strains

All PCR primers are listed in **Table 1**. The 3′ end and downstream regions of the coding genes SEN1521 (232 bp) and SE1522 (267 bp) were amplified from the genomic DNA of *S.* Enteritidis 13A with the primer pairs of T1-F and T1-BC (Barcode)-P1-R, and T3-P2-F and T3-R, respectively (termed, upstream and downstream fragments, respectively) (**Figure 1**). The T1-BC-P1-R primer contained a barcode of six random nucleotides and the sequence overlapping with 5′ end of the Km resistance gene (P1). The T3-P2-F primer contained the sequence overlapping with 3′ end of the Km gene (P2). The Km resistance gene (1,496 bp) was amplified from the plasmid pKD4 with the primer pair of P1 and P2. The PCR assays were conducted by combining approximately 0.1 μg of purified genomic DNA or plasmid along with 1 μl of 2.5 U/μl Pfu polymerase (Agilent Technologies), 5 μl of 10 × cloned Pfu polymerase buffer, 4 μl of 2.5 mM dNTPs (TaKaRa), and 1 μl of 1.2 μM of each primer resulting in a total volume of 50 μl. The DNA Engine® Thermal Cycler (Bio-Rad, Hercules, CA, USA) was used with the following amplification cycles: 94°C for 2 min; 30 cycles of 94°C for 30 s, 58°C for 60 s, 72°C for 60 s per 1 kb; and 72°C for 10 min for the final extension. Each PCR product was gel purified and eluted in 25 μl EB buffer (10 mM Tris–Cl; pH 8.5) for preparation of templates to be used for overlapping extension PCR. Overlapping extension PCR was employed to join the three fragments (upstream fragment plus a barcode + Km resistance gene + downstream fragment) together with the primers T1-F and T3-R (**Figure 1**). After running the agarose gel for confirmation of the correct size, electroporation was used to introduce the overlapping PCR fragments into *S.* Enteritidis carrying pKD46 plasmid. A number of transformants selected on LB agar plates supplemented with Km were first analyzed by PCR for the presence of the barcode plus kanamycin resistance gene in the correct genomic locus with the primers BC-F and BC-R and, if positive, analyzed for barcode sequences

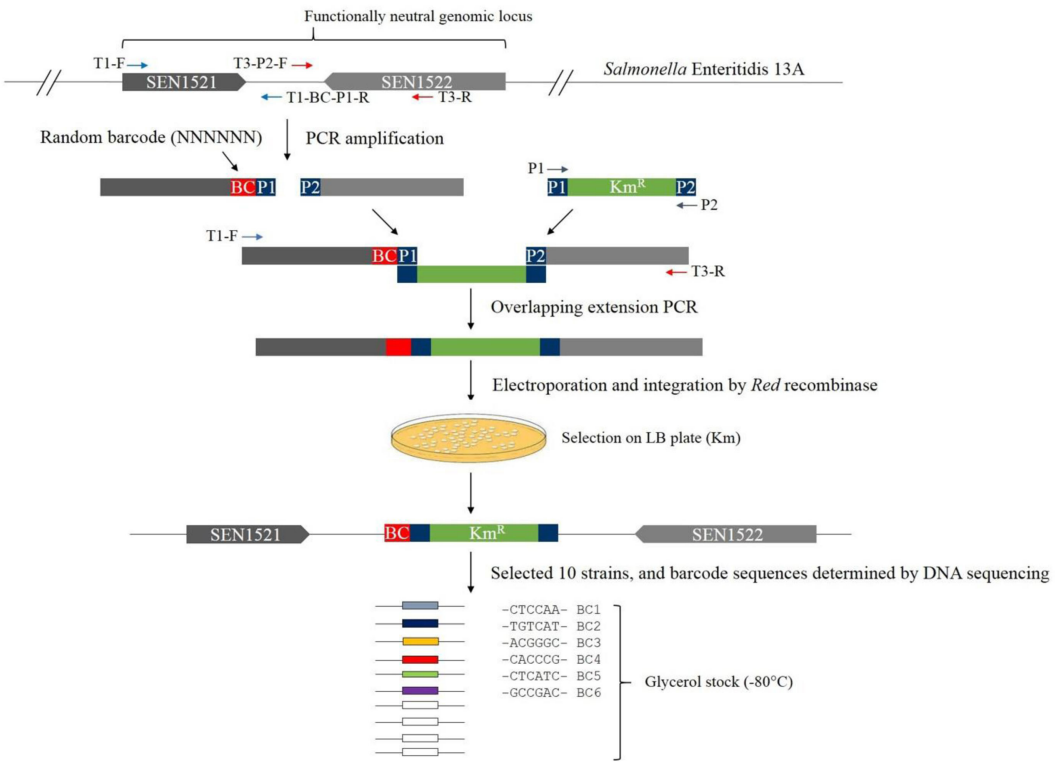

FIGURE 1 | Schematics of the construction of barcode-tagged *Salmonella* Enteritidis 13A strains.

TABLE 1 | Oligonucleotides used in this study.

Primers for construction of barcode-tagged strains (5′→3′)	
T1-F	GCAAGGTTGGTGTCTGTCCT
T1-BC-P1-R	GAAGCAGCTCCAGCCTACACNNNNNNATTATTGTTAATTTATTCTT
P1	GTGTAGGCTGGAGCTGCTTC
P2	ATGGGAATTAGCCATGGTCC
T3-P2-F	GGACCATGGCTAATTCCCATAAAGGTTAAGCAGTGACCCA
T3-R	GTTGATGGACTGGGTTCGTT
BC-F	AGCGTCCTGAAATAATAAAAGAA
BC-R	CGGACTGGCTTTCTACGTGT
Illumina index forward primers (5′→3′) 6 nt-index sequences are underlined	
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATCACGGCGTCCTGAAATAATAAAAGAATAAA	
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCGATGTGCGTCCTGAAATAATAAAAGAATAAA	
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTTAGGCGCGTCCTGAAATAATAAAAGAATAAA	
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTGACCAGCGTCCTGAAATAATAAAAGAATAAA	
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTACATGTGCGTCCTGAAATAATAAAAGAATAAA	
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGCCAATGCGTCCTGAAATAATAAAAGAATAAA	
Illumina index reverse primers (5′→3′) 6 nt-index sequences are underlined	
CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTATCACGGAAGCAGCTCCAGCCTACAC	
CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTCGATGTGAAGCAGCTCCAGCCTACAC	
CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTTTAGGCGAAGCAGCTCCAGCCTACAC	
CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTTGACCAGAAGCAGCTCCAGCCTACAC	
CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTACATGTGAAGCAGCTCCAGCCTACAC	
CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTGCCAATGAAGCAGCTCCAGCCTACAC	
CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTCAGATCGAAGCAGCTCCAGCCTACAC	
CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTACTTGAGAAGCAGCTCCAGCCTACAC	
CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTGATCAGGAAGCAGCTCCAGCCTACAC	

Underline sequences indicate Illumina index adapter.

by Sanger sequencing of the PCR products (41). Finally, we isolated and confirmed 10 barcode-tagged strains carrying unique barcodes. Six of them were used in this study, and the barcodes in the respective strains were BC1 (CTCCAA), BC2 (TGTCAT), BC3 (ACGGGC), BC4 (CACCCG), BC5 (CTCATC), and BC6 (GCCGAC).

Chicken Infection Experiments

All animal procedures in this study were conducted in accordance with the protocol approved by the University of Arkansas Institutional Animal Care and Use Committee. In all experiments, day-of-hatch broiler chicks were obtained from Cobb-Vantress (Siloam Springs, AR, USA). To test the utility of the barcode-tagged strains for quantitative tracking of *Salmonella* transmission, we set up three independent experiments as described below.

Oral Infection into Seeder Chickens Experiment 1

Six chickens were randomly selected for oral infection with *Salmonella* barcode-tagged strains (referred to as seeder chickens hereafter) on day 1. Three of the chickens (seeder chickens #1–3) were orally infected at low dose (10^3 CFUs) with BC1, BC2, and BC3 strains, respectively. The other three chickens (seeder chickens #4–6) were orally infected at high dose (10^5 CFUs) with BC4, BC5, and BC6 strains, respectively. The other 10 chickens were not infected with any barcode-tagged strains and were referred to as contact chickens (# 7–16). Seeder and contact chickens were housed together for 14 days. On day 7, post-infection three contact chickens (# 7–9) were euthanized, and cecal contents were removed and stored at −20°C for genomic DNA isolation. Each bird foot was washed thoroughly in 5 ml of PBS buffer in a sterile Ziploc bag, and bacterial cells from the rinse were subsequently harvested *via* centrifugation at 4,468 × *g* for 10 min. The bacterial pellets were stored at −20°C and used for genomic DNA isolation. On day 14, four seeder chickens (chick 1, 4, 5, 6; chick #2 and 3 were not sampled) and four contact chicks (chick 10–13) were also euthanized and cecal contents as well as foot wash were collected for DNA isolation as described previously.

Consumption of Contaminated Feed Experiment 2

The same six barcode-tagged strains were used to inoculate a balanced antibiotic-free corn/soybean-based diet at two different levels: at low dose (10^3 CFUs) with BC1, BC2, and BC3 strains, respectively, and at high dose (10^5 CFUs) with BC4, BC5, and BC6 strains, respectively. To minimize the volume of the liquid inoculum, the cell suspension of each barcode-tagged strain was concentrated to contain the target cell number in 1 µl inoculum. We spotted 1 µl of inoculum for each of six barcode-tagged strains on the surface of the feed (1.36 kg) placed in the feeder using a pipette and left it without any mixing to simulate the way *Salmonella* would contaminate feed in the real situation. Sixteen chickens were allowed to consume this contaminated feed for 48 h. After 2 days, the contaminated feed was replaced by *Salmonella*-free feed and water *ad libitum*. On day 7 and 14, two and four chickens were euthanized, respectively. For each euthanized bird, both ceca and foot-wash samples were collected and processed by the same procedures described previously.

Drinking Water Administration Experiment 3

This experiment was setup essentially in the same way as Experiment 2, except that the six barcode-tagged strains were added to and mixed in 11.36 l of drinking water. Chickens ($n = 16$) were allowed to drink *ad libitum* this contaminated water for 48 h. After 2 days, the contaminated water was replaced with *Salmonella*-free fresh water. On days 7 and 14, four chicks were euthanized, respectively. Cecal and foot-wash samples were collected and processed by the same procedure described previously.

Illumina Sequence Sample Preparation

Genomic DNA was isolated from each sample using QIAamp DNA MiniKit (Qiagen). The concentration of purified DNA was measured by a Qubit®3.0 Fluorometer (ThermoFisher Scientific). Subsequently, the barcode regions in the extracted genomic DNA of each sample were amplified using the primers BC-F and BC-R (**Table 1**), and G2 PCR mixture (Promega) with an initial incubation of 2 min at 95°C followed by 35 cycles of 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C followed by a 10 min extension at 72°C. The PCR products of 191 bp were purified by using a QIAquick PCR purification kit (Qiagen) for use as a template in the next round of PCR. The second step PCR was conducted to attach Illumina-specific sequences along with the combinatorial sample index sequences (6 nt) on both ends using the Illumina index forward and reverse primers shown in **Table 1**. A total of nine Illumina index forward and six Illumina index reverse primers were used, allowing up to 54 (9×6) samples to be sequenced simultaneously. The resulting amplicons of 167 bp were purified by ethanol purification method and were pooled together to generate an amplicon library for MiSeq sequencing with single-end read option *via* 150 cycles.

Analysis of DNA Sequencing Results

Custom Perl script was used to perform the following data analysis: first, the barcode regions of 57 bp in the sequence reads from Illumina MiSeq data were extracted. The 12 bp-index sequences were obtained by extracting and combining forward index sequence (6 bp) and reverse index sequence (6 bp) and used to sort the barcode reads to different samples. The six different barcodes were subsequently extracted and used to determine the relative abundance of different barcode-tagged strains in each sample.

RESULTS AND DISCUSSION

Quantitative Profiling of Barcode-Tagged Strains

A total of 1,461,014 sequence reads of 150 bp were obtained from the MiSeq sequencing run. The sequence reads were binned into different files according to the combinatorial index sequences corresponding to the samples from the three experiments. If any reads did not match perfectly to one of the original six barcode sequences, they were subsequently deleted. Since the read numbers reflect only relative frequency of each barcode-tagged strain in a given sample, the original read numbers were converted to calculate the percentage of each barcode-tagged strain in each sample.

Experiment 1: *Salmonella* Transmission after Oral Infection

The results of transmission of the SE barcode-tagged strains in the cecal content and foot wash of seeder chickens on day 14 from Experiment 1 are summarized in **Table 2**. For cecal samples of seeder bird #1, which was infected with BC1 strain at low dose (10^3 CFUs), the BC1 strain was the predominant colonizer (46.37%); however, the other strains challenged at a higher dose (10^5 CFUs) were also recovered from cecal content of chicken 1: BC3 (20.40%), BC4 (29.63%), and BC5 (3.59%). These results suggest that a significant mixed infection by different *S.* Enteritidis BC strains could occur when the chick was infected by barcode-tagged strains at low dose and subsequently comingled with other infected chickens. The barcode-tagged strains used in this study are isogenic strains with the identical genome sequence except for the barcode region. Therefore, it is possible that the multiple barcode-tagged strains may be recognized as the same strains from each other and/or by the host, leading to avoidance of the exclusion mechanism(s) observed among different strains as has been described previously in chickens and mammals (42–44). In the seeder chickens #4, #5, and #6 infected by respective barcode-tagged strains at high dose, the barcode-tagged strains used for infection were the dominant strains (93.21, 98.56, and 99.94%, respectively) in the ceca (**Table 2**). It appears that barcode-tagged

TABLE 2 | Relative abundance of the *Salmonella* Enteritidis (SE) barcode-tagged strains in seeder chickens on day 14 in the cecal content and foot wash from Experiment 1.

	BC1	BC2	BC3	BC4	BC5	BC6
Ceca content						
Chicken 1 BC1/10^3	46.37%	0.00%	20.40%	29.63%	3.59%	0.01%
Chicken 4 BC4/10^5	0.04%	0.004%	0.00%	93.21%	6.74%	0.01%
Chicken 5 BC5/10^5	0.04%	0.01%	0.27%	0.00%	98.56%	1.12%
Chicken 6 BC6/10^5	0.03%	0.00%	0.004%	0.00%	0.03%	99.94%
Foot wash						
Chicken 1 BC1/10^3	0.03%	6.37%	0.00%	0.00%	0.02%	93.57%
Chicken 4 BC4/10^5	0.42%	0.01%	0.00%	0.01%	99.55%	0.00%
Chicken 5 BC5/10^5	0.04%	0.003%	0.00%	36.77%	52.97%	10.21%
Chicken 6 BC6/10^5	0.03%	0.005%	0.00%	0.002%	21.10%	78.86%

Six chickens were randomly selected for oral infection with Salmonella barcode-tagged strains on day 1. Chickens 1 through 3 were orally infected with 10^3 CFUs with BC1, BC2, and BC3 strains, respectively. Chickens 4 through 6 were orally infected with 10^5 CFUs with BC4, BC5, and BC6 strains, respectively. At 14 days post challenge, cecal content or foot wash sample was collected from each chicken and used for isolation of genomic DNA. Following PCR and MiSeq analyses of barcode regions, the number of the sequence reads corresponding to different barcodes were used to determine the relative abundance (%) of each SE barcode strain from each chick. Chickens 2 and 3 orally gavaged with BC 2 and BC 3 were not sampled in this experiment.

strains introduced at high dose saturated all potential colonization niches, thus impeding colonization by other strains. This phenomenon observed in the chicks infected by a high dose of *Salmonella* is consistent with the colonization inhibition theory (42, 43). In conclusion, these results suggest that the outcome of cecal colonization in terms of the number of barcode-tagged strains colonizing the ceca is dose dependent, and a high dose beyond a certain threshold level results in dominant colonization by a single strain.

Contamination of feet by dominant barcode-tagged strains occurred for the seeder chickens #1, #4, and #6 (93.57% of BC6, 99.55% of BC5, and 78.86% of BC6, respectively), but they were not necessarily the same strains used for infection of the same chickens (**Table 2**). In the case of seeder bird #5, the foot was contaminated by three strains, BC4, BC5, and BC6 strains (36.77, 52.97, and 21.10%, respectively) among which BC5 was the one used for oral infection of the bird. The vast majority of the strains contaminating feet were those used for infection at high dose (BC4, BC5, and BC6), which indicated that high dose of *Salmonella* BCs is widely disseminated in the environment and thus may frequently be isolated from the feet. However, there is no correlation between the orally infected strain and dominant strain occurring on the feet. It is possible that the major strain isolated from the feet is from the environment instead of coming from chick itself.

Figure 2 shows the results of transmission of the *S.* Enteritidis barcode-tagged strains in the oral infection model in contact chickens. For the contact chickens, almost all (99%) of the barcode-tagged strains colonizing ceca on day 7 were strains administered at high dose, namely BC4-6. However, on day 14, a more diverse set of barcode-tagged strains were detected from the ceca of contact chickens, including a greater proportion of the barcode-tagged strains that were used to infect seeder chickens at low dose (BC1-3). It seems that the contact chickens are more likely to be colonized by the strains initially used for infection at high dose, but they eventually become colonized in the ceca also by the strains originating from the low dose as time progresses (**Figure 2**). In contrast, foot-wash samples from all contact chickens did not reveal any obvious trends as compared to those observed in cecal samples. On day 7, BC3 strain, which was administered at low dose, was the only strain (100%) contaminating the foot of the contact bird #7. Conversely, the feet of the contact chickens #8 and 9 were colonized mainly by the two strains, BC4 and BC6, which were used for infection at high dose. After the passage of time, the barcode-tagged strain populations on the feet of the contact chickens became more diverse on day 14. Comparing the relative abundance between days 7 and 14 indicated that the barcode-tagged strains that were used for infection at low dose increased the chances to contaminate the feet with the exception of BC3, which was not detected on the feet of any bird on day 14.

Experiment 2: *Salmonella* Transmission after Infection through Contaminated Feed

The results of transmission of the SE barcode-tagged strains in a feed contamination model (Experiment 2) are shown in **Figure 3**. On day 7, the ceca from the two chickens were colonized mainly

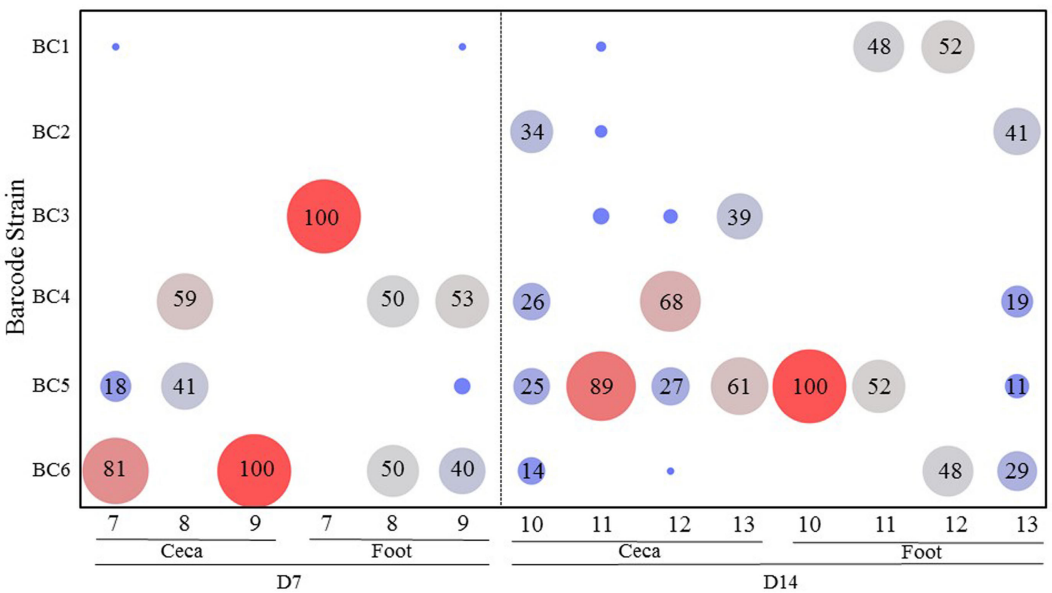

FIGURE 2 | Transmission of the *Salmonella* barcode-tagged strains in contact chickens in oral infection model. In Experiment 1, six seeder chickens were infected by different dose of SE barcode strains (BC1, BC2, and BC3 are used for infection of three chickens at 10^3 CFUs; BC4, BC5, and BC6 are used for infection of other three chickens at 10^5 CFUs). Other 10 chickens were roomed together with these six seeder chickens and named as contact chickens. Three contact chickens were euthanized on day 7, and four contact chickens were euthanized on day 14. The cecal tonsil and foot wash samples were collected from each chicken by aseptic technique. *x*-axis represents different contact chickens from Experiment 1, and *y*-axis represents different SE barcode strains. The number in bubble presents the relative abundance of each barcode strain in each chicken. Bigger size and red color means the higher relative abundance, and smaller size and blue color means lower relative abundance.

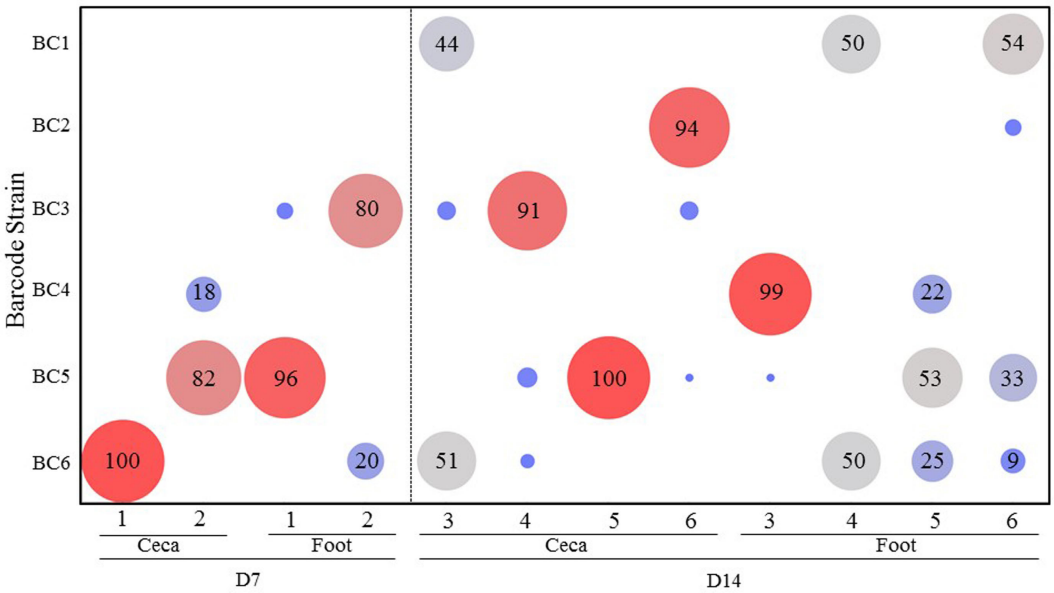

FIGURE 3 | Transmission of the *Salmonella* barcode-tagged strains in feed contamination model. In Experiment 2, the feed was contaminated by six SE barcode strains at two doses (BC1, BC2, and BC3 were introduced into the feed at 10^3 CFUs, and BC4, BC5, and BC6 were at 10^5 CFUs on day 1). Two chickens were euthanized on day 7, and the other four chickens were euthanized on day 14. The cecal and foot wash samples were collected from each chicken by aseptic technique and used for isolation of genomic DNA. Following PCR and MiSeq analyses of barcode regions, the number of sequence reads corresponding to different barcodes were used to determine the relative abundance (%) of each SE barcode strain from each sample. *x*-axis represents different chickens from Experiment 2, and *y*-axis represents different SE barcode strains. Bigger size and red color means the higher relative abundance, and smaller size and blue color means lower relative abundance.

by the barcode-tagged strains that were introduced at the higher dose. On day 14, the ceca from the birds #4 and 6 were predominantly colonized by BC3 (91%) and BC2 (94%) (both introduced at a low dose), respectively, while bird #5 was exclusively colonized by BC5. On day 14, only bird #3 showed colonization by multiple strains, mostly BC1 (44%) and BC6 (51%) strains. By comparing the combined percentages of the low versus high dose strains in the ceca at day 7 (0 versus 100%) and day 14 (60 versus 40%), it is apparent that the strains introduced to feed at a low dose eventually colonized the ceca, but it required a much longer period of time when compared to the strains introduced at high dose. Greater diversity of the strains was also detected at day 14 as compared to day 7 for the feet samples with the exception of bird #3 (**Figure 3**).

Experiment 3: *Salmonella* Transmission after Infection through Contaminated Drinking Water

Figure 4 summarizes the results of transmission of the SE barcode-tagged strains in water contamination model (Experiment 3). When the chicks were infected through contaminated drinking water, only three barcode-tagged strains (BC2, BC3, and BC6), representing both the strains that had been introduced at low and high dose, were recovered from the ceca on days 7 and 14. Strain BC6 (high dose), which was the predominant cecal colonizer, was also detected as the predominant strain contaminating the feet. Interestingly, BC1 (a low-dose challenge strain), even though it was not detected in the ceca of any chicken at any time, was recovered as the predominant strain in the feet of the chickens (**Figure 4**). Since only 8 chickens were analyzed out of the total of 16 chickens, BC1 is the predominant colonizer in at least one of the remaining chickens that was not used for sample collection.

CONCLUSION

Salmonella transmission in chicken flocks has already been the subject of several studies in which the *Salmonella* strains introduced to the flock were identified and quantified by culturing on selective agar plates and confirmed by biochemical and serological methods (45–50). In the studies conducted by De Vylder et al. (47) and Thomas et al. (48, 49), single *Salmonella* Enteritidis strains were used to analyze different aspects of *Salmonella* transmission within the laying hen flocks. These approaches have been useful in understanding the impact of different phage type strains or housing system on the frequency of horizontal transmission (47, 50, 51) or measuring different parameters of *Salmonella* transmission (48). However, a detailed picture of transmission involving interactions among multiple strains or serotypes cannot be investigated using the culture methods, due to the inability to differentiate multiple strains based on the culture methods.

Several investigators have studied the persistence of horizontal fecal shedding of *Salmonella* Enteritidis in experimentally infected

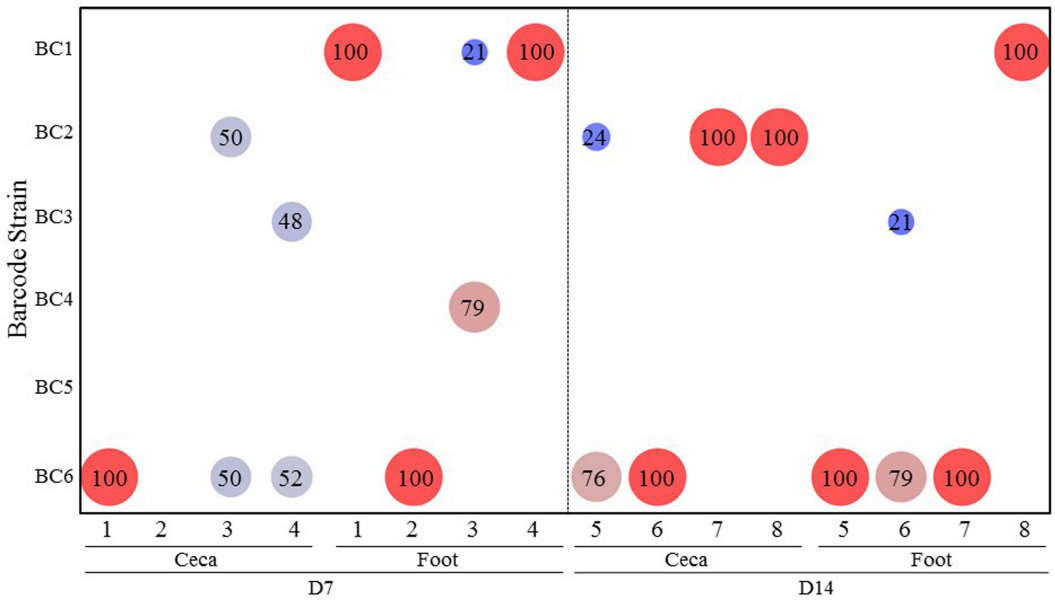

FIGURE 4 | Transmission of the *Salmonella* barcode-tagged strains in water contamination model. In Experiment 3, the water was contaminated by 6 SE barcode strains at two doses (BC1, BC2, and BC3 were introduced into the water at 10^3 CFUs, and BC4, BC5, and BC6 were at 10^5 CFUs on day 1). Four chickens were euthanized on day 7, and the other four chickens were euthanized on day 14. The cecal and foot wash samples were collected from each chicken by aseptic technique and used for isolation of genomic DNA. Following PCR and MiSeq analyses of barcode regions, the number of sequence reads corresponding to different barcodes were used to determine the relative abundance (%) of each SE barcode strain from each sample. *x*-axis represents different chickens from Experiment 2, and *y*-axis represents different SE barcode strains. Bigger size and red color means the higher relative abundance, and smaller size and blue color means lower relative abundance.

laying hens housed on different commercial conditions (50, 51). However, these studies are still limited to reflect the complexity of the environmental conditions that *Salmonella* is exposed to during transmission in a poultry farm. The other weakness of culture method approaches is that the isolated strains may be from environment rather than the strain externally introduced as a part of an experimental infection, thus handicapping the ability to differentiate the corresponding strain. Even though the strain might be confirmed as an experimental strain by further characterization, the result can only indicate the presence of the strain and reliable quantification is not possible.

In order to quantitatively track the *Salmonella* transmission routes from environment to flock, we constructed a series of barcode-tagged strains, which carry distinct barcode tags that would allow them to be identified and quantified accurately by high-throughput sequencing of the barcode regions. Similar methods of barcode tagging have been applied to understand the transmission dynamics within the infected hosts for *Salmonella* (52, 53), other pathogenic bacteria (54), and viruses (55). However, to our knowledge, this is the first report on the application of the barcode-tagged strains to study transmission dynamics within a population of the host animals. In this study, we used the barcode-tagged strains of *S.* Enteritidis to understand the transmission dynamics of *Salmonella* in a quantitative manner after initial introduction through oral infection or consumption of contaminated feed or drinking water.

In the current study, six barcode-tagged *S.* Enteritidis strains were employed to infect six chickens (seeder chickens) orally in oral infection experiment. In contaminated feed and water study, the same six barcode-tagged strains were introduced into feed or water in each isolator. Following the exposure *via* different routes, the corresponding distributions of the six different barcode-tagged strains at different colonization sites (ceca and feet) were analyzed at different time points post-infection.

Utilizing PCR and Illumina MiSeq analyses, the population structure could be assessed and representative transmission figures could be constructed. The results are important for understanding the patterns of *S.* Enteritidis dissemination in poultry and are revealed by demonstrating that a higher dose of *S.* Enteritidis has a greater opportunity to infect flocks. In addition, the data from this study suggest that colonization-inhibition by competing *Salmonella* is somewhat dosage dependent. Based on qPCR result for quantification of the combined load of all barcode-tagged strains (data not shown), it appears that recovery of *S.* Enteritidis barcode-tagged strains introduced orally were not different among the seeder chickens and contact chicks in both cecal and foot-wash samples on day 14. All barcode strains combined in the cecal samples remained stable on days 7 and 14 in Experiment 1, while those from foot-wash samples increased 10-fold in the three experiments after time had elapsed.

To better establish the implications for commercial poultry production settings, larger scale experiments are needed to assess additional environmental and host factors. However, the current experiment demonstrated the proof of concept that the use of barcode-tagged strains is a novel and an effective approach to understand the dynamics of *Salmonella* transmission within a chicken flock and can provide valuable insights for the potential

to develop and optimize measures that protect host animals from infection with *Salmonella*. Studies to evaluate and confirm previous work published by our laboratory (18–20, 24) that demonstrate the importance of airborne transmission of *Salmonella* versus oral infection as well as the competitive exclusion concept of *Salmonella* versus *Salmonella* (43, 56) or cross protection (44, 57, 58) using these SE barcode-tagged strains are currently in progress.

AUTHOR CONTRIBUTIONS

YK, YY, SR, and GT edited and wrote the manuscript and edited the drafts.

ACKNOWLEDGMENTS

We appreciate Dr. Si Hong Park (Center for Food Safety, Department of Food Science, University of Arkansas at Fayetteville) for his technical assistance. We also appreciate Dr. Billy M. Hargis, the director of the Poultry Health Laboratory (University of Arkansas at Fayetteville), for sharing his insights and expertise for design and interpretation of the chicken experiments.

FUNDING

This research was partially funded by the Arkansas Biosciences Institute.

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

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 15 February 2016
doi: 10.3389/fvets.2016.00010

Leaky Gut and Mycotoxins: Aflatoxin B1 Does Not Increase Gut Permeability in Broiler Chickens

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OPEN ACCESS

Edited by:
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Reviewed by:
Justin C. Fowler, University of Georgia, USA
Adebukunola Olufunmilayo Lala, Federal University of Agriculture, Nigeria

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 10 October 2015
Accepted: 01 February 2016
Published: 15 February 2016

Citation:
Galarza-Seeber R, Latorre JD, Bielke LR, Kuttappan VA, Wolfenden AD, Hernandez-Velasco X, Merino-Guzman R, Vicente JL, Donoghue A, Cross D, Hargis BM and Tellez G (2016) Leaky Gut and Mycotoxins: Aflatoxin B1 Does Not Increase Gut Permeability in Broiler Chickens. Front. Vet. Sci. 3:10. doi: 10.3389/fvets.2016.00010

Previous studies conducted in our laboratory have demonstrated that intestinal barrier function can be adversely affected by diet ingredients or feed restriction, resulting in increased intestinal inflammation-associated permeability. Two experiments were conducted in broilers to evaluate the effect of three concentrations of Aflatoxin B1 (AFB1; 2, 1.5, or 1 ppm) on gastrointestinal leakage and liver bacterial translocation (BT). In experiment 1, 240 day-of-hatch male broilers were allocated in two groups, each group had six replicates of 20 chickens (n = 120/group): Control feed or feed + 2 ppm AFB1. In experiment 2, 240 day-of-hatch male broilers were allocated in three groups, each group had five replicates of 16 chickens (n = 80/group): Control feed; feed + 1 ppm AFB1; or feed + 1.5 ppm AFB1. In both experiments, chickens were fed starter (days 1–7) and grower diets (days 8–21) *ad libitum* and performance parameters were evaluated every week. At day 21, all chicks received an oral gavage dose of FITC-d (4.16 mg/kg) 2.5 h before collecting blood samples to evaluate gastrointestinal leakage of FITC-d. In experiment 2, a hematologic analysis was also performed. Liver sections were aseptically collected and cultured using TSA plates to determine BT. Cecal contents were collected to determine total colony-forming units per gram of Gram-negative bacteria, lactic acid bacteria (LAB), or anaerobes by plating on selective media. In experiment 2, liver, spleen, and bursa of Fabricius were removed to determine organ weight ratio, and also intestinal samples were obtained for morphometric analysis. Performance parameters, organ weight ratio, and morphometric measurements were significantly different between Control and AFB1 groups in both experiments. Gut leakage of FITC-d was not affected by the three concentrations of AFB1 evaluated ($P > 0.05$). Interestingly, a significant reduction in BT was observed in chickens that received 2 and 1 ppm AFB1. An increase ($P < 0.05$) in total aerobic bacteria, total Gram negatives, and total LAB were observed in chickens fed with 2 and 1.5 ppm of AFB1 when compared with Control and 1 ppm chickens. The integrity of gut epithelial barrier was not compromised after exposure to the mycotoxin.

Keywords: aflatoxin B1, bacterial translocation, broilers, gut leakage, performance

INTRODUCTION

In the winter of 1959, the British cargo ship Rosetti, unloaded a shipment of peanut meal from Brazil to England, which was utilized as a protein supplement in the diets of poultry and other domestic animals. By summer of 1960, an outbreak of an unknown disease killed several species of poultry including turkeys, ducklings, and pheasants. In all, 500 cases were reported involving the deaths of more than 100,000 turkeys. This was the first report of Turkey "X" Disease (1, 2). Exhaustive research led to the discovery of aflatoxins, secondary metabolites of *Aspergillus flavus* and *Aspergillus parasiticus*, as the etiological agents and the development of mycotoxicology (3–5). More recent studies demonstrated that aflatoxins are potent carcinogenic compounds (6–11). About 14 different types of aflatoxins are produced in nature (10, 12), but aflatoxin B1 (AFB1) produced by *A. flavus* and *A. parasiticus* is considered the most toxic (13, 14). In spite of 55 years of continuous research on aflatoxins, several areas of aflatoxicosis remain yet to be investigated. It is particularly interesting that studies on poultry aflatoxicosis have not kept pace with the research in mammals, and there still exists an incomplete description of aflatoxicosis in avian species, especially when searching for scientific publications related to the effect(s) of aflatoxins on the gastrointestinal tract (GIT).

The GIT is the first organ coming into contact with mycotoxins from the diet and should be expected to be affected by AFB1 with greater potency as compared to other organs. Nevertheless, literature regarding the effects of AFB1 on the GIT is particularly confusing. Few researchers have look at morphometric changes following dietary administration of aflatoxins in chickens, turkeys, and ducks, but results from those studies contradict each other, particularly when looking at villi high and villi to crypt ratio (10, 11, 15–20). Similarly, contradictive results arise from the effects of AFB1 on digestibility of amino acids, energy utilization, and absorption of macronutrients (18, 20–27).

Aflatoxins are absorbed very quickly into the blood from the GIT, followed by an extensive transformation into metabolites primarily in the liver (9, 28, 29). Contrary to the studies on mucosal damage and nutrient absorption caused by AFB1, there is an universal agreement that beside the carcinogenic and hepatotoxic effects on the liver, dietary aflatoxins reduce weight gain, feed intake (FI), increase feed conversion ratio (FCR), and are immunosuppressive (12, 30, 31).

Today, only a few reports could be found in databanks, in which the issue of barrier function and intestinal permeability has been reported. From recent studies by Yunus et al. (19) in broilers, it has been suggested that the absorptive surface of small intestine declines during a chronic exposure to low levels of AFB1. However, in that study, broilers compensated for the reduced absorptive surface by increasing the length of the small intestine (19). In the second study, transepithelial electrical resistance (TEER), used as an important indicator of barrier function of intestinal epithelial cells (IEC), showed that AFB1 was only moderately affect during acute exposure to the toxin (10). As far as we can tell, the only study of the effect of AFB1 on possible damage to tight junctions (TJs) was performed by Caloni et al. (32) who demonstrated that AFB1 does not affect the integrity of TJ proteins or barrier damage *in vitro*.

We have previously shown that intestinal barrier function can be adversely affected by poorly digested diets, feed restriction, or dexamethasone resulting in increased intestinal inflammation-associated permeability in poultry (33–36). The purpose of the present investigation was to evaluate the effect of three doses of aflatoxin B1 on growth, physiological parameters, and gut permeability in broiler chickens.

MATERIALS AND METHODS

Animal Source, Diets, and Experimental Design

Two experiments were conducted several weeks apart using two hundred and forty 1-day-old male broiler chicks (Cobb-Vantress, Silom Springs, AR, USA) raised in floor pens. Unmedicated corn-soybean-based broiler starter and medicated (with coccidiostat) corn-soybean-based broiler grower diets were prepared according to the broiler's recommendations (37). Experiments were conducted to evaluate the effect of three concentrations of AFB1 (2 ppm in experiment 1 and 1.5 or 1 ppm in experiment 2) on systemic fluorescein isothiocyanate-dextran (FITC-d; 3–5 kDa) levels and liver bacterial translocation (BT) as indicators of increased gut epithelial leakage. AFB1 was provided by Dr. George E. Rottinghaus, Veterinary Medical Diagnostic Laboratory, University of Missouri, Columbia, MO, USA. AFB1 was produced through the fermentation of rice and the aflatoxin content was measured by spectrophotometric analysis. The aflatoxin within the rice powder consisted of 74.62% AFB1, 22.38% AFG1, 2.48% AFB2, and 0.49% AFG2, based on total aflatoxin in the rice powder. Diets containing AFB1 were analyzed, and the presence of parent AF was confirmed by high-performance liquid chromatography with fluorescence detection (HPLC-FLD) method by using a Romer Derivatization Unit (Romer Labs, Inc., MO, USA). AFB1 was added to the diets and mixed thoroughly in a graded sequence to specified concentrations. The birds were given diets with or without supplemental AFB1 and water *ad libitum*. All animal handling procedures were in compliance with the Institutional Animal Care and Use Committee at the University of Arkansas. In experiment 1, broilers were allocated randomly to two groups, each group had six replicates of 20 chickens ($n = 120$/group): Control feed or feed + 2 ppm AFB1. In experiment 2, broilers were allocated randomly to three groups, each group had five replicates of 16 chickens ($n = 80$/group): Control feed; feed + 1 ppm AFB1; or feed + 1.5 ppm AFB1. In both experiments, chickens were fed starter (days 1–7) and grower diet (days 8–21) *ad libitum* until the end of the experiment at day 21. In each experiment, each pen was used as a replicate and also as an experimental unit per treatment to evaluate body weight (BW), body weight gain (BWG), FI, and FCR. These growth performance parameters were obtained every week. At the end of experiment 2, blood samples were collected from the wing vein into tubes with heparin as anticoagulant for differential cell counts. In both experiments, 21-day-old chickens received an oral gavage dose

of FITC-d (4.16 mg/kg) 2.5 h before collecting blood samples to evaluate passage of FITC-d. Chickens were humanely killed by CO_2 asphyxiation. Blood was collected from the femoral vein to obtain serum for FITC-d determination (as described below) and serum clinical chemistry (in experiment 2 only) with a Corning clinical chemistry analyzer (Chiron Corporation, San Jose, CA, USA). Liver sections ($n = 12$ chickens/treatment) were aseptically collected to determine BT, and cecal contents were collected to determine total colony-forming units per gram of Gram-negative bacteria, lactic acid bacteria (LAB), or anaerobes by plating on a selective media as described below.

Determination of Hematological Parameters

Differential counts of blood samples collected from experiment 2 were determined using a Cell-Dyne 3500 System (Abbott Laboratories, Chicago, IL, USA) that had been standardized for differential counts of poultry blood cells. Hematologic measurements of heparin anticoagulated blood included total numbers of white blood cells (WBC), heterophils, lymphocytes, monocytes, eosinophils, and basophils. Heterophil/lymphocyte ratios (H/L), an indicator of stress in birds (38), were calculated by dividing the number of heterophils in 1 mL of peripheral blood by the number of lymphocytes. Total counts of red blood cells, hemoglobin (HGB), hematocrit (HCT)%, mean corpuscular volume (MVC), and mean corpuscular hemoglobin (MCH) were also determined. Additionally, in experiment 2, liver, spleen, and bursa of Fabricius were removed and cleaned of adherent tissues. The weight of these organs was measured and expressed as percentage of BW (organs weight/final BW) × 100.

Serum Determination of FITC-d

Blood samples were kept at room temperature for 3 h and centrifuged (1,000 × *g* for 15 min) to separate the serum from the red blood cells. FITC-d levels of undiluted serum were measured at excitation wavelength of 485 nm and emission wavelength of 528 nm (Synergy HT, Multi-mode microplate reader, BioTek Instruments, Inc., VT, USA). Fluorescence measured was then compared to a standard curve with known FITC-d concentrations. Gut leakage for each bird was reported as microgram of FITC-d/mL of serum (35, 36).

Bacterial Translocation

The number of birds used was based on published studies, in which similar variables were measured (34, 39). Briefly, the right half of the liver was removed from each chicken, collected in sterile bags, homogenized, weighed, and 1:4 wt/vol dilutions were made with sterile 0.9% saline. Ten-fold dilutions of each sample from each group were made in a sterile 96-well Bacti flat bottom plate, and the diluted samples were plated on tryptic soy agar plates (TSA, catalog no. 211822, Becton Dickinson, Sparks, MD, USA).

Determination of Microbial Level in Ceca

Both ceca were aseptically removed, placed into sterile bags, and homogenized. Samples were weighed, and 1:4 wt/vol dilutions were made with sterile 0.9% saline. Ten fold dilutions of each sample from each group were made in a sterile 96-well Bacti flat bottom plate, and the diluted samples were plated on four different culture media to evaluate the total number of LAB in deMan Rogosa Sharpe (Difco™ Lactobacilli MRS Agar VWR Cat. No. 90004-084 Suwanee, GA, USA); total recovered Gram-negative bacteria in MacConkey; total anaerobes in tryptic soy agar with sodium thioglycolate plates (TSA, catalog no. 211822, Becton Dickinson, Sparks, MD, USA); and total yeast in Sabouraud Glucose Agar Base with antibiotics, tetraciclina, and 100-mg sodium benzyl penicillin (HiMedia Laboratories Pvt. Ltd., Mumbai, India).

Histology and Morphometric Analysis of Intestine

Intestinal sections from duodenum (~1-cm section was collected from the middle of the descending duodenum), and ileum (0.5-cm section was obtained from the mid-ileum at Meckel's diverticulum) were fixed in 10% neutral buffered formalin and embedded in paraffin, sectioned (5 μm thick), set on a glass slide, and stained with hematoxylin and eosin (H&E), and then examined by light microscopy. Photomicrographs of randomly selected fields of each intestinal sample were acquired using a microscope equipped with a Leica DFC450C camera and Leica v.3.8.Software (Leica Application Suite) and used for morphometric analysis. ImageJ 1.47v software (http://rsb.info.nih.gov/ij/) was used for the morphometric measurements of villus length, villus width, and crypt depth. Under a magnification of 2×, 10 villi per bird per section were measured, with a total of five birds per group. Villus length was measured from the top of the villus to the upper part of the lamina propria. Crypt depth was measured from the base upwards to the region of transition between the crypt and villus (40). Villus width was measured at the widest area of each villus, whereas villus:crypt ratio was determined dividing villus height into crypt depth values. Villus surface area was calculated using the formula $(2\pi)(VW/2)(VL)$, where VW = villus width and VL = villus length (41).

Statistical Analysis

All data were subjected to analysis of variance as an entirely randomized design using the General Linear Models procedure of SAS (42). Data were expressed as mean ± SE. Significant differences among the means were determined by using Duncan's multiple-range test at $P < 0.05$.

RESULTS

Performance Parameters

Body weight of chickens fed 2 ppm of AFB1 was not affected in the first week; however, BW was significantly ($P < 0.05$) reduced by 18.36 and 34.89% during the second and third weeks of age, respectively, when compared with Controls (**Table 1**). BW gain and FI were also affected by AFB1 consumption with a reduction of 20% for both variables during the second week and 37 and 49%, respectively, in the third week. FCR only showed a significant difference in the third week with an improvement in the AFB1 group when compared with Controls (**Table 1**). Administration

TABLE 1 | Effect of dietary administration of 2, 1.5, and 1 ppm of aflatoxin B1 on body weight (BW), body weight gain (BWG), feed intake, and feed conversion ratio at 7, 14, and 21 days in broiler chickens or experiments 1 and 2.

	Experiment 1		Experiment 2		
Parameters	**Control**	**2 ppm AFB1**	**Control**	**1 ppm AFB1**	**1.5 ppm AFB1**
BW (g/Broiler)					
Day 7	144.79 ± 1.85[a]	142.05 ± 1.04[a]	136.82 ± 2.87[a]	134.92 ± 2.44[a]	133.34 ± 2.74[a]
Day 14	385.88 ± 5.02[a]	315.42 ± 5.40[b]	337.03 ± 9.38[a]	309.76 ± 2.21[b]	298.95 ± 5.03[b]
Day 21	771.55 ± 8.61[a]	502.28 ± 7.90[b]	690.45 ± 19.36[a]	581.99 ± 8.54[b]	511.03 ± 11.47[c]
BWG (g/Broiler)					
Days 0–7	97.83 ± 1.71[a]	95.07 ± 1.03[a]	93.24 ± 2.71[a]	90.40 ± 2.9[a]	88.62 ± 2.89[a]
Days 7–14	338.88 ± 4.85[a]	268.45 ± 5.07[b]	293.48 ± 9.20[a]	265.22 ± 2.11[b]	254.13 ± 4.74[b]
Days 14–21	724.60 ± 8.46[a]	455.30 ± 7.92[b]	646.65 ± 18.94[a]	537.47 ± 8.37[b]	466.22 ± 11.19[c]
Feed intake (g/Broiler)					
Days 0–7	132.1 ± 1.92[a]	127.44 ± 1.62[a]	131.35 ± 3.17[a]	128.34 ± 2.94[a]	126.42 ± 3.44[a]
Days 7–14	505.65 ± 5.86[a]	405.94 ± 6.12[b]	405.49 ± 13.15[a]	406.08 ± 6.40[a]	399.36 ± 14.80[a]
Days 14–21	966.15 ± 17.74[a]	489.09 ± 16.53[b]	790.56 ± 40.09[a]	670.32 ± 17.08[b]	570.14 ± 53.87[c]
Feed conversion ratio					
Days 0–7	1.35 ± 0.01[a]	1.34 ± 0.01[a]	1.41 ± 0.02[a]	1.42 ± 0.01[a]	1.43 ± 0.02[a]
Days 7–14	1.49 ± 0.02[a]	1.51 ± 0.01[a]	1.39 ± 0.06[b]	1.53 ± 0.01[a]	1.57 ± 0.03[a]
Days 14–21	1.33 ± 0.02[a]	1.08 ± 0.04[b]	1.23 ± 0.09[a]	1.25 ± 0.02[a]	1.22 ± 0.09[a]

[a–c]Superscripts within rows indicate significant (P < 0.05) difference within each experiment.

TABLE 2 | Effect of 1 and 1.5 ppm of aflatoxin B1 on body weight ratios for liver, spleen, and bursa of Fabricius in 21-day-old broiler chickens.

Treatment	Liver ratio (%)	Spleen ratio (%)	Bursa of Fabricius ratio (%)
Control	3.24 ± 0.09[b]	0.11 ± 0.01[b]	0.15 ± 0.01[b]
1 ppm AFB1	3.60 ± 0.19[a,b]	0.16 ± 0.02[a]	0.20 ± 0.02[a]
1.5 ppm AFB1	4.23 ± 0.34[a]	0.15 ± 0.01[a]	0.18 ± 0.02[a,b]

Experiment 2.
Mean ± SE from 10 chickens.
[a,b]Superscripts within columns indicate significant difference at P < 0.05.

TABLE 3 | Effect of 2 ppm of aflatoxin B1 (experiment 1) or 1 and 1.5 ppm of aflatoxin B1 (experiment 2) on total bacterial and yeast counts from cecum samples in broiler chickens at 21 days.

Diet	Ceca (Log10 cfu/g of tissue)			
	Total aerobic bacteria	**Total Gram-negative bacteria**	**Total lactic acid bacteria**	**Total yeast**
Experiment 1				
Control	6.41 ± 0.19[a]	6.08 ± 0.22[b]	5.75 ± 0.21[b]	3.13 ± 0.20[a]
2 ppm AFB1	6.83 ± 0.29[a]	7.00 ± 0.21[a]	6.56 ± 0.13[a]	3.33 ± 0.07[a]
Experiment 2				
Control	6.98 ± 0.23[b]	6.51 ± 0.37[b]	6.91 ± 0.14[a]	2.74 ± 0.33[a]
1 ppm AFB1	7.25 ± 0.22[b]	7.04 ± 0.24[a,b]	6.33 ± 0.15[b]	3.36 ± 0.18[a]
1.5 ppm AFB1	7.82 ± 0.17[a]	7.66 ± 0.15[a]	7.22 ± 0.16[a]	2.86 ± 0.33[a]

Data are expressed as mean ± SE from 12 chickens.
[a,b]Superscripts within columns indicate significant difference at P < 0.05.

of 1 and 1.5 ppm of AFB1 also decreased BW by 8 and 11% during the second week and 16 and 26% in the third week, respectively, compared with Controls. This reduction was proportionally similar in BWG being 10 and 13% lower for 1 and 1.5 ppm of AFB1 during the second week, and 17 and 28% for 1 and 1.5 ppm during the third week. FI was not affected by AFB1 consumption during the first 2 weeks; however, there was a reduction of 15 and 28% in fed intake in chickens that consumed 1 and 1.5 ppm of AFB1, respectively, during the last week (**Table 1**). FCR varied accordingly in the three diet groups during the whole experiment except the second week where Control group had a more efficient ratio compared to the AFB1 groups (**Table 1**). In experiment 2, the liver weight ratio was significantly increased in chickens that received 1.5 ppm when compared with Control (**Table 2**). However, spleen ratio was increased in both groups of chickens that received 1 or 1.5 ppm of AFB1 when compared with Controls. Bursa ratio was increased only in chickens that received 1 ppm (**Table 2**).

Total Bacterial Counts in Cecum

In experiment 1, chicks receiving 2 ppm of AFB1 had an increase in the number of total Gram-negative bacteria and total LAB, but the total numbers of aerobes were similar between chickens that received 2 ppm of AFB1 and Control chickens (**Table 3**). In experiment 2, the total number of aerobic bacteria and total Gram negatives were higher in 1.5 ppm AFB1 group. Conversely, the number of total LAB was reduced in chickens fed with 1 ppm AFB1. No difference was observed in total yeast count between groups in neither of both experiments (**Table 3**).

Hematology

In experiment 2, a significant heterophilia with a marked lymphopenia was observed in both groups that received AFB1 (**Table 4**). Consequently, an increase in the heterophils-to-lymphocyte ratio was also observed in those groups when compared with Controls. No significant differences were found in the numbers of monocytes, eosinophils, or basophils (data not shown). Hemoglobin, MVC, and MCH were significantly decreased in chickens that consumed 1.5 ppm of AFB1 when compared with Controls.

These values were not affected in chickens that received 1 ppm when compared with Controls (**Table 4**).

Bacterial Translocation and FITC-d Leakage

Chickens receiving a diet with 2 ppm of AFB1 had a significant reduction in BT to the liver when compared to Control chickens (**Table 5**). Interestingly, there were no differences in serum levels of FITC-d levels between Control and treated chickens. On the other hand, in experiment 2, chicks fed 1.5 ppm AFB1 did not show significant differences in BT when compared with Control chickens, but no bacteria recovery was observed from livers of chickens fed with 1 ppm AFB1. Nevertheless, similar to experiment 1, no significant differences were observed in the levels of serum FITC-d between chicks that received 1 or 1.5 ppm of AFB1 and Control chickens (**Table 5**).

TABLE 4 | Effect of 1 and 1.5 ppm of aflatoxin B1 on blood parameters and serum chemistry in broiler chickens at 21 days.

Hematological parameters	Treatments		
	Control	1 ppm AFB1	1.5 ppm AFB1
White blood cells	30.02 ± 4.57[a]	27.89 ± 2.50[a]	37.20 ± 4.23[a]
Heterophils	13.21 ± 1.38[b]	26.39 ± 2.04[a]	28.62 ± 2.70[a]
Lymphocytes	77.15 ± 2.07[a]	62.58 ± 3.31[b]	58.08 ± 2.11[b]
Heterophils lymph. ratio (HLR)	0.18 ± 0.02[b]	0.45 ± 0.05[a]	0.51 ± 0.06[a]
Red blood cells	1.81 ± 0.09[a]	1.70 ± 0.04[a]	1.68 ± 0.06[a]
Hemoglobin (HGB)	5.98 ± 0.17[a]	5.56 ± 0.18[a]	4.90 ± 0.13[b]
Hematocrit (HCT)%	44.95 ± 2.41[a]	42.07 ± 1.33[a]	39.23 ± 1.29[a]
Mean corpuscular volume (MVC)	248.1 ± 2.83[a]	247.0 ± 3.23[a]	234.4 ± 3.19[b]
Mean corpuscular hemoglobin (MCH)	33.58 ± 1.15[a]	32.63 ± 0.56[a,b]	29.42 ± 0.81[b]

Experiment 2.
Mean ± SE from 10 chickens.
[a,b]Superscripts within rows indicate significant difference at P < 0.05.

TABLE 5 | Effect of 2 ppm of aflatoxin B1 (experiment 1) or 1 and 1.5 ppm of aflatoxin B1 (experiment 2) on liver bacterial translocation and serum FITC-d levels in broiler chickens at 21 days.

Diet	Liver bacterial translocation[c] (log10 cfu/g of tissue)	FITC-d[c] (μg/mL of serum)
Experiment 1		
Control	2.77 ± 0.50[a]	0.34 ± 0.01[a]
2 ppm AFB1	1.13 ± 0.49[b]	0.39 ± 0.05[a]
Experiment 2		
Control	1.51 ± 0.46[a]	0.34 ± 0.02[a]
1 ppm AFB1	0.00 ± 0.00[b]	0.31 ± 0.02[a]
1.5 ppm AFB1	1.30 ± 0.47[a]	0.31 ± 0.01[a]

[a,b]Superscripts within columns indicate significant difference at P < 0.05.
[c]Data are expressed as mean ± SE, n = 12 birds/treatment.

Morphometric Analysis

Villus length in both duodenum and ileum sections was significantly increased in a dose-related fashion in chickens that received 1 and 1.5 ppm of AFB1 when compared with Control chickens (**Table 6**). However, a significant reduction in duodenum crypt depth was observed in chickens that received 1 and 1.5 ppm of AFB1 when compared with Control chickens. On the other hand, similar changes in ileum crypt depth were found in chickens that received 1.5 ppm of AFB1 when compared with Control or 1 ppm chickens. Changes in duodenum villus height/crypt depth ratio were inconsistent between doses of AFB1 in this study.

In the ileum, this relationship was increased in chickens that received 1 ppm, followed by chicks that received 1.5 ppm of AFB1 and Control chickens had the lower villus height/crypt depth ratio. The surface area of the duodenum was significantly higher in chicks that received 1.5 ppm of AFB1, but no changes in ileum surface area were observed between the three groups (**Table 6**).

DISCUSSION

Aflatoxins have several effects in poultry, including poor performance, liver pathology, immunosuppression, and changes in relative organ weights (30, 31, 43, 44). Our results were consistent with these previous studies demonstrating dose-related effects on reduction of BW, BWG, FI, and feed conversion as well as increase relative weights of liver, spleen, and bursa of Fabricius.

In spite of the indicated antimicrobial potential of AFB1, we found few reports regarding the effects of the toxin on gut microbial populations. Kubena et al. (44) reported a significant increase in total volatile fatty acids at 5 days of age in chickens that received 2.5 and 7.5 ppm of AFB1, suggesting changes in LAB populations (45, 46). In other studies, *Lactobacillus* spp. have been noted to change under the influence of AFB1; however, these changes did

TABLE 6 | Morphometric analysis of duodenum and ileum samples from broiler chickens at 21 days.

Parameters	Duodenum			Ileum		
	Control	1 ppm AFB1	1.5 ppm AFB1	Control	1 ppm AFB1	1.5 ppm AFB1
Villus length (μm)	382.41 ± 5.03[c]	398.40 ± 2.01[b]	437.00 ± 7.50[a]	164.32 ± 3.75[c]	175.42 ± 3.13[b]	199.78 ± 3.42[a]
Villus width (μm)	45.22 ± 1.66[a]	45.83 ± 1.38[a]	47.74 ± 1.50[a]	38.93 ± 0.68[a]	37.69 ± 1.33[a]	32.57 ± 0.78[b]
Crypt depth (μm)	31.83 ± 1.03[a]	26.01 ± 0.79[b]	24.35 ± 0.15[b]	23.24 ± 0.49[b]	21.51 ± 0.69[b]	26.67 ± 0.67[a]
Villus height/crypt depth ratio	12.45 ± 0.33[c]	16.03 ± 0.48[b]	18.06 ± 0.39[a]	7.11 ± 0.12[c]	8.49 ± 0.24[a]	7.58 ± 0.10[b]
Villus surface area (mm^2)[d]	0.054 ± 0.019[b]	0.057 ± 0.001[b]	0.066 ± 0.002[a]	0.020 ± 0.005[a]	0.021 ± 0.009[a]	0.020 ± 0.007[a]

Experiment 2.
[a–c]Superscripts within rows within intestinal section indicate significant difference at P < 0.05.
[d]Surface was calculated as [2π × (villus width/2) × (villus height)] (41).

not warrant any beneficial effects of AFB1 on intestinal microbial population (47).

In the present study, AFB1 significantly increased the total number of Gram-negative bacteria in chickens fed with 2 and 1.5 ppm and numerically in chickens fed with 1 ppm, and a similar trend was observed in the total number of LAB for chickens receiving 2 and 1.5 ppm of AFB1. However, chickens that received 1 ppm showed a significant reduction of total LAB but higher total number of aerobic bacteria when compared with Control chickens. Interestingly, little information about the outcomes of AFB1 on gut microbiome is available. In one study, Kubena et al. (44) reported that 2.5 ppm of AFB1 increased the production of total volatile fatty acids in broilers, which suggest higher number of total LAB populations. In the present study, no differences were observed in total yeast counts between groups in neither of both experiments; nevertheless, we could not find any other report to compare our results. Perhaps, such inconsistent results may be a reason of the lack of publications reporting yeast evaluation. Interestingly, it has been showed that fermentation patterns of *Saccharomyces cerevisiae* also change under the influence of AFB1 (48). AFB1 has also been reported to change fermentation patterns with increase gas production, due to fermentation of other carbohydrates of LAB, that affects negatively the cheese industry (46, 47, 49). Several investigators have reported the effects that aflatoxins cause to heterophilia lymphopenia and hemolytic anemia in poultry (6, 10, 30, 50, 51). In experiment 2, a dramatic increase in the heterophils occurred while the lymphocytes were reduced. Consequently, an increase in the heterophils-to-lymphocyte ratio was also observed in those groups when compared with Control chickens. A similar response of circulating leukocytes was also found when a physiological stress was applied to chickens (38). In aflatoxicosis, the spleen is enlarged due to the hemolytic anemia (52) and some reports indicate that the spleen of chickens is almost doubled in size (53). In experiment 2, spleens of chickens that received 1 and 1.5 ppm were significantly larger when compared with Control. The elevated WBC counts caused by both doses of AFB1 also support the clinical presentation of hemolytic anemia. Additionally, hemoglobin, MVC, and MCH were significantly increased in chickens that consumed 1.5 ppm of AFB1 when compared with Control chickens, confirming that aflatoxicosis causes a hemolytic anemia in chickens as has been previously reported (30, 51, 52, 54, 55).

We have previously shown that intestinal inflammation can be induced by diet ingredients or stress, affecting intestinal permeability (33–36). As the largest barrier in the body, IEC are responsible for absorption of water and nutrients, but they also prevent the entry of antigens into the blood (56–58).

Contrary to our initial hypothesis, 2 ppm of AFB1 did not increase intestinal permeability, as was evidenced by a significant reduction in BT or similar levels of serum FITC-d when compared with Control chickens. It is possible that the inflammation of the liver that is characterized by infiltration of heterophils and other inflammatory cells may handle cleaning any bacterial leakage that arrives from the porta system to the liver. Those results encouraged us to repeat and extend the experiment with lower doses of AFB1 and by comparing the morphometric changes between Control and treated groups. Our findings from experiment 2 showed that chickens fed 1.5 ppm AFB1 showed a numerical reduction in BT when compared with Control chickens, but no bacteria were recovered from livers of chickens fed with 1 ppm AFB1. Also, similar to experiment 1, no significant differences were observed in the levels of serum FITC-d between chicks that received 1 or 1.5 ppm of AFB1 and Control chickens.

Increased intestinal leakage is also associated with BT in the portal circulation (59, 60). Likewise, FITC-d is a bulky molecule (3–5 kDa) which is not observed under normal conditions. Nevertheless, if TJs between epithelial cells are altered, FITC-d can be detected in serum, indicating damage to the TJs following FITC-d gavage administration (61). It has been reported that AFB1 does not destroy TJs (32), it has only minor effects on the gut-associated lymphoid tissue (GALT) (62), confirming that AFB1 does not induce inflammation in the GIT.

Literature reports on the effects of AFB1 on histology of GIT are limited and not conclusive (10, 11, 18, 27, 63). However, it is important to mention that the few studies that have evaluated the effect of AFB1 on intestinal histology are reports using different concentrations of AFB1, different avian species, different ages, as well as time of AFB1 administration. Interpretation of our morphometric results was also inconclusive. Nevertheless, the GIT is highlighted as a dynamic organ that is able to adapt to a chronic AFB1 as has been demonstrated by several scientists (18, 20–23, 26, 27). In summary, the results of the present study suggest that AFB1 does not increase gut leakage as is evidenced by the lack of increase permeability of FITC-d in the serum. On the other hand, further studies are needed to clarify the BT and morphometric results with AFB1.

AUTHOR CONTRIBUTIONS

RS: contributions to conception and design, acquisition of data, and analysis and interpretation of data; JL: contributions to conception and design, acquisition of data, and/or analysis and interpretation of data; LB: contributions to conception and design, and/or analysis and interpretation of data; VK: contributions to conception and design and acquisition of data; AW: acquisition of data; XV: final approval of the version to be submitted, drafting the article, or revising it critically for important intellectual content; RG: acquisition of data; JV: acquisition of data; AD: final approval of the version to be submitted; DC: acquisition of data; BH: final approval of the version to be submitted; and GT: contributions to conception and design, acquisition of data, and analysis and interpretation of data.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the excellent technical assistance of Sonia Tsai (Poultry Production and Product Safety Research Unit, USDA, Agricultural Research Service, Poultry Science Center, University of Arkansas, Fayetteville) and all the professional staff, who work at the Interlibrary Loan Service of the University of Arkansas, for their unconditional help and support.

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

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 25 February 2016
doi: 10.3389/fvets.2016.00016

Rearing Room Affects the Non-dominant Chicken Cecum Microbiota, While Diet Affects the Dominant Microbiota

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The combined effect of environment and diet in shaping the gut microbiota remains largely unknown. This knowledge, however, is important for animal welfare and safe food production. For these reasons, we determined the effect of experimental units on the chicken cecum microbiota for a full factorial experiment where we tested the combined effect of room, diet, and antimicrobial treatment. By Illumina Deep sequencing of the 16S rRNA gene, we found that diet mainly affected the dominant microbiota, while the room as a proxy for environment had major effects on the non-dominant microbiota (p = 0.006, Kruskal–Wallis test). We, therefore, propose that the dominant and non-dominant microbiotas are shaped by different experimental units. These findings have implications both for our general understanding of the host-associated microbiota and for setting up experiments related to specific targeting of pathogens.

Keywords: chicken, microbiota, 16S rRNA gene, cecum, *Clostridium perfringens*

OPEN ACCESS

Edited by:
Guillermo Tellez, University of Arkansas, USA

Reviewed by:
Filip Boyen, Ghent University, Belgium
Morgan Brian Farnell, Mississippi State University, USA

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *01 December 2015*
Accepted: *10 February 2016*
Published: *25 February 2016*

Citation:
Ludvigsen J, Svihus B and Rudi K (2016) Rearing Room Affects the Non-dominant Chicken Cecum Microbiota, While Diet Affects the Dominant Microbiota. Front. Vet. Sci. 3:16. doi: 10.3389/fvets.2016.00016

INTRODUCTION

The gut microbiota plays a crucial role for the host health through providing essential metabolites and vitamins, in addition to immune/gut maturation and protection toward pathogen colonization (1). Despite this crucial role, our knowledge about the ecological driving forces shaping the gut microbiota is limited. Although it is well known that diet and antimicrobial compounds can affect the gut microbiota, the influence of the environment is still largely unknown (2). This represents a major challenge when setting up experiments involving antimicrobial and/or dietary perturbations of the gut microbiota.

Here, we evaluated the effect of different experimental units in a full factorial experimental design, where both the microbiota composition and the level of *Clostridium perfringens* were determined for the chicken cecum microbiota. The experimental units evaluated were room as a proxy for environment, diet, and antimicrobial treatment. For the microbiota composition, we used Illumina deep sequencing of the 16S rRNA gene (3), while the level of *C. perfringens* was determined by real-time PCR (4).

The rationale for the choice of chicken microbiota and *C. perfringens* association is that chickens are kept in large flocks, with the potential for rapid, large-scale pathogen transmission (5). Since chickens do not have contact with the adult population other than from bacteria potentially colonizing the egg shell, they are prone to colonization by the environmental microbiota (6–8). *C. perfringens* represents a major challenge in poultry production (9). Traditionally, prophylactic use of antibiotics

has been applied in *C. perfringens* control, but due to the spread of antibiotic resistance, prophylactic use of antibiotics is now banned or will be banned in most countries. Challenges related to banning antimicrobial compounds, however, are both the lack of alternatives for pathogen control and the lack of knowledge about the ecology of chicken gut microbiota (10).

MATERIALS AND METHODS

Experimental Design

A total of 360 male broiler chickens of the breed Ross 308 were used in the experiment. Half of these were vaccinated against coccidiosis upon arrival by spraying Paracox-8 (Schering-Plough Ltd., UK) on the feed, and each of these two groups were then distributed among 12 pens (15 birds per pen) divided between two rooms (6 pens per room per treatment). The physical environment should be identical in the two rooms. All chickens received commercial starter diets until 7 days of age, where the non-vaccinated chickens were also given the antimicrobial narasin (Monteban, Elanco Animal Health, USA) throughout the experiment to prevent coccidiosis. Narasin, however, also have cross-inhibition toward *C. perfringens* (11). After 7 days, each of the treatment groups was split in two, and half of the pens continued to receive a commercial diet, while the other half received an equal portion of a barley/oats/wheat experimental diet. The rationale for the experimental diet was to utilize locally produced grains to reduce transportation costs. This resulted in four treatment groups with six pens per groups with three pens per room. After 4 days of adaptation to the new diets, at 11 days of age, birds were weighed in groups. Birds were also weighed prior to slaughter at 34 days of age. At 35 days of age, the chickens were slaughtered and the cecal contents from 3 birds per pen (72 birds in total) were collected for microbiota analyses. The birds were killed by cervical dislocation, and one randomly selected cecum was immediately dissected out from each animal. The experiments were conducted following Norwegian legislation and guidelines. The experimental design is schematically outlined in **Figure 1**.

Microbiota Analyses

The complete content of one cecum for each chicken was suspended 1:3 in STAR buffer (Roche, Switzerland). The samples were then immediately frozen at −20°C, with further processing within 1 month.

Thawed samples were vortexed, and 500 µl of the liquid phase was transferred to tubes with acid-washed glass beads (Sigma-Aldrich, <106 µm; 0.25 g). Subsequently, the samples were processed twice in a MagNaLyzer (Roche, Switzerland) at 6500 rpm for 20 s with cooling using the MagNaLyzer cooler Wein-between to disrupt the cells. Cell debris was removed by centrifugation at 19,000 × *g* for 5 min. Subsequent DNA extraction was done using MagMiniLGC kit (LGCgenomics, UK), following the manufacturer's recommendations using a KingFisher Flex (Thermo Scientific, USA) DNA extraction robot.

A nested approach was used for the 16S rRNA gene Illumina sequencing. The first PCR was run for 25 cycles using the primers and protocol developed by Yu et al. (12). The PCR product was then diluted 1:100, with subsequent 10 PCR cycles following the protocol by Naseribafrouei et al. (3) using Illumina MiSeq V3 kit (Illumina, USA). Resulting 300 bp paired-end data were analyzed using the QIIME pipeline (13). Sequences were paired-end joined (*fastq-join*) and quality filtered based on average sequence quality score more than 25. Then, sequences were clustered with 97% identity level using *usearch* v7 (14, 15). Taxonomic assignments were done using the Greengenes (16) and the RDP database (17).

For the categorical variables room, diet, and antimicrobial treatment, we used partial least square discriminant (PLS-DA) analyses for relating the variables with the operational taxonomic unit (OTU) table, while for *C. perfringens* we used partial least square (PLS) analyses. In all cases, we used Venetian Blinds cross validation and the average microbiota within each pen as explanatory variables. These analyses were done using the PLS toolbox (Eigenvector, USA), running in the Matlab environment (Mathworks Inc., USA).

We used Simpson's *D* and observed species as alpha diversity measures whereas we used Bray–Curtis and Jaccard indexes for beta diversity analyses. The non-parametric Kruskal–Wallis test was used to test the significance of the differences detected for the diversity measures (Minitab Inc., USA).

For the quantitative PCR, we first quantified the 16S rRNA gene using the previously described PRK primers (12). For *C. perfringens* detection, we used real-time PCR targeting the toxin gene cpe (154 bp), as described previously by Rinttila et al. (4). Both the rtPCRs were run on a LightCycler 480 (Roche, Switzerland) with evagreen PCR chemistry (Solis BioDyne, Estonia). The chickens were scored positive for the respective toxin genes given that the PRK PCR gave a ct below 20 and toxin gene PCR below <35, while the corresponding negative assignments were the cases where PRK PCR gave a ct below 20 and the toxin gene PCR gave a ct of 35, or above. The rationale for the qualitative assignments was the expected low quantitative levels of *C. perfringens*. ANOVA (Minitab) was used to analyze the relationship between the qPCR data, room, diet, and antimicrobial treatment.

RESULTS

Growth Characteristics and Mortality

Analysis of variance showed no significant effects on weight gain, with an average weight gain of 2.2 kg from day 11 to 34. A large numerical difference in mortality was observed during the first 11 days of life. The chickens that did not receive narasin showed a mortality of 11%, while the chickens that received narasin showed a mortality of 0.5%. From 11 to 34 days of age, the numerical difference decreased: 1.8% for the narasin group and 4.4% for the non-narasin group. As similar difference was observed for the experimental diet as compared to the commercial (average 4.7 vs. 1.8%, respectively) diet. Room did not appear to have any appreciable effect on mortality.

Microbiota Composition

A total of 4.7 Gbp 16S rRNA gene sequence data with 84% above Q30 was generated by Illumina sequencing. After assembly and quality filtering, average number of sequences per sample was

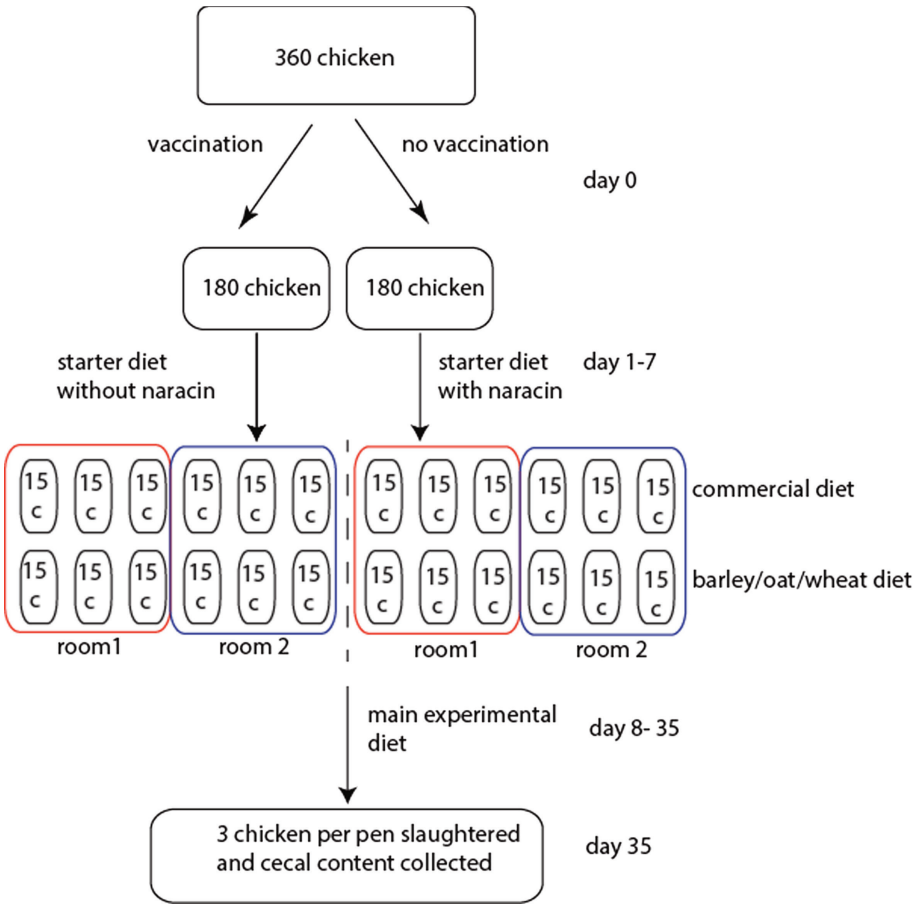

FIGURE 1 | **Schematic outline of the experimental design**. For the rooms, the boxes represent pens with the respective number of chickens.

49,037, with only 1 out of 72 samples with <4000 sequences. We therefore rarefied the samples to 4000 sequences prior to further analysis.

Analyses of the taxonomic assigned data showed that the overall microbiota composition was dominated by *Firmicutes* at the phylum level, *Clostridia* at the class level, while at the order level most of the sequences were unclassified, suggesting a high level of poorly characterized bacteria (Figure S1 in Supplementary Material).

Due to the high number of taxonomically unassigned sequences, we pursued our further analyses at the OTU level using the phylogenetic tree as a proxy for taxonomy. These analyses revealed an overall large observed microbial species richness ($n = 273$), with only a few dominant OTUs (**Figure 2**). For the whole tree, there were two lineages that could not be taxonomically assigned beyond the class level. These were denoted Clostridiales I and II, respectively (**Figure 2**). Comparison of the relative distribution of OTUs with that of the expected log normal distribution confirmed an overrepresentation for seven OTUs with an average abundance >3% (Figure S2 in Supplementary Material). All the seven overrepresented OTUs have previously been identified in poultry, but they generally lack closely related taxonomically assigned sequences (Table S1 in Supplementary Material).

Effect of Room, Feed, Antimicrobial Treatment, and Pen on the Microbiota Composition

We first evaluated the effect of pen by comparing the variance for each OTU between the three birds per pen with the variance between the three pens within the treatment groups. For 76% of the OTUs, the variance was lower between the pens within the same treatment group than among birds in the same pen.

Using PLS-DA, we obtained an overall significant association between the experimental factors and microbiota, with a respective cross-validated accuracy of classification for room, diet, and antimicrobial treatment of 0.70, 0.78, and 0.60. This means that the microbiota composition can be predicted based on the experimental factors. There were no internal correlations between the OTUs important for these associations ($p > 0.1$, Spearman correlation), with a relatively complex pattern in which closely related OTUs have opposite influences in the classification models (**Figure 2**). This means that closely related bacteria can have opposite relationships to the factors investigated.

We found no significant associations for alpha diversity (Simpson's *D* and observed species), whereas for beta diversity, we found strong associations for room and the Jaccard index (presence

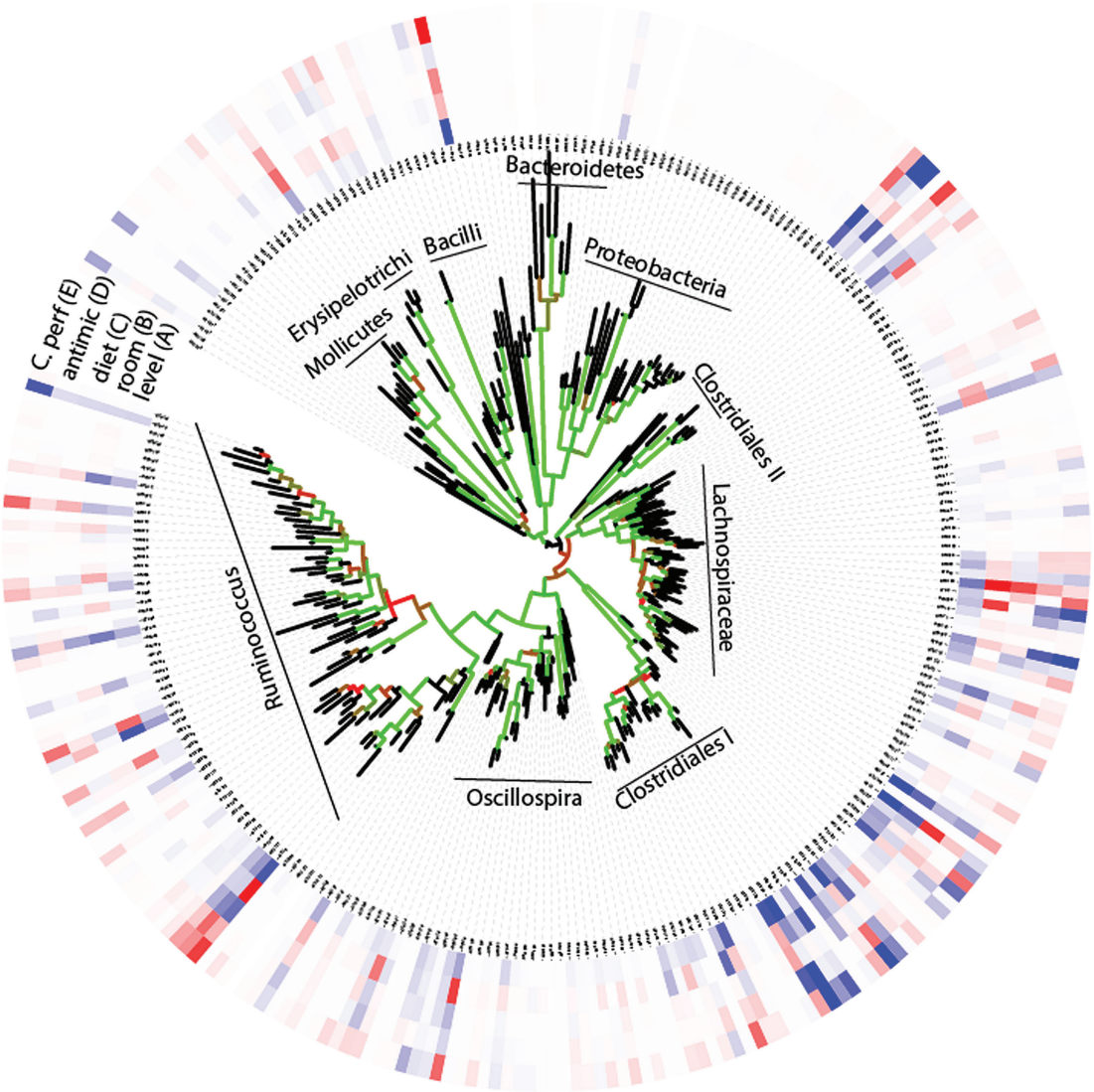

FIGURE 2 | OTU associations (A) with relative composition, (B–D) experimental factors, and (E) *C. perfringens*. (A) The inner circle represents the relative composition in percentage, while the other circles (B–E) represent loadings (OTU importance) in the respective regression models. The color code for the circles are given by that blue represents high (greater than 3) and red low (less than −3) values. The tree shows the phylogenetic association of the OTUs, with the Bootstrap support being given by the color code of the branches (black <60%, red 60–80%, and green >80%).

absence of OTU's), the Bray–Curtis index to diet (taking into account OTU levels) showed the strongest association (**Figure 3**).

Since the non-dominant microbiota have a larger impact on the Jaccard index than the dominant, and because the Bray–Curtis index is mostly influenced by dominant OTUs, we compared the levels of the OTUs that show significant false discovery corrected associations with the experimental factors (Table S2 in Supplementary Material). These comparisons showed that the average level of the OTUs associated with room differences was significantly lower than for those associated with diet (**Figure 4**).

Associations to *C. perfringens*

Using *plc* as a proxy for *C. perfingens*, we established a significant association with antimicrobial treatment, where narasin showed a major reduction in *C. perfringens* prevalence (63 vs. 17%, $p = 0.001$ ANOVA test for non-treated and narasin-treated chickens, respectively). The effect was independent of room.

The direct correlation between *C. perfringens* and the microbiota was investigated by PLS regression. A four component model showed the best correlation ($R^2 = 0.94$ for calibration and 0.04 for validation). Mapping the loadings onto the OTU-derived phylogenetic tree shows OTUs with both positive and negative *C. perfringens* associations across the tree (**Figure 2**). OTU 20 (within the Clostridiales I cluster in **Figure 2**) showed the largest influence on the model (loading = 23.3). This OTU also show a significant direct positive correlation with *C. perfringens* ($p = 0.002$, Spearman correlation). However, there were no single OTUs with pronounced negative associations to *C. perfringens*.

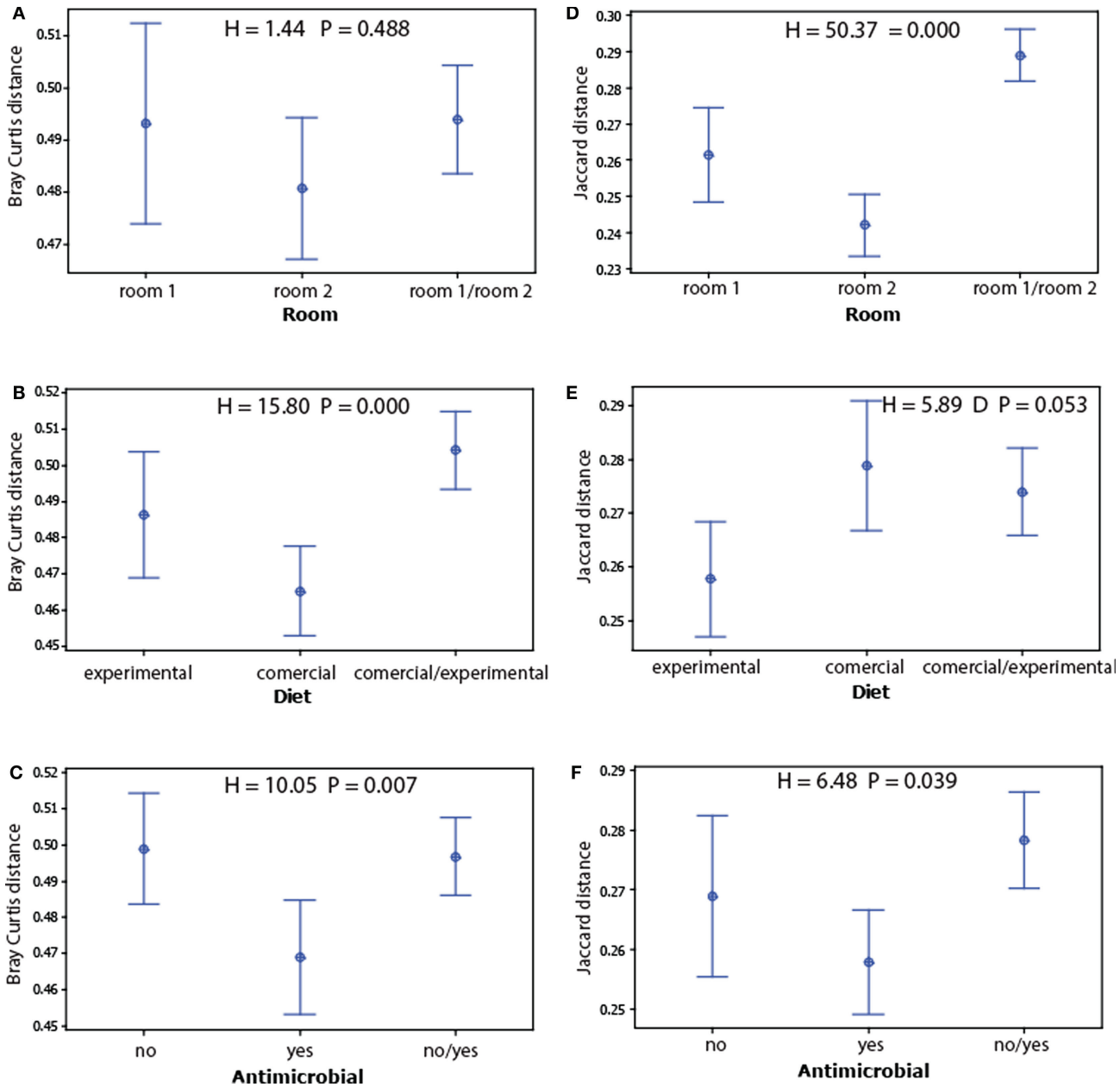

FIGURE 3 | Main beta diversity associations of the microbiota with the experimental factors. The beta diversity within and between the factors levels for each treatment were determined for Bray–Curtis **(A–C)** and Jaccard distances **(D–F)**. The dots represent mean values, while the error bars represent the 95% confidence intervals. The overall significance for each model was tested by Kruskal–Wallis test.

DISCUSSION

Our main finding was the presence of a diverse but low-abundant microbiota associated with the experimental unit room, while diet mainly affected the high-abundant microbiota. Since the physical environment is designed to be similar in the two rooms, the differences detected could potentially be due to differences in the room microbiota, and as a consequence a difference in microbial exposure (18). A likely source for the potential room associated microbiota is clostridial spores, since these can easily survive decontamination (19).

The high abundant microbiota – being more abundant in the population than expected from a lognormal distribution – seemed host specific (OTUs were poultry associated as determined by Blast searches). The distinct distribution patterns of high- and low-abundant species resemble a common pattern in many ecosystems (20). Related distributions have also been observed for the human gut microbiota, with a common core of

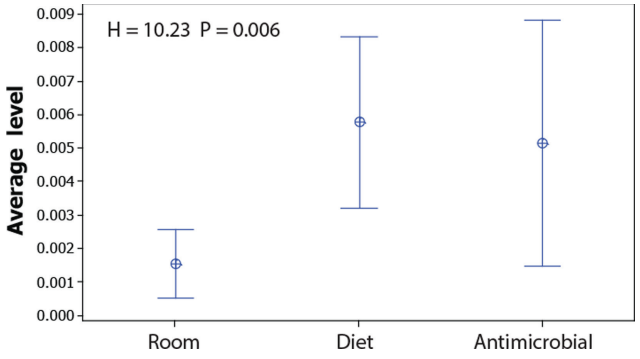

FIGURE 4 | Abundance of OTUs significantly associated with room, diet, and antimicrobial treatment. The dots represent the mean values, while the error bars represent the 95% confidence interval for the OTUs showing significant false discovery corrected associations with the respective factors (raw data presented in Table S2 in Supplementary Material). The overall significance was tested by Kruskal–Wallis test.

bacteria shared among most individuals (21, 22). A host-specific microbiota has also been identified from cataloging mouse microbiota (23). Taken together, our results contribute to the support of a model advocating the importance of a host-specific microbiota (24).

Several closely related OTUs show opposite relationship to the experimental factors investigated. This highlights the importance of high-resolution analyses of the microbiota. Furthermore, we identified two abundant clusters of OTUs denoted Clostridiales I and II with no closely related counterparts in the databases. The dominance of *Clostridium* spp. in the chicken cecum has previously been noted in several studies (6–8). However, despite the importance of clostridia in the chicken gut, this class is poorly characterized taxonomically (25). This renders the risk of overseeing or misinterpreting effects when using taxonomic model-based approaches.

Since there are no direct transmission routes of bacteria from mother to offspring for chickens other than potentially through the egg shell, most of the host-associated microbiota is probably transmitted at a later stage than egg laying. *Clostridia* are generally spore-forming and widely transmitted at the farm level (26). Thus, spores could be the main vector for host-specific clostridia colonization for chickens. The importance of spores in establishing a host-specific microbiota has also recently been noted for humans (27, 28).

The antimicrobial effect of narasin on *C. perfringens* seems independent of the effect on the cecum microbiota, because the OTUs correlating with narasin treatment are not the same as those correlating with *C. perfringens*. This may be due to the fact that the main reservoir of *C. perfringens* is in the small intestine, with the main effect of narasin being in the small intestine because of the mucus association of bacteria. Furthermore, the effect of narasin could also potentially be indirect There were, however, OTUs that correlated directly both positively and negatively with *C. perfringens* in the cecum independent of the narasin treatment. In the cecum, most of the bacteria are lumen associated, which may explain the different interaction pattern here. The strongest positive correlation was detected for an OTU within the uncharacterized Clostridiales I group. Since no information is available for these clostridia, we cannot deduce potential mechanisms for the correlations, or whether intervention strategies through the microbiota would be feasible.

The main conclusion from our work is that the experimental units affect the dominant and non-dominant microbiota differently. These differences need to be considered when investigating the effect of dietary and antimicrobial interventions.

AUTHOR CONTRIBUTIONS

JL conducted the analytical work, BS designed and carried out the chicken experiments, and KR wrote the paper and analyzed the data.

ACKNOWLEDGMENTS

We thank Nortura for financial support and Frank Sundby for help with the chicken analyses.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fvets.2016.00016

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

Nortura is a meat producing company. None of the authors are associated with Nortura, and there were no restrictions on data reporting.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 14 December 2015
doi: 10.3389/fvets.2015.00076

West Nile Virus Challenge Alters the Transcription Profiles of Innate Immune Genes in Rabbit Peripheral Blood Mononuclear Cells

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The peripheral innate immune response to West Nile virus (WNV) is crucial for control of virus spread to the central nervous system. Therefore, transcriptomes encoding the innate immune response proteins against WNV were investigated in peripheral blood mononuclear cells (PBMCs) of New Zealand White rabbits, a recently established novel rabbit model for WNV pathogenesis studies. PBMCs were challenged with an Australian WNV strain, $WNV_{NSW2011}$, *in vitro*, and mRNA expression of selected immune response genes were quantified at 2-, 6-, 12-, and 24-h post-infection (pi) using qRT-PCR. Compared to mock-inoculated PBMCs, WNV-stimulated PBMCs expressed high levels of interferon (IFN) alpha (IFNA), gamma (IFNG), IL6, IL12, IL22, CXCL10, and pentraxin 3 (PTX3) mRNA. Likewise, TLR1, 2, 3, 4, 6, and 10 mRNA became up-regulated with the highest expression seen for TLR3, 4, and 6. TLRs-signaling downstream genes (MyD88, STAT1, TRAF3, IRF7, and IRF9) subsequently became up-regulated. The high expression of IFNs, TLR3, TLR4, TRAF3, STAT1, IRF7, and IRF9 are in accordance with antiviral activities, while expression of TNFA, HO1, iNOS, caspase 3, and caspase 9 transcripts suggests the involvement of oxidative stress and apoptosis in WNV-stimulated rabbit PBMCs, respectively. The level of $WNV_{NSW2011}$ RNA increased at 24-h pi in PBMCs challenged with virus *in vitro* compared to input virus. The expression dynamics of selected genes were validated in PBMCs from rabbits experimentally infected with WNV *in vivo*. Higher expression of IFNA, IFN beta (IFNB), IFNG, TNFA, IL6, IL22, PTX3, TLR3 and TLR4, IRF7, IRF9, STST1, TRAF3, caspase 3, and caspase 9 were seen in PBMCs from WNV-infected rabbits on day 3 post-intradermal virus inoculation compared to PBMCs from uninfected control rabbits. This study highlights the array of cytokines and TLRs involved in the host innate immune response to WNV in the rabbit leukocytes and suggests that these cells may be a useful *in vitro* model for WNV infection study.

Keywords: West Nile virus, rabbit, PBMCs, innate immune response, cytokines, TLRs

OPEN ACCESS

Edited by:
Guillermo Tellez, University of Arkansas, USA

Reviewed by:
Kieran G. Meade, Teagasc, Ireland
Amanda Jane Gibson, Royal Veterinary College, UK

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *30 September 2015*
Accepted: *30 November 2015*
Published: *14 December 2015*

Citation:
Uddin MJ, Suen WW, Prow NA, Hall RA and Bielefeldt-Ohmann H (2015) West Nile Virus Challenge Alters the Transcription Profiles of Innate Immune Genes in Rabbit Peripheral Blood Mononuclear Cells. Front. Vet. Sci. 2:76. doi: 10.3389/fvets.2015.00076

INTRODUCTION

Since the first isolation of the Australian strain of West Nile virus, the Kunjin strain (WNV_{KUN}), in 1960 in North Queensland (1), it has been found to be endemic in Australia (2). WNV_{KUN} belongs to lineage 1 of WNV, which also includes the highly pathogenic neuro-invasive New York 99 strain (WNV_{NY99}) (3). WNV_{KUN} causes mainly asymptomatic infection and only a small number of mild human cases have been documented with no reported fatalities in Australia (2). In contrast, since its introduction into New York in 1999, WNV_{NY99} has spread rapidly throughout the USA with close to 40,000 human cases of WNV disease and more than 1,600 deaths reported in the USA between 1999 and 2014 (4). WNV has also spread to other parts of the Americas, and also to Europe, Asia, and the Middle East (5). In addition to human infections, the WNV_{NY99} virus has caused significant morbidity and mortality in horses and birds, with more than 20,000 equine cases and hundreds of thousands of avian deaths (6). Relative to the WNV_{NY99} strain, WNV_{KUN} exhibits much reduced virulence in humans, animals, and birds (2). Equine disease associated with WNV_{KUN} infection was rare, however, in early 2011 following extensive flooding in the Murray–Darling river basin and other inland river systems, an unprecedented outbreak of equine encephalitis occurred in south-eastern Australia involving more than 1,000 horses (7). Genomic sequencing of viruses isolated from a horse succumbing to encephalitis in the 2011 outbreak revealed the etiological agent to be a variant strain of WNV, most closely related to WNV_{KUN} and subsequently named $WNV_{NSW2011}$ (7). As the virus strain may still be circulating and the human and equine populations in Eastern Australia remain susceptible to WNV_{KUN} (8, 9), it is important to get a better understanding of the host response to this virus.

Peripheral blood mononuclear cells (PBMCs) are among the first immune components to encounter WNV following a mosquito bite. It has been postulated that the PBMC may serve as target cells for initial replication of WNV and play a role in subsequent viral dissemination (10). Additionally, primary PBMC cell culture has been proposed to be a potentially useful model of a natural WNV host (10). To survive virus infection, the host must recognize invasion and develop an effective antiviral immune response. This response is initiated in infected cells after detection of non-self pathogen-associated molecular patterns (PAMPs). PAMPs motifs are detected by specific, conserved host molecular patterns – pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs), which trigger signaling cascades that induce the activation of interferon (IFN) regulatory factors (IRFs) and nuclear factor kappa B (NFκB), leading to expression of antiviral molecules, including type I IFNs (IFNA and IFNB), and hundreds of different IFN-stimulated effector genes (ISGs). ISG products include additional antiviral effector molecules and immunomodulatory cytokines that serve to restrict virus replication and modulate the immune response (11).

The signaling pathways thought to detect entry and infection by WNV and initiate a protective IFN response have mostly been studied in mice (12–18) with only limited studies in the horse (19). Based on those studies, a partial signaling pathway has been proposed by which WNV and other flaviviruses are detected, and the effector mechanisms that contribute to protective cell-intrinsic immunity executed (11, 20). Nevertheless, there appears to be some discrepancies between the protective immune mechanisms between humans and mice (21, 22) and even more so between mice and horses (23, 24). In order to overcome the latter problem, we have recently established a New Zealand White (NZW) rabbit model for WNV-infection (25). Physiologically, rabbits resemble horses by being hindgut fermenters and 10–20% of feral rabbits may be exposed to WNV in any season throughout Australia (8). Previously, gene expression has been performed in selected tissues (thalamus and cerebrum) of horse (19), mouse (26), NZW rabbits (25), and in human cells and tissues infected with WNV (27). However, there is a gap in knowledge of the expression patterns of immune-related genes during the early time points following infection. Therefore, this study aimed to characterize the innate immune response in rabbit PBMCs, exposed *in vitro* to $WNV_{NSW2011}$ using transcriptomic analysis of immunologically important genes. Selected transcripts were subsequently confirmed in the *in vivo* WNV-infection model, supporting the contention that *in vitro* studies of PBMCs responses are a useful surrogate model for acute WNV infection in natural hosts and relevant animal model.

MATERIALS AND METHODS

Preparation of WNV

The $WNV_{NSW2011}$ strain used in this study was isolated from a 10% weight/volume brain homogenate of an encephalitic horse during the 2011 Australian arboviral outbreak. The virus isolate was passaged initially in C6/36 cells (*Aedes albopictus* mosquito cells), followed by 1× in Vero (African green monkey kidney) cells and 3× final passages in C6/36 cells cultured with 10% fetal bovine serum (FBS) at 28°C before use. Detailed characterization of the mouse virulence of $WNV_{NSW2011}$ has been described in Frost et al. (7). The mock inoculum consisted of tissue culture medium only. The virus stock was stored at −80°C. The titer of the stock was quantified using standard plaque assay on Vero cells, as described previously (25, 28). The $WNV_{NSW2011}$ was diluted to 1×10^6 PFU/50 μL for use in PBMCs infection *in vitro* experiments.

PBMCs Isolation, Culture, and Challenged with Viruses

EDTA-stabilized blood samples were collected from WNV-seronegative NZW rabbits ($n = 3$) (25). All animal procedures had received prior approval from the University of Queensland

Abbreviations: ACTB, actin, beta; CXCL10, C–X–C motif chemokine 10 [also known as Interferon gamma-induced protein 10 (IP-10)]; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HO1, heme oxygenase 1; IFNα, interferon alpha; IFNβ, interferon beta; IFNγ, interferon gamma; IL12, interleukin IL22; IL6, interleukin 6; iNOS, inducible nitric oxide synthase; IRF, interferon regulatory factor; Kunjin, australian strain of west nile virus; MyD88, myeloid differentiation 88; PPIA, peptidylprolyl isomerase A (also known as cyclophilin A); PTX3, pentraxin 3; STAT1, signal transducer and activator of transcription 1; TLR, toll-like receptor; TNFα, tumor necrosis factor alpha; TRAF3, TNF receptor-associated factor 3.

Animal Ethics Committee (SVS/369/12/ARC), and all procedures were performed under xylazine sedation and anesthesia (25). Blood was collected by cardiac bleed of anesthetized rabbits into EDTA-coated collection tubes (Vacuette®, Greiner Bio One, Australia) immediately prior to their euthanasia. The PBMCs were isolated from the blood using Ficoll-Histopaque (Sigma) as described previously (29). The viability of the purified PBMCs was assessed using the trypan blue exclusion method and was always >90%. The cells were counted using a hemocytometer and the concentration was adjusted to 1×10^6 cells/0.5 ml in RPMI-1460 supplemented with L-glutamine (2 nM), streptomycin (50 μg/mL), penicillin (50 U/mL), and 10% FBS (29). The cells were cultured in 24-well cell culture plates (Costar Corning, the Netherlands) seeded with 500 μL of cell suspension per well and incubated at 37°C with 5% CO_2. Remaining PBMCs (fresh-isolated PBMCs) were pelleted and kept at −80°C for RNA isolation. After 1 h of incubation, the PBMCs were challenged by adding 50 μL of $WNV_{NSW2011}$ to each well to give a final multiplicity of infection (MOI) of one. The dose of virus was chosen based on the typical dose inoculated by mosquitoes (30). Additional 450 μL medium was added to each well to make up the final volume of 1 ml. One well of PBMCs per animal was harvested at 2, 6, 12, and 24 h after virus infection, respectively. The harvested cells (WNV-stimulated PBMCs) were pelleted and kept at −80°C until subjected to RNA isolation. For complete harvesting of adherent PBMCs, detachment with lidocaine HCl (12 mM) was performed (31). To ensure the complete harvesting of cells, wells were checked using an inverted microscope. Duplicate wells of uninfected cells (mock-inoculated PBMCs) were cultured in 500 μL of RPMI culture media supplemented with 10% FBS. They were harvested and treated in a similar manner at each time point.

PBMCs Isolation from Infected Rabbits

In order to compare the transcriptional profile of in vitro challenged PBMCs against those from in vivo challenged rabbits blood samples were obtained from $WNV_{NSW2011}$-infected New Zealand White (NZW) rabbits ($n = 3$) on day 3 pi, and mock-infected NZW rabbits ($n = 3$) on day 6 post-mock (medium only) inoculation (25). Collected blood was immediately processed for PBMCs isolation, as described above.

RNA Isolation and Transcriptome Quantification

Total RNA was isolated from PBMCs using miRNeasy RNA isolation kit (Qiagen Pty Ltd., Australia) and on-column DNA digestion (Qiagen) was performed following the manufacturer's instructions. The quantity and quality of RNA was measured using NanoDrop 1000 spectrophotometer (Thermo Scientific, Australia). The isolated RNA was subjected to PCR with GAPDH primers without a reverse transcription step and run in gel to check for DNA-contamination. None was found to be contaminated (data not shown). The isolated RNA was kept at −80°C for further transcriptome analysis using quantitative real time PCR (qRT-PCR). Primers for cytokines, TLRs and downstream genes, apoptosis and oxidative stress-related genes, and two normalizer genes (*GAPDH* and *PPIA*) were designed from FASTA products of the GenBank mRNA sequences for *Oryctolagus cuniculus* using the Primer3 program (32). No suitable normalizer genes has been reported in rabbits PBMCs yet, but *GAPDH* and *PPIA* are reported to be appropriate stably expressed normalizer genes in PBMCs in pigs (33). The $WNV_{NSW2011}$ (WNV_{KUN})-specific primers (34) and primers of some cytokines (35) were described earlier. Details of the primers are given in **Table 1**.

To quantify the mRNA expression for target and reference genes, qRT-PCR was performed using the Rotor Gene Corbett 6000 quantitative real-time PCR system (Qiagen). A one step qRT-PCR was performed using Rotor-Gene SYBR Green RT-PCR Kit (Qiagen). Each run contained each RNA sample and a no-template control. qRT-PCR was set up using 1 μL of RNA template, 5.25 μL of deionized RNase free H_2O, 0.5 μM of upstream and downstream primers, 0.25 μL Rotor-Gene RT mix (RT mix), and 12.5 μL of 2× Rotor-Gene SYBR Green RT-PCR master mix (MM) (Qiagen) in a total reaction volume of 20 μL. The cycling conditions included reverse transcription step (10 min at 55°C), PCR initial activation step (5 min at 95°C), and a two-step cycling protocol with a denaturation step and a combined annealing/extension step. The two-step thermal cycling conditions were 5 s at 95°C followed by 10 s at 60°C (40 cycles). An amplification-based auto-threshold (Rotor-Gene Q Series Software, Qiagen) and adaptive baseline were selected as algorithms. Melting curve analysis was performed to detect the specificity of the PCR reaction. Each sample was run twice, and the average value was used as expression value. The qRT-PCR products of selected genes (e.g., GAPDH) on agarose gel. The gel documentation showed amplification only at the anticipated product length (i.e., 126 bp for GAPDH) (data not shown). Gene-specific expression was measured as relative to the geometric mean of the expression of two normalizer genes (*GAPDH* and *PPIA*) (**Table 1**). The delta Ct (ΔCt) ($\Delta Ct = Ct_{target} - Ct_{normaliser\ genes}$) values were calculated as the difference between target gene and reference genes, and expression was calculated as $2^{(-\Delta\Delta Ct)}$ (36). To compare the magnitude of gene expression, the fold change was calculated. For this purpose, the delta delta Ct (ΔΔCt) values were calculated as follows: $\Delta\Delta Ct = \Delta Ct_{WNV} - \Delta Ct_{mock}$. The bar graphs (**Figures 1–5A**; **Figure 7**) show the expression of genes in WNV-stimulated PBMCs over mock-inoculated PBMCs (fold change: the normalized expression value of a gene in WNV-stimulated cells/the normalized expression value of a gene in mock-inoculated cells). In addition, accounting for the effects of culture conditions on gene transcription in WNV- and mock-inoculated rabbit PBMCs, the normalized expression of genes from PBMCs harvested at each time point was compared to their respective expression levels before either WNV inoculation or mock inoculation. The ΔΔCt values were calculated by subtracting ΔCt of genes in fresh-isolated PBMCs from the ΔCt of genes in WNV- or mock-inoculated PBMCs at each time-point. The average expression values of the mRNA levels were considered for further analysis.

Statistical Analysis

The technical replications were averaged. The impact of virus-challenge (treatment) and duration of incubation (time points) were evaluated using the SAS software package v. 9.2 (SAS Institute, Cary, NC, USA). For this purpose, the GLM (general

TABLE 1 | List of primer sequences used in this study.

Gene	Primer set[a]	Amplicon size (bp)	GenBank accession number
Kunjin	F: AACCCCAGTGGAGAAGTGGA[b] R: TCAGGCTGCCACACCAAA	70	D00246
IFNA	F: TGCTTGCAGGACAGACATGA R: ATCTCGTGGAGCACAGAGAT	95	XM_002708065.1
IFNB	F:TCCAACTATGGCACGGAAGTCT[c] R:TTCTGGAGCTGTTGTGGTTCCT	89	XM_002707968
IFNG	F:TGCCAGGACACACTAACCAGAG[c] R:TGTCACTCTCCTCTTTCCAATTCC	127	NM_001081991
TNFA	F:CTGCACTTCAGGGTGATCG[c] R:CTACGTGGGCTAGAGGCTTG	94	NM_001082263
IL6	F: CTACCGCTTTCCCCACTTCAG[c] R: TCCTCAGCTCCTTGATGGTCTC	135	NM_001082064.2
IL12/IL23P40	PS211: CTCCGAAGAAGATGGCATTACC[c] PS212: TCTCCTTTGTGGCAGGTGTATTG	126	XM_002710347
IL22	PS567: ACCTCACCTTCATGCTGGCTAA PS568: CATGGAACAGCTCATTCCCAAT	84	XM_002711248
CXCL10	F: ATAGAAGCATCCTGAGCCCA R: GAACTGCAAACTGAGGCCAA	86	XM_002717106.1
PTX3	F: TTCCCCATGCGTTCCAAGAA R: GTGGCTTTGACCCAAATGCA	95	XM_002716328.1
HO1	F: ACTGCCGAGGGTTTTAAGCT R: GGTTCTCCTTGTTGTGCTCA	88	XM_002711415.1
iNOS	F: GACGTCCAGCGCTACAATATCC[c] R: GATCTCTGTGACGGCCTGATCT	102	XM_002718780
Caspase 3	F: AAGCCGACTTCCTGTATGCA R: CGTACTCTTTCAGCATGGCA	111	NM_001082117.1
Caspase 9	F: AAACGTGGATTTGGCGTACG R: TGCTGCTGAAGTTCACGTTG	80	XM_002722329.1
TLR1	F: TGTGTCCCACAATGAGCTGT R: GGCAGAGCATCAAACGCATT	93	XM_002709270.1
TLR2	F: GCTGCGCAAGATCATGAACA R: TTTATGGCGGCCCTCAAGTT	96	NM_001082781.1
TLR3	F: ATGACCTGCCCACCAACATA R: TTCTGGCTCCAGCTTTGAGA	140	NM_001082219.1
TLR4	F: AGGCTGTTGGTGGAAGTTGA R: TGCTTATCTGACAGGTGGCA	91	NM_001082732.2
TLR6	F: CATTGAGCACAACGCAGTGT R: AGCTCGCATGTACAGTGGAA	108	XM_002709388.1
TLR10	F: ACACCGGTAATGCACTTGGA R: TAAGCAAGGTGTCTGGCCAT	85	XM_002709387.1
MyD88	F: GCCAGTGAGCTCATCGAGAA R: TCACACTCCTTGCTCTGCAG	80	XM_002723869.1
IRF1	F: AGCACTGTCACCACATAGCA R: TCATCTGTCGCAGCTTCAGA	120	NM_001171347.1
IRF7	F: AAGTGCAAGGTGTACTGGGA R: AGCTCTTGGAAGAAGGTGCT	119	XM_002724304.1
IRF9	F: TAACTGAGGCTGCTGTGCAA R: ACACGCCCGTTGTAGATGAA	103	XM_002718097.1
TRAF3	F: TGGCTATAAGATGTGCGCCA R: ACTCTCCACGCATGATGACA	95	XM_002721716.1

(Continued)

TABLE 1 | Continued

Gene	Primer set[a]	Amplicon size (bp)	GenBank accession number
STAT1	F: TTCAACATCCTGGGCACACA R: TGCCAGCGTTCTTCTGTTCT	112	XM_002712346.1
GAPDH	F: TGACGACATCAAGAAGGTGGTG** R: GAAGGTGGAGGAGTGGGTGTC	126	NM_001082253
PPIA	F: AGGGCATGAGCATTGTGGAA R: TCCACAGTTGGCAATGGTGA	86	NM_001082057.1

[a]*Annealing temperature was 60°C for all the primer sets.*
[b]*Primer set is adopted from Ref. (34).*
[c]*Primer set are adopted from Ref. (35).*

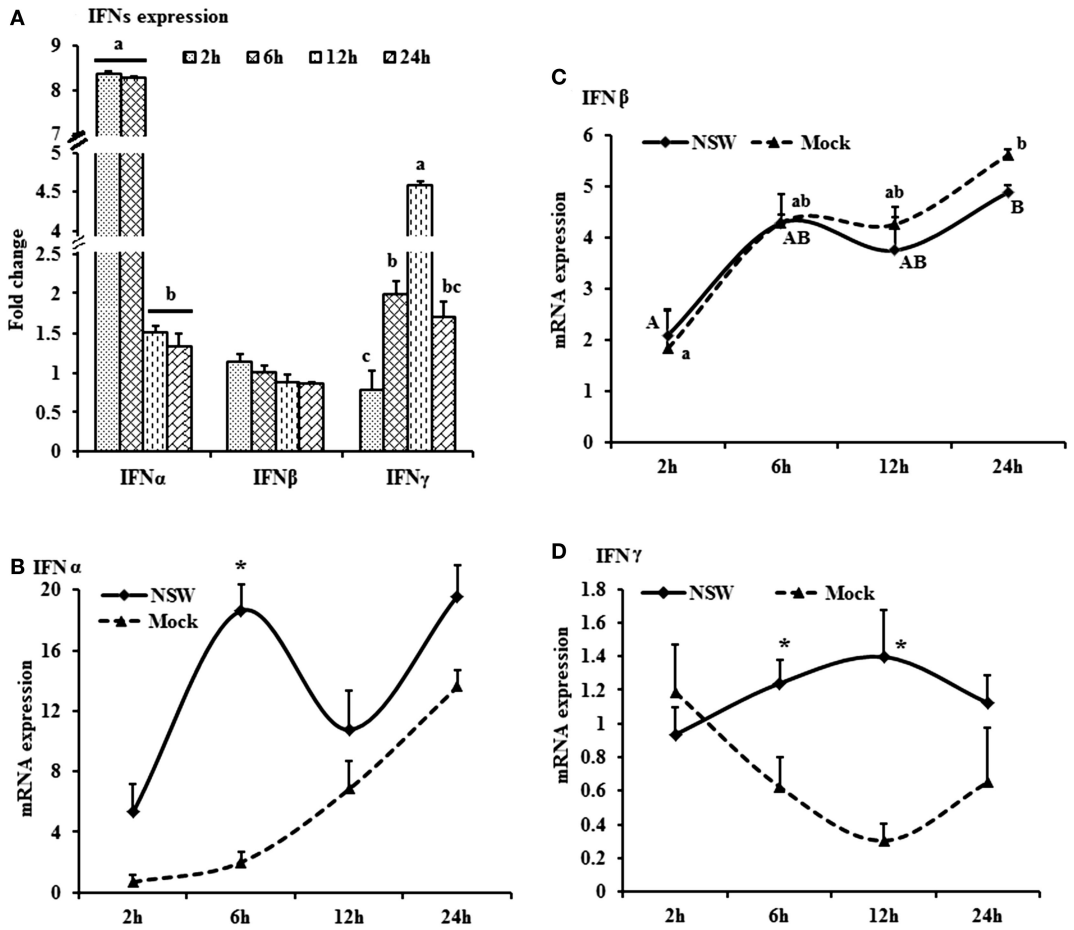

FIGURE 1 | Differential expression of interferon genes in response to West Nile virus. **(A)** Interferons mRNA expression in fold change. The ΔΔCt (ΔΔCt = $\Delta Ct_{WNV} - \Delta Ct_{mock}$) values were calculated by subtracting the ΔCt of genes in mock-inoculated PBMCs ($n = 3$). The bar graph showed the expression of genes in WNV-infected PBMCs over mock-inoculated PBMCs (fold change: the normalized expression value of a gene in WNV-stimulated cells/the normalized expression value of a gene in mock-inoculated cells). Bars without common superscripts (a,b; a,c; b,c) denote statistical difference among time points ($p < 0.05$). **(B–D)** Relative expression of interferons mRNA, accounting for the effects of culture conditions on gene transcription in WNV- and mock-inoculated rabbit PBMCs ($n = 3$). To compare the normalized expression of IFNs genes from PBMCs harvested at each time point to their respective expression levels before either WNV inoculation or mock inoculation, the ΔΔCt values were calculated by subtracting ΔCt of genes in fresh-isolated PBMCs from the ΔCt of genes in WNV- or mock-inoculated PBMCs at each time-point (for WNV-stimulated PBMCs, $\Delta\Delta Ct_{WNV} = \Delta Ct_{WNV} - \Delta Ct_{fresh}$; and for mock-inoculated PBMCs, $\Delta\Delta Ct_{mock} = \Delta Ct_{mock} - \Delta Ct_{fresh}$). A time-dependent relative expression patterns of **(B)** *IFNA*, **(C)** *IFNB*, and **(D)** *IFNG* mRNA in WNV-stimulated rabbit PBMCs at different time points. Line graphs without common superscript differ significantly ($p < 0.05$). Upper case letter denotes difference of a gene expression among the time points in WNV-challenged cells; lower case letter denotes difference of a gene expression among the time points in mock-inoculated cells. *indicates the difference of a gene expression between WNV- and mock-challenged cells in the same time point ($p < 0.05$).

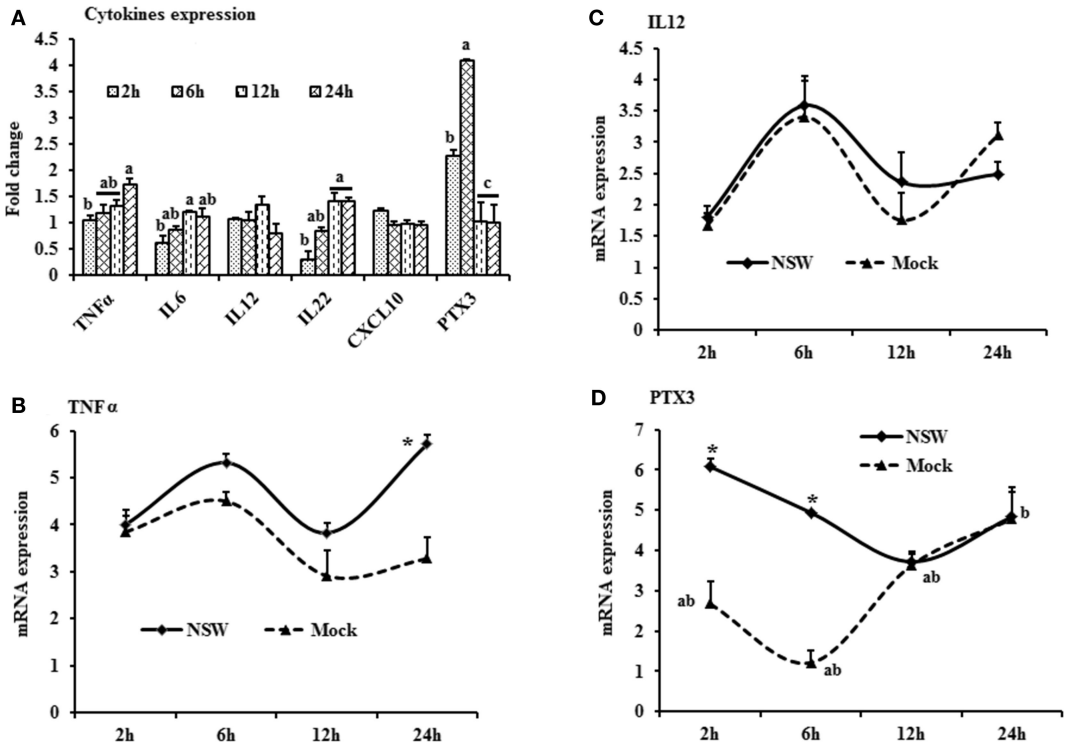

FIGURE 2 | Differential expression of inflammatory cytokine genes in response to West Nile virus. **(A)** Cytokines mRNA expression in fold change. The ΔΔCt (ΔΔCt = $\Delta Ct_{WNV} - \Delta Ct_{mock}$) values were calculated by subtracting the ΔCt of genes in mock-inoculated PBMCs ($n = 3$). The bar graph showed the expression of genes in WNV-infected PBMCs over mock-inoculated PBMCs (fold change: the normalized expression value of a gene in WNV-stimulated cells/the normalized expression value of a gene in mock-inoculated cells). Bars without common superscripts (a,b; a,c; b,c) denote statistical difference among time points ($p < 0.05$). **(B–D)** Relative expression of cytokines mRNA, accounting for the effects of culture conditions on gene transcription in WNV- and mock-inoculated rabbit PBMCs ($n = 3$). To compare the normalized expression of cytokine genes from PBMCs harvested at each time point to their respective expression levels before either WNV inoculation or mock inoculation, the ΔΔCt values were calculated by subtracting ΔCt of genes in fresh-isolated PBMCs from the ΔCt of genes in WNV- or mock-inoculated PBMCs at each time-point (for WNV-stimulated PBMCs, $\Delta\Delta Ct_{WNV} = \Delta Ct_{WNV} - \Delta Ct_{fresh}$; and for mock-inoculated PBMCs, $\Delta\Delta Ct_{mock} = \Delta Ct_{mock} - \Delta Ct_{fresh}$). A time-dependent relative expression patterns of **(B)***TNFA*, **(C)** *IL12*, and **(D)** *PTX3* mRNA in WNV-challenged rabbit PBMCs at different time points. Line graphs without common superscript differ significantly ($p < 0.05$). Upper case letter denotes difference of a gene expression among the time points in WNV-challenged cells; lower case letter denotes difference of a gene expression among the time points in mock-inoculated cells. *indicates the difference of a gene expression between WNV- and mock-challenged cells in the same time point ($p < 0.05$).

linear model; Proc GLM) procedure and the implemented analysis of variance (ANOVA) statistic were used. Pairwise comparisons were performed between the time points and treatment groups using Tukey's multiple comparisons in SAS, where *P* value was simultaneously adjusted. Besides, student's *t*-test was applied when treatment groups were compared. The data were expressed as means ±SD and values of $p < 0.05$ were considered to indicate statistically significant differences.

RESULTS

Expression Dynamics of Cytokines

The mRNA levels of cytokine *IFNA*, *IFNB*, *IFNG*, *IL6*, *IL12*, *CXCL10*, *IL22*, and *TNFA* showed a time-dependent expression pattern in rabbit PBMCs in response to WNV infection (**Figures 1A** and **2A**). When gene expressions in WNV-infected PBMCs were expressed in fold change with regards to the expression of genes in control (mock-inoculated) PBMCs (**Figures 1A** and **2A**), *IFNA* expression was up-regulated (8.4-folds) between 2- and 6-h pi and then declined (**Figure 1A**). The highest expression of *IFNB* gene was detected at the beginning of PBMCs–virus interaction and then declined gradually over time (**Figure 1A**). *IFNG* mRNA expression was increased over time and peaked (4.6 times) at 12-h pi (**Figure 1A**). While *IL6* and *IL22* mRNA expression increased over time, pentraxin 3 (*PTX3*) expression was significantly increased at the earlier hours of virus stimulation (**Figure 2A**).

When cytokine mRNA expressions in WNV-infected and mock-inoculated PBMCs were calculated with regards to the expression in freshly isolated PBMCs (fresh-PBMCs), *IFNA* mRNA expression was significantly higher at 6 h, then declined to the expression level in control at 12-h pi (**Figure 1B**). *IFNB* mRNA showed similar expression pattern in both infected and control cell lines (**Figure 1C**). With the exception at initial cell–pathogen interaction, *IFNG* (**Figure 1D**) and *TNFA* (**Figure 2B**) mRNA expression in virus-infected PBMCs was greater than in the mock-inoculated PBMCs. *IL12* expression in both the infected- and mock-inoculated PBMCs exhibited a similar pattern

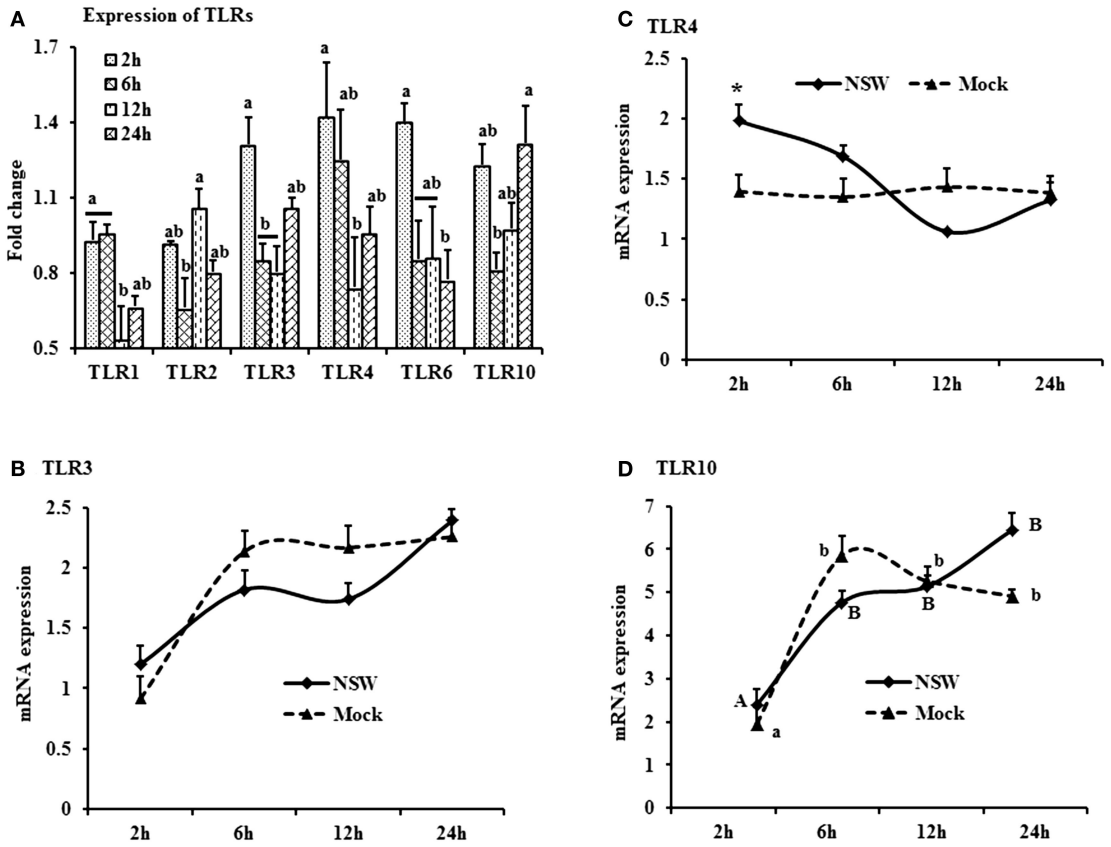

FIGURE 3 | Differential expression of Toll-like receptor genes in response to West Nile virus. **(A)** TLrs mRNA expression in fold change. The ΔΔCt ($\Delta\Delta Ct = \Delta Ct_{WNV} - \Delta Ct_{mock}$) values were calculated by subtracting the ΔCt of genes in mock-inoculated PBMCs ($n = 3$). The bar graph showed the expression of genes in WNV-infected PBMCs over mock-inoculated PBMCs (fold change: the normalized expression value of a gene in WNV-stimulated cells/the normalized expression value of a gene in mock-inoculated cells). Bars without common superscripts (a,b; a,c; b,c) denote statistical difference among time points ($p < 0.05$). **(B–D)** Relative expression of TLRs mRNA, accounting for the effects of culture conditions on gene transcription in WNV- and mock-inoculated rabbit PBMCs ($n = 3$). To compare the normalized expression of TLRs genes from PBMCs harvested at each time point to their respective expression levels before either WNV inoculation or mock inoculation, the ΔΔCt values were calculated by subtracting ΔCt of genes in fresh-isolated PBMCs from the ΔCt of genes in WNV- or mock-inoculated PBMCs at each time-point (for WNV-stimulated PBMCs, $\Delta\Delta Ct_{WNV} = \Delta Ct_{WNV} - \Delta Ct_{fresh}$; and for mock-inoculated PBMCs, $\Delta\Delta Ct_{mock} = \Delta Ct_{mock} - \Delta Ct_{fresh}$). A time-dependent relative expression patterns of **(B)** *TLR3*, **(C)** *TLR4*, and **(D)** *TLR10* mRNA in WNV-challenged rabbit PBMCs at different time points. Line graphs without common superscript differ significantly ($p < 0.05$). Upper case letter denotes difference of a gene expression among the time points in WNV-challenged cells; lower case letter denotes difference of a gene expression among the time points in mock-inoculated cells. *indicates the difference of a gene expression between WNV- and mock-challenged cells in the same time point ($p < 0.05$).

of expression, peaking at 6-h pi then declined (**Figure 2C**). *PTX3* gene expression was significantly up-regulated in the early hours of WNV infection, then declined to the expression level in mock-inoculated PBMCs (**Figure 2D**).

Expression Patterns of TLRs and Associated Genes

The TLR-family genes showed similar patterns of expression characteristics with higher genes involvement at the beginning as well as at 24 h of post-virus stimulation, except *TLR2* and *TLR6* (**Figure 3A**). *TLR3*, *4*, and *6* mRNA expressions was higher in virus-stimulated PBMCs, compared to the mock-inoculated PBMCs (**Figure 3A**). When mRNA expressions in WNV-stimulated and mock-inoculated PBMCs were calculated with regards to the expression in fresh PBMCs, *TLR3* and *TLR4* expression was up-regulated at the initial hour pi in virus-stimulated PBL compared to mock-inoculated PBMCs, then declined (**Figures 3B,C**). *TLR10* mRNA expression was up-regulated in both WNV-stimulated and mock-inoculated PBMCs (**Figure 3D**).

When mRNA expressions in WNV-stimulated PBMCs were expressed in fold change with regards to the expression of mRNA in mock-inoculated PBMCs, *MyD88* expression was initially up-regulated then declined, whereas *IRF7* mRNA expression increased over time (**Figure 4A**). *STAT1* mRNA expression peaked at 24-h pi in WNV-stimulated PBMCs compared to mock-inoculated PBMCs (**Figure 4A**). *TRAF3* mRNA expression increased over time, peaking at 24-h pi (**Figure 4B**), and *STAT1* mRNA expression was significantly up-regulated at 24-h pi (**Figure 4C**) in virus-stimulated PBMCs. *IRF7* mRNA expression

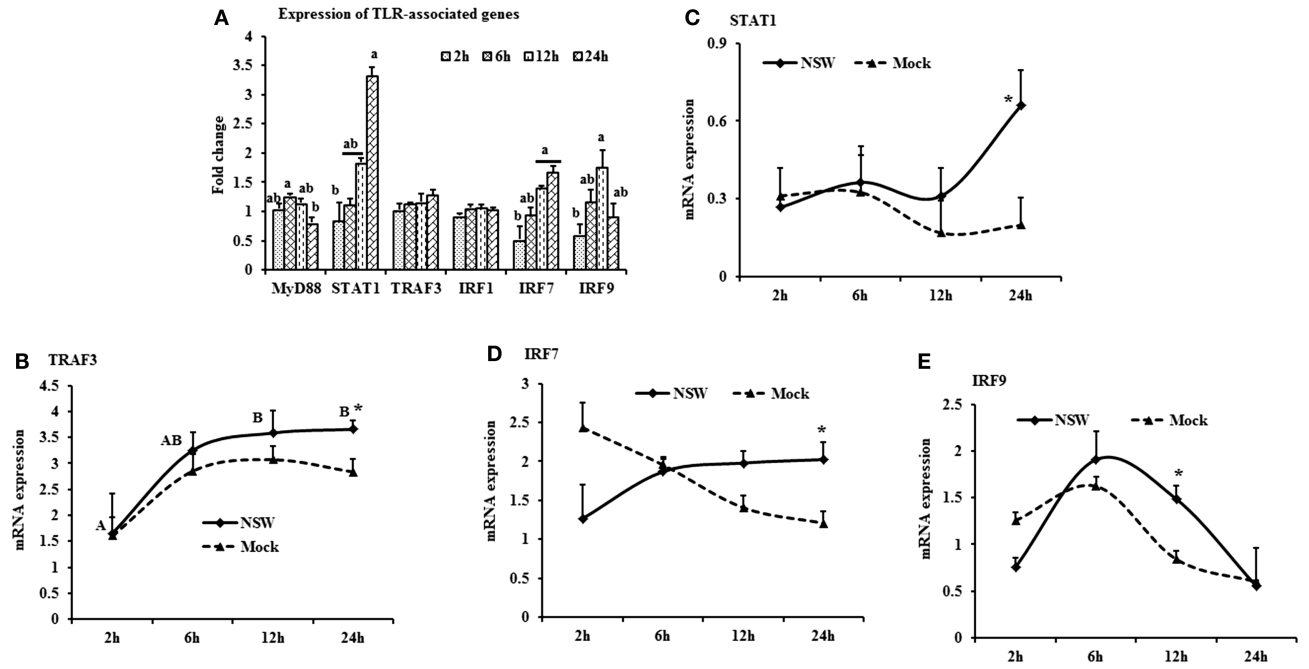

FIGURE 4 | Differential expression of the TLR downstream genes in response to West Nile virus. **(A)** TLR-associated genes mRNA expression in fold change. The ΔΔCt (ΔΔCt = $\Delta Ct_{WNV} - \Delta Ct_{mock}$) values were calculated by subtracting the ΔCt of genes in mock-inoculated PBMCs ($n = 3$). The bar graph showed the expression of genes in WNV-infected PBMCs over mock-inoculated PBMCs (fold change: the normalized expression value of a gene in WNV-stimulated cells/the normalized expression value of a gene in mock-inoculated cells). Bars without common superscripts (a,b) denote statistical difference among time points ($p < 0.05$). **(B–E)** Relative expression of TLRs-associated genes mRNA, accounting for the effects of culture conditions on gene transcription in WNV- and mock-inoculated rabbit PBMCs ($n = 3$). To compare the normalized expression of TLR-associated genes from PBMCs harvested at each time point to their respective expression levels before either WNV inoculation or mock inoculation, the ΔΔCt values were calculated by subtracting ΔCt of genes in fresh-isolated PBMCs from the ΔCt of genes in WNV- or mock-inoculated PBMCs at each time-point (for WNV-stimulated PBMCs, $\Delta\Delta Ct_{WNV} = \Delta Ct_{WNV} - \Delta Ct_{fresh}$; and for mock-inoculated PBMCs, $\Delta\Delta Ct_{mock} = \Delta Ct_{mock} - \Delta Ct_{fresh}$). A time-dependent relative expression patterns of **(B)** *TRAF3*, **(C)** *STAT1*, **(D)** *IRF7*, and **(E)***IRF9* mRNA in WNV-challenged rabbit PBMCs at different time points. Line graphs without common superscript differ significantly ($p < 0.05$). Upper case letter denotes difference of a gene expression among the time points in WNV-challenged cells; lower case letter denotes difference of a gene expression among the time points in mock-inoculated cells. *indicates the difference of a gene expression between WNV- and mock-challenged cells in the same time point ($p < 0.05$).

between the earlier and later hours of stimulation showed opposite pattern in response to WNV stimulation in rabbit PBMCs (**Figure 4D**). *IRF9* showed a similar pattern of expression both in WNV-stimulated and mock-inoculated PBMCs with a significant upregulation at 12-h pi (**Figure 4E**).

Oxidative Stress and Apoptosis-Related Genes Expressions

Oxidative stress-related *HO1* gene expression became up-regulated over time, whereas iNOS mRNA expression decreased over time after a slight peak at 6-h pi in virus-stimulated PBMCs (**Figure 5A**). The expression of the apoptosis-associated gene *caspase 3* increased over time, whereas *caspase 9* mRNA expression was up-regulated at initial hours pi, then declined (**Figure 5A**).

When mRNA expressions in WNV-stimulated and mock-inoculated PBMCs were calculated with regards to the expression in fresh-PBMCs, *HO1* mRNA expression increased over time in both WNV-stimulated and mock-inoculated PBMCs (**Figure 5B**), whereas *iNOS* expression was higher in virus-stimulated PBMCs at 6-h pi (**Figure 5C**). Both *caspase 3* and *9* mRNA expressions were higher in virus-stimulated PBMCs at 12-h pi, then declined (**Figures 5D,E**).

Kinetics of Virus-Specific Gene Expression in Rabbit PBMCs

When viral RNA expression was compared among the virus-stimulated PBMCs samples, the WNV-specific transcript expression was increased at 24-h pi compared to the expression at 2- and 6-h pi (**Figure 6**). Notably, viral RNA could not be detected in *in vitro* mock-inoculated PBMCs or in fresh PBMCs samples or in PBMCs collected from *in vivo* virus infected or control rabbits.

Validation of Transcripts Expressions in PBMCs from Virus-Infected Rabbits

Despite the lack of detectable viral RNA in PBMCs collected on day 3 pi from $WNV_{NSW2011}$-infected rabbits, the cells had upregulation of *IFNA*, *IFNB*, *TNFA*, *IL22* and *PTX3* (**Figure 7A**), *TLR3* and *IRF7* (**Figure 7B**), and *caspase 9* (**Figure 7C**) mRNA expression when compared to PBMCs from mock-infected control rabbits. *IFNA*, *IFNB*, *TNFA*, and *PTX3* mRNA expression was 8-,

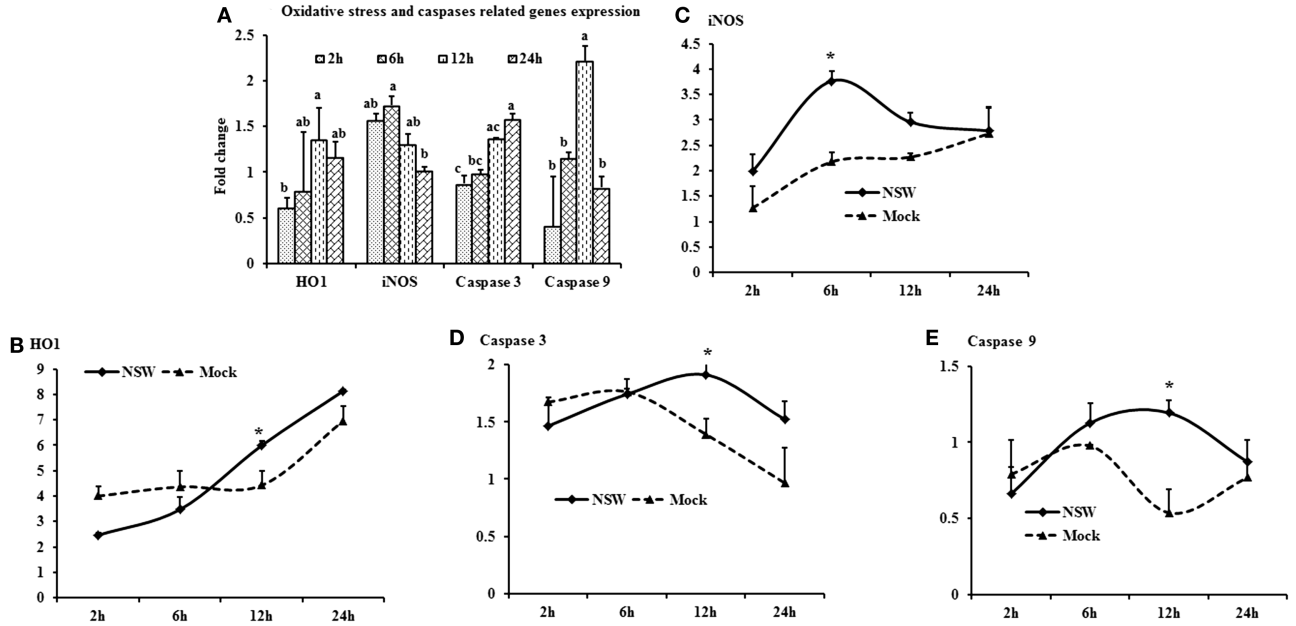

FIGURE 5 | Differential expression of oxidative stress and apoptosis-related genes in response to West Nile virus. **(A)** Oxidative stress and apoptosis-related genes mRNA expression in fold change. The ΔΔCt (ΔΔCt = $\Delta Ct_{WNV} - \Delta Ct_{mock}$) values were calculated by subtracting the ΔCt of genes in mock-inoculated PBMCs ($n = 3$). The bar graph showed the expression of genes in WNV-infected PBMCs over mock-inoculated PBMCs (fold change: the normalized expression value of a gene in WNV-stimulated cells/the normalized expression value of a gene in mock-inoculated cells). Bars without common superscripts (a,b; a,b,c) denote statistical difference of a gene expression among time points ($p < 0.05$). **(B–E)** Relative expression of oxidative stress and apoptosis-related genes mRNA, accounting for the effects of culture conditions on gene transcription in WNV- and mock-inoculated rabbit PBMCs ($n = 3$). To compare the normalized expression of oxidative stress- and apoptosis-related genes from PBMCs harvested at each time point to their respective expression levels before either WNV inoculation or mock inoculation, the ΔΔCt values were calculated by subtracting ΔCt of genes in fresh-isolated PBMCs from the ΔCt of genes in WNV- or mock-inoculated PBMCs at each time-point (for WNV-stimulated PBMCs, $\Delta\Delta Ct_{WNV} = \Delta Ct_{WNV} - \Delta Ct_{fresh}$; and for mock-inoculated PBMCs, $\Delta\Delta Ct_{mock} = \Delta Ct_{mock} - \Delta Ct_{fresh}$). A time-dependent relative expression patterns of **(B)** *HO1*, **(C)** *iNOS*, **(D)** *Caspase 3* and **(E)** *Caspase 9* mRNA in WNV-challenged rabbit PBMCs at different time points. Line graphs without common superscript differ significantly ($p < 0.05$). Upper case letter denotes difference of a gene expression among the time points in WNV-challenged cells; lower case letter denotes difference of a gene expression among the time points in mock-inoculated cells. *indicates the difference of a gene expression between WNV- and mock-challenged cells in the same time point ($p < 0.05$).

2.8-, 3.2-, and 3.5-folds, respectively, higher in PBMCs from the virus-infected rabbits compared to the PBMCs from the control animals (**Figure 7A**). Remarkably, *TLR3* and *IRF7* transcripts were up-regulated by 13 and 44 times, respectively, in PBMCs from $WNV_{NSW2011}$-infected rabbits compared to PBMCs from mock-infected rabbits (**Figure 7B**).

DISCUSSION

The economic burden of non-lethal WNV disease in horses and humans is substantial (37–39). Due to the cost and logistic limitations of using horses for pathogenesis studies, we have recently established an alternative small animal model in laboratory NZW rabbits to study the host–pathogen interactions (25). Tracking changes in the gene expression following viral infection is paramount to understand the host–pathogen interactions including the host–immune responses and pathogenesis. In this study, the expression patterns of selected genes involved in the innate immune response are documented in rabbit blood mononuclear cells following *in vitro* and *in vivo* challenge with an equine-virulent, Australian strain of WNV. The innate immune component includes Toll-like receptors, acute phase proteins, and cytokines expressed by different cell types including blood leukocytes. Among the ten members of the TLR family (TLR1-10), TLR3, 7, 8, and 9 are reported to recognize viral genomic components (40). TLR3 has been associated with the direct recognition of double-stranded viral RNA, while TLR7 and TLR8 target single-stranded viral RNA (40). The involvement of TLR3 (12) and TLR7 (18) have been extensively studied for WNV infection and recognition in mice (15), however, TLR7 and TLR8 are reported to be absent and pseudogenized, respectively, in rabbit (*O. cuniculus*) (41). Furthermore, TLR9 recognizes unmethylated viral-CpG DNA leaving TLR3 the only available TLR for the recognition of viral RNA in the rabbit. In case of WNV infection, peripheral inflammatory responses are initiated through the TLR3 (11, 15). In this study, upregulation of *TLR3* mRNA in *in vitro* virus challenged rabbit PBMCs was detected, suggestive of the involvement of this molecule in the WNV-induced innate immune response.

Although there was only a marginal and transient upregulation of the expression of *MyD88* mRNA under the present culture conditions, a study documented that MyD88 is involved in the restriction and spread of WNV in mice (16). *MyD88*-deficient mice showed elevated viral burden, and increased WNV replication was observed in *MyD88* deficient macrophages and subsets

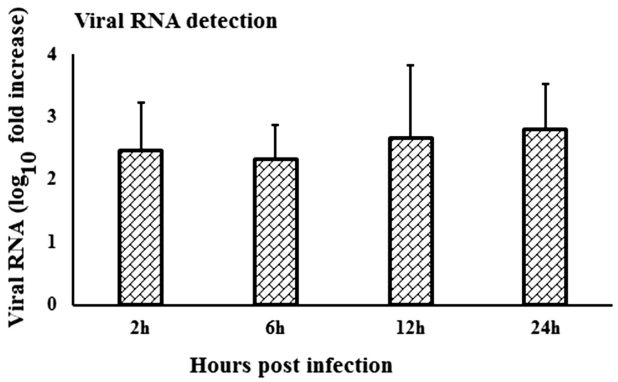

FIGURE 6 | Kinetics of viral RNA expression in *in vitro* WNV-infected PBMCs. *$WNV_{NSW2011}$* RNA expression in *in vitro* virus-infected PBMCs at different time points was been quantified using qRT-PCR. The ΔΔCt ($\Delta\Delta Ct = \Delta Ct_{WNV} - \Delta Ct_{mock}$) values were calculated by subtracting the ΔCt of genes in mock-inoculated PBMCs ($n = 3$). Ct for the mock-inoculated PBMCs (undetected) was set to 40. The bar graph showed the expression of genes in WNV-infected PBMCs over mock-inoculated PBMCs (fold change: the normalized expression value of a gene in WNV-stimulated cells/the normalized expression value of a gene in mock-inoculated cells).

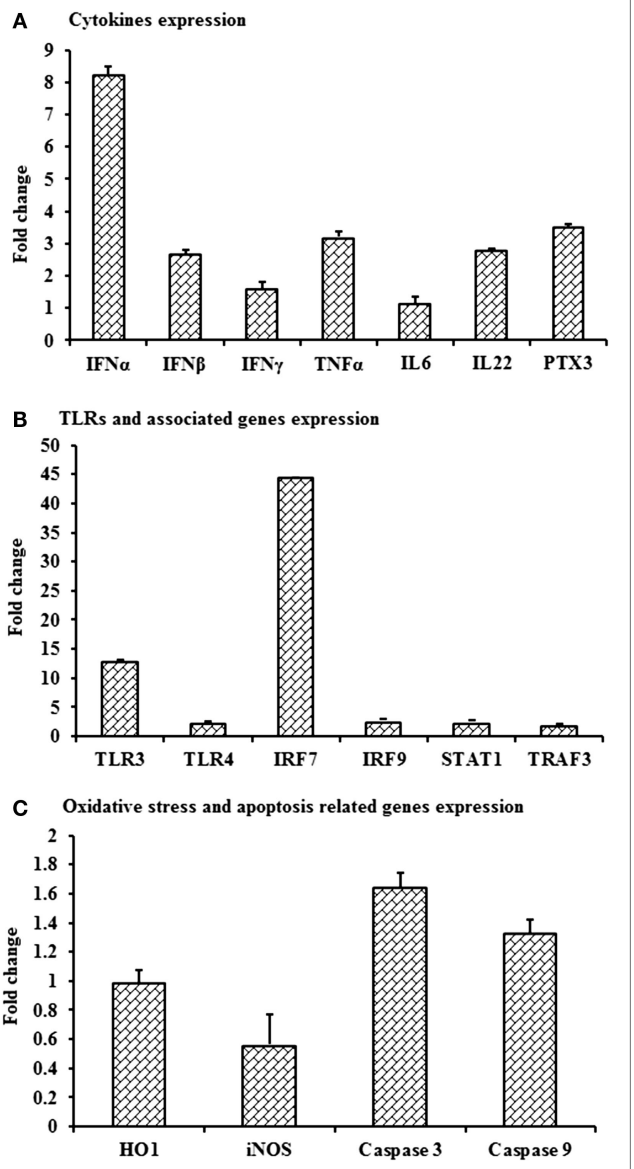

FIGURE 7 | Differential expression of mRNA level of selected immune molecules in *in vivo* WNV-infected rabbits. Expression of **(A)***IFNA*, *IFNB*, *IFNG*, *TNFA*, *IL6*, *IL22* and *PTX3*, **(B)** *TLR3*, *TLR4*, *IRF7*, *IRF9*, *STAT1*, and *TRAF3*, and **(C)** *HO1*, *iNOS*, *Caspase 3*, and *Caspase 9* mRNA in $WNV_{NSW2011}$-infected rabbit PBMCs at day 3 post-inoculation in fold change. The ΔΔCt ($\Delta\Delta Ct = \Delta Ct_{WNV} - \Delta Ct_{mock}$) values were calculated by subtracting the ΔCt of genes in uninfected control rabbit PBMCs ($n = 3$). The bar graph showed the expression of genes in WNV-infected PBMCs over uninfected control rabbit PBMCs (fold change: the normalized expression value of a gene in *in vivo* WNV-infected rabbit cells/the normalized expression value of a gene in uninfected control rabbit cells). Data are presented as mean ± SD.

of neurons in cell culture [reviewed by Ref. (11)]. This implies that WNV, after being recognized by TLRs, initiates the downstream signaling cascades of the MyD88 and TRAF dependent pathways. Even in the face of some disparity in the utilization of adaptor proteins by different TLRs, their downstream signaling cascades converge on the transcription factor NFκB. TLR engagement initiates rapid signaling events that lead to activation of the transcription factors NFκB and IRF3, and production of cytokines including type I IFNs (11). The upregulation of *IFNA* was only transient in the *in vitro* WNV-stimulated PBMCs compared to mock-inoculated PBMCs. Induction of IFNα genes occurs mainly via the transcriptional activity of IRF7 (11). Recently, over expression of *TLR2*, *TLR3*, *TLR5*, *MyD88*, *STAT1*, *CXCL10*, *IL6*, *IL12*, and *TNFA* has been quantified in various tissues collected from *in vivo* WNV-infected mice (26). Higher mRNA expression of adaptor molecule MyD88, STAT1, TRAF3, IRF7, and 9 is suggestive of the involvement of these molecules in the rabbit PBMCs innate immune response to WNV. STAT1 is the key molecule controlling the course of IFN stimulation and kinetics of ISG expression. The type I IFN response depends on the phosphorylation patterns of STAT1 and is important for the WNV-induced immune response (42). Our results corroborate this, as *STAT1* mRNA increased over time in the *in vitro* WNV-infected rabbit PBMCs. WNV has been reported to block the phosphorylation of STAT1 as a means of immune evasion (11), leading to the blocking of IRF9 expression. However, IRF9 mRNA expression was up-regulated in *in vitro* WNV-stimulated rabbit PBMCs, suggesting that the reported immune evasion mechanisms either do not occur in rabbit PBMCs or at least not under the present culture conditions.

Productive replication of WNV has previously been demonstrated in *in vitro*-infected horse PBMCs by viral growth curve and qRT-PCR for WNV RNA (10). These authors reported that peak virus titer was reached at 6-day pi and high titers were maintained through 10- to 15-day pi (10). Rawle et al. (43) compared the growth kinetics of $WNV_{NSW2011}$ and WNV_{NY99} using plaque assay and found that $WNV_{NSW2011}$ did not replicate in human blood monocyte-derived dendritic cells as they extended the

experiment from 24- to 72-h pi, whereas WNV_{NY99} successfully replicated in these cells during the entire duration of the experiment. Since other studies (10, 43) did not quantify viral RNA during the first 24-h pi, a comparison to our findings of viral RNA detection is precluded. These differences in growth kinetics in leukocytes between $WNV_{NSW2011}$ and WNV_{NY99} may be ascribed to virus characteristics *sensu stricto* or may be explained by their respective ability to induce protective innate immune responses. In case of *in vivo* NZW rabbit infection, the draining lymph node was found to be the main site for peripheral replication of $WNV_{NSW2011}$ following foodpad inoculation, with peak levels reached on day 3 pi (25). In addition, virus antigen was detected in pleomorphic leukocytes, macrophages, and/or dendritic cells in the paracortical zone of draining popliteal lymph nodes and in the leukocytes in multiple sites of the deep dermis of the injected footpad (25). It is important to note that the present study was limited to 24 h post-*in vitro* stimulation of rabbit PBMCs, as the study aimed to decipher the patterns of early responses of selected genes involved in innate immune response to WNV. Further studies will be required to characterize the virus growth kinetics in rabbit PBMCs over extended time periods but that was beyond the scope of the present study.

Higher expression of IRF7 mRNA *in vitro* stimulation of PBMCs and in PBMCs from rabbits 3 days post-*in vivo* infection is suggestive of its involvement in WNV-induced innate immune responses in the rabbit. TLR3 activation leads to the induction of IRF7 which triggers IRF3 and NFκB to produce IFNs (11). A deficiency of *IRF7* completely abrogated the *IFN*α response while no effect on *IFNB* gene induction was observed in IRF7−/− macrophages in mouse (13). *IFNB* mRNA expression was unaffected in *in vitro* virus-induced PBMCs, which may be explained by the previous findings that *IRF3* and *IRF7* only partially regulate the *IFNB* gene and ISG expression in macrophages (13). The upregulation of *IRF7* and IFNA mRNA expression was significantly higher in *in vitro* WNV-stimulated rabbit PBMCs compared to mock-inoculated PBMCs, suggesting the involvement of these downstream molecules in WNV infection.

Unexpectedly, mRNA expression of *TLR4* and *TLR6*, which recognizes bacterial pathogens, was found to be significantly up-regulated in this study. Antiviral activity of TLR3 and TLR4 has been detected in human microglial cells (44). Single-stranded RNA viruses such as respiratory syncytial virus (RSV) are documented to activate the innate immune response through TLR2 and TLR6 in murine macrophages (45). However, an irregular pattern of *TLR2* expression was found in WNV-stimulated rabbit PBMCs. Nevertheless, this is in agreement with previous studies that demonstrated varying TLR expression in different cell types (46). TLR2 is reported to recognize Epstein–Barr virus (EBV) in human monocyte (47). It is important to note that RSV and EBV are negative sense ssRNA and DNA virus, respectively, whereas WNV is a positive sense ssRNA virus. Recently, upregulation of TLR2 and TLR3 within 1-day pi has been reported in WNV-infected mouse tissues (26), which coincided with our findings in PBMCs.

Recognition of WNV through TLR signaling pathways via MyD88 and TRIF adaptor molecules induced the IRF family and NF-κB genes. NF-κB binds to transcription sites and induces an array of genes that are responsible for production of acute phase proteins, iNOS, coagulation factors, and pro-inflammatory cytokines. Some of the major immune pathways identified to be up-regulated by microarray in the equine brain following experimental WNV infection included the *IL15*, *IL22*, *IL9*, and *IFN* signaling pathways (19), while in mice *IFNB*, *TNFA*, and *IL6* may be key factors (17). In this study, *TNFA* mRNA was up-regulated in *in vitro* WNV-stimulated rabbit PBMCs, suggesting that *TNFA* may be an important component of the WNV-induced innate immune response. Although rabbit PBMCs have not been studied before, the role of macrophages in WNV infection has been reviewed earlier (11). Activation of macrophages in response to WNV infection also promotes the release of type I IFN, TNFA, IL1B, IL8, and other cytokines, thus reducing viral replication in cell culture [reviewed by Ref. (11)]. TNFα is strongly induced by TLR activation and consequently, cellular activation by TNFα could potentially induce TLR gene expression and provide a means for enhancing cellular responsiveness to microbial ligands recognized by those TLRs (40). TNFα mRNA expression was found to be up-regulated over time and peaked at 24-h pi, which is similar to findings by Kwon et al. (48), who found that *TNFA* was significantly up-regulated in horse monocytes 12 and 20 h after challenge with synthetic poly I:C.

IFNG mRNA expression was up-regulated from 6 to 24 h after *in vitro* WNV stimulation of rabbit PBMCs. A dominant protective antiviral role of IFNG against WNV has been documented to occur in peripheral lymphoid tissues (49). A notable difference in the levels of type I and II interferon was reported in the brain in $WNV_{NSW2011}$-infected rabbits, but their expressions were invariable in draining lymph nodes (25). A lack of IFNG production or signaling was reported to increase vulnerability to lethal WNV infection in mice, with a rise in mortality, a decrease in survival time, higher viremia and greater viral replication in lymphoid tissues (49). γδT cells require IFNG to limit the dissemination of WNV and treatment of primary dendritic cells with IFNG-reduced WNV replication (49). However, it remains to be determined which cell subset in the rabbit PBMCs were responsible for the IFNG mRNA expression. *IL22* mRNA was up-regulated over time in rabbit PBMCs in response to *in vitro* WNV stimulation. IL22 is expressed by a wide range of immune cells, including T and NK cells, and engagement of the IL22 receptor leads to STAT3 and STAT1 signaling (50). Notably, relatively high expression of *IL22* mRNA by rabbit PBMCs following *in vivo* infection of the animals may help explain that no viral RNA was detected in their PBMCs at 3-day pi. The expression of *IL22* was consistent with a previous study detecting upregulation of *IL22* mRNA in WNV infected horse lymphoid tissues (19).

PTX3 was one of the most (4.2-folds) up-regulated genes in rabbit PBMCs infected with WNV *in vitro*. Microarray expression analysis showed that *PTX3* was the gene displaying the most pronounced expression in thalamus and cerebrum of horses experimentally infected with WNV (19). PTX3 is a soluble, acute phase protein (soluble pattern recognition receptor; PRMs) and recognizes PAMPs (51). PTX3 is produced by a variety of cells and tissues, most notably dendritic cells and macrophages, in response to TLR engagement and inflammatory cytokines. This

molecule has many functions, including an integral role in the pathway of PRRs in recognition of viruses and bacteria (52). The *PTX3* gene is induced by *IL1B* and *TNFA*, and functions in phagocytosis and opsonization of antigens, as well as in the inflammatory response (52). Human and murine PTX3 bound to influenza virus and mediated a range of antiviral activities, including inhibition of hemagglutination, neutralization of virus infectivity, and inhibition of viral neuraminidase (53). The exact role of *PTX3* in WNV-infection remains to be elucidated, but our results suggest a potential antiviral role in some species, notably rabbit (**Figure 2**).

Apoptosis is a highly conserved mode of programed cell death, mediated by the activation of caspases. WNV is reported to induce apoptosis in human brain derived glia cells in culture by the activation of caspase 3, 8, and 9 (54). WNV proteins such as envelope (E) and non-structural protein 3 (NS3) have been shown to induce caspase-dependent apoptosis when transfected into cells (55). Apoptosis is induced through the mitochondrial pathway resulting in caspase 9 and caspase 3 activation in mouse brain cells *in vitro* (55). WNV NS3 induced host cell apoptotic pathways involving caspase 8 and 3 in different cell types (56), but so far there has been no study of caspase expressions either in rabbit or equine PBMCs following WNV infection. Higher expression of *caspase 3* and *9* mRNA both in *in vitro*-infected rabbit PBMCs and in PBMCs from the *in vivo* rabbit model are in accordance with other studies establishing that caspase 3, 8, and 9-dependent apoptosis is involved in WNV infection. It remains to be shown that the rabbit cells proceed to undergo apoptosis following WNV exposure *in vitro*.

HO1 mRNA expression was increased at 12- to 24-h post-WNV infection of PBMCs *in vitro*, whereas iNOS mRNA expression was up-regulated at 6-h pi. In contrast, neither *iNOS* nor *HO1* appeared to be affected in the PBMCs from WNV-infected rabbits. The discrepancy might be due to the time difference between the *in vitro* and the *in vivo* study. Monocytes infiltrating into the brain of mice in WNV-induced encephalitis produced nitric oxide (NO) (57). Macrophages have been reported to control JEV infection directly through the production of NO and other reactive oxygen intermediates (58). Activation of HO1 by a natural substrate, hemin, effectively enhanced the ability of human macrophages to resist infections by several pathogens, including dengue virus, WNV and poxvirus (59). Similarly high expression of *TNFA* and *HO1* might suggest that oxidative stress protects rabbit PBMCs from WNV infection both *in vitro* and *in vivo*.

CONCLUSION

Expression patterns of selected genes involved in the innate immune response to WNV have been documented in this study using rabbit PBMCs as an *in vitro* model. Expression of selected genes was validated in the WNV-infected rabbit *in vivo*. A rabbit model has several advantages over the mouse model, the more commonly used model for WNV, by mimicking the course of infection in the horse better, including viremia, virus distribution, and morbidity. Therefore, the presented data on the genes pivotal in WNV infection in a novel rabbit cell model will help to focus on candidate markers for further study. Specifically, a pan-genomic approach using Next-Generation Sequencing would have yielded much deeper insights into the differential expression in response to WNV.

AUTHOR CONTRIBUTIONS

MU conceived and designed the experiments, performed the experiments, analyzed the data, drafted, and edited the manuscript. WS conceived and designed the experiments, performed the experiments, analyze the data, and edited the manuscript. NP and RH conceived and designed the experiments and edited the manuscript. HB-O conceived and designed the experiments, analyzed the data, drafted, and edited the manuscript.

FUNDING

This study was supported by a grant from the Australian Research Council (ARC-LP120100686; RH, HB-O et al.). MU is supported by a UQ Postdoctoral Fellowship. WS is supported by an Australian Postgraduate Award.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fvets.2015.00076

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Conflict of Interest Statement: All authors read and approved the final manuscript. The authors declare that they have no competing interests.

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