Composition and inclusion of probiotics in broiler diets alter intestinal permeability and spleen immune cell profiles without negatively affecting performance

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ABSTRACT

Probiotic feed additives with potential to enhance performance, health, and immunity have gained considerable popularity in commercial broiler production. The study objectives were to measure broiler performance, gut integrity, and splenic immune cell profiles in birds fed one of 2 probiotics at 2 inclusion levels. Nine hundred and sixty Ross 708 broilers (12/ pen) were randomly assigned to no additive control, 0.05 or 0.10% LactoCare (*Lactobacillus reuteri)*, or 0.05 or 0.10% LactoPlan (*Lactobacillus plantarum)* dietary treatments for 6 wk. On d27, a 20-pen subset was utilized for a fluorescein isothiocyanate dextran (FITC-d) assay, where half of the pens were subject to a 12-h feed restriction (FR) pre-gavage. Serum collected from blood drawn 1-h post-gavage was analyzed for relative fluorescence of FITC-d absorbed across the intestinal barrier as a gut leakiness indicator. On d42, spleens from 8 birds/ treatment were collected for immune cell profile analysis by multicolor flow cytometry. Although performance outcomes were not affected by dietary treatment, FITC-d absorption post-FR was increased 57% in the 0.05% LactoPlan treatment, and was decreased by 12.6% in the 0.05% LactoCare diet, 12% in the 0.10% LactoCare diet, and 22% in the 0.10% LactoPlan diet compared to the control. This indicates a positive impact in barrier integrity maintenance due to 0.05% and 0.10% LactoCare and 0.10% LactoPlan diet following a challenge. Immune cell profiles varied between the two probiotic compositions, with an approximately 50% reduction in splenic innate immune cells (monocyte/macrophage$^+$) in birds fed LactoPlan ($P < 0.0001$) and greater overall percentages of CD45$^+$ leukocytes and CD3$^+$ T-cells in birds fed 0.10% LactoCare ($P < 0.0001$). LactoPlan diets shifted splenic T-cell populations in favor of CD8α^+ cytotoxic T-cells (T_C; $P= 0.007$), while higher inclusions (0.10%) of either probiotic increased the percentage of activated CD4$^+$ helper T-cells (T_H; $P < 0.0001$). These results indicate that compositionally different probiotics had varying effects on the gut permeability and splenic immune cell profiles in

broiler chickens, particularly at higher inclusion rates, but observed changes to underlying physiology did not negatively impact performance outcomes. The ability of a probiotic to alter gut permeability and immune cell profile therefore may depend on the compositional complexity of the product as well as inclusion rate.

Key words: broiler, gut integrity, immune cell profile, performance, probiotic

INTRODUCTION

Modern broiler producers continue to seek methods to improve feed efficiency and growth performance. Prophylactic probiotic feed additives are one tool to improve production in reduced or no-antibiotic production systems (Lutful Kabir, 2009; Abdelrahman, et al., 2014). Lactic-acid producing bacteria promote healthy gut bacterial colonization and general systemic immunity (Marteau and Rambaud, 1993; Gareau, et al., 2010). *Lactobacillus plantarum* and *Lactobacillus reuteri* are two such commensal bacteria fed in broiler diets that have resulted in improved ADG, FCR, and body weight gain through enhanced gut health parameters, including decreased intestinal viscosity and increased villus height and crypt depth (Liu, et al., 2007; Yu, et al., 2007 Peng, et al., 2016). Additionally, solid-state fermented probiotics have been shown to improve weight gain and feed efficiency, likely through increased energy and protein retention (Shim, et al., 2010). In addition to improved production parameters, *L. plantarum* increased peripheral lymphocyte proliferation and increased expression of interferon-gamma, interleukin (IL)-6 and IL-10 in the ileal mucosa while *L. reuteri* increased levels of serum immunoglobulin (Ig) A, IgG, and IgM in healthy broilers (Shen, et al., 2014; Wu, et al., 2019; Salim, et al., 2013). Further, *L. plantarum* and *L. reuteri* administration have resulted in improved responses to pathogenic bacterial challenge, such as *E. coli* and *Brachyspira pilosicoli*, by reducing pathologies associated with infection in the intestine and increasing serum IgG (Mappley, et al., 2013; Ding, et al., 2019). While changes to the immune system in both healthy and pathogen-challenged birds have been noted, these alterations are typically examined as levels of downstream products (i.e. cytokines and Ig) while changes to underlying immune cell populations are studied less frequently at both the local and systemic levels.

Fluorescein isothiocyanate dextran (FITC-d) gavage is a validated gut-integrity parameter that quantifies intestinal permeability in broilers (Tellez, et al., 2014; Vicuña, et al., 2015; Baxter, et al., 2017). Dextran is a large molecule that is not typically absorbed across the intestine into the bloodstream; however, if the gut is challenged by stress or damage and tight junction integrity is reduced, dextran will translocate into the bloodstream. A feed restriction model is known to cause stress in broilers, triggering this translocation (Kuttappan, et al., 2015, Baxter, et al, 2017, Maguey-Gonzales, et al., 2018). FITC-labeled dextran allows for quantification of absorbed dextran in serum pulled from blood drawn 1-h post-FITC-d gavage (Baxter, et al., 2017). Therefore, the objectives were to determine if differing solid-state fermented *L. plantarum* and *L. reuteri* inclusion rates improved broiler performance outcomes, decreased gut leakage during feed restriction, or altered systemic immune cell populations as measured in the spleen.

MATERIALS AND METHODS

Animals and Housing

All animal protocols in this study were approved by the Iowa State University Institutional Animal Care and Use Committee. Nine hundred and sixty Ross 708 broilers were obtained from a commercial hatchery (International Poultry Breeders Hatchery, Bancroft, Iowa) and transported to the Iowa State Poultry Research and Teaching Farm on day of hatch for a 6-wk grow-out period. The birds were housed on re-used litter (fourth time) in 80 1.2 by 1.2 m pens, with 12 birds/pen (0.12 m^2/bird), with *ad libitum* access to feed and water. Average ambient temperatures are listed from the starter, grower, and finisher periods respectively: 85.08 ± 4.44° F, 80.96 ± 6.19° F, and 77.05 ± 7.05° F. Birds were adjusted from 24 h light on d0-7 (30-40 lux) to 20 h

light (20-30 lux) from d8-42. Chicks were brooded with 1 heat lamp/pen using 125-watt heat bulbs (Sylvania, Wilmington, MA) for the first wk.

Dietary Treatments and Performance

Starter, grower, and finisher diets were formulated according to Ross 708 production guidelines (Table 1). Five dietary treatment groups were assigned: 1) basal diet without probiotic inclusion; 2) basal + 0.05% LactoCare, a solid-state fermented *Lactobacillus reuteri* probiotic; 3) basal + 0.10% LactoCare; 4) basal + 0.05% LactoPlan, a solid-state fermented *Lactobacillus plantarum* probiotic; or 5) basal + 0.10% LactoPlan, (Nutraferma Biotech, Sioux City, IA). Each diet was randomly assigned to 16 pens. Birds were fed a starter diet wk 1-2, grower wk 3-4, and finisher wk 5-6. Pen weights were collected upon placement (d0) and conclusion of each 2-wk performance period. Feed disappearance was recorded throughout and used to calculate feed intake, weight gain, and Gain:Feed.

FITC-Dextran Administration

On d26, a subset of 20 out of 80 pens were weighed, and 10 of those pens were subjected to a 12-h feed restriction (FR) to provide a gut-integrity challenge. On d27, FITC-d (3,000-5,000 molecular weight) was administered by oral gavage to all 12 birds/pen in 10 pens (5 FR, 5 non-FR) at an inclusion of 8.32 mg/kg based on individual bird weights taken on d26. Two additional pens (1 FR, 1 non-FR)/dietary treatment (10 pens total) were used as serum blank controls as outlined in Baxter et al. (2017).

Blood drawn 1 h post-gavage was collected into serum separation tubes, allowed to clot at room temperature, and centrifuged at 1000 × G for 15 minutes. Serum was then transferred into amber tubes, diluted 1:5 in saline, and stored at -20˚C until analysis. Standard curves were set by

diluting FITC-d (8.32 mg/kg) in blank serum at 6400 ng/mL, 3200 ng/mL, 1600 ng/mL, 800 ng/mL, 400 ng/mL, 200 ng/mL, 100 ng/mL, and 0 ng/mL (blank serum only). All serum samples (diluted 1:5 in saline) were plated on black 96-well plates in duplicate (100 µl/well). Duplicate blanks of FR non-FITC-d and non-FR non-FITC-d were included on each plate. The plates were read at 485 and 528 nm excitation and emission wavelengths, respectively. The $[(raw\ value * slope + intercept) * 5]$ was used to calculate relative fluorescence (ng/mL).

Flow Cytometry

Spleens collected from 8 broilers/ treatment at d42 were gently homogenized in PBS and passed through a 70µm strainer. Cells were counted using a hemocytometer and frozen at -80°C in heat inactivated chicken serum (Equitech-Bio Inc., Kerrville, TX) supplemented with 7.5% DMSO until flow cytometric analysis. Prior to extracellular staining, cells were thawed and counted before being aliquoted into 12 × 75mm polystyrene flow cytometry tubes. Two different staining panels were used on each spleen to obtain data for innate and adaptive immune cell populations. Staining panel #1 consisted of mouse anti-chicken CD45 FITC (clone LT40; mouse IgMκ), TCRγδ PE (clone TCR-1; mouse $IgG_1\kappa$), Bu-1 Alexa-Fluor (AF) 647 (clone AV20; mouse $IgG_1\kappa$), CD4 AF700 (clone CT-4; mouse $IgG_1\kappa$), CD8α Pacific Blue (clone CT-8; mouse $IgG_1\kappa$), and CD28 biotin (clone AV7; mouse $IgG_1\kappa$) with a Brilliant Violet (BV) 785-conjugated streptavidin (SA) secondary stain. Panel #2 contained mouse anti-chicken CD1.1 FITC (clone CB3; mouse $IgG_1\kappa$), monocyte/macrophage PE (clone KUL01; mouse $IgG_1\kappa$), TCRαβ/Vβ2 Spectral Red (clone TCR-03; mouse $IgG_1\kappa$), CD8α AF700 (clone CT-3; mouse $IgG_1\kappa$), CD3 Pacific Blue (clone CT-3; mouse $IgG_1\kappa$), and CD4 biotin (clone CT-4; mouse $IgG_1\kappa$) with a BV510-conjugated SA secondary stain. All antibodies were sourced from Southern Biotech

(Birmingham, AL), while BV-conjugated SA was purchased from BioLegend (San Diego, CA). Isotype controls (fluorescence-minus-one staining protocol) were used to account for non-specific binding by each antibody.

For extracellular staining, 0.5µl of each antibody and 0.2µl of corresponding isotype controls were diluted in PBS and 50µl of each stain mix was added to the appropriate cell aliquot. Cells were incubated in the dark for 30 min at 4°C before being washed in PBS. Secondary staining of biotin-conjugated antibodies in both stain mixes was done by diluting 0.3µl of fluorochrome-conjugated SA in 50µl PBS and allowing the cells to incubate for 30 min in the dark at 4°C. Cells were washed and resuspended to a final volume of 300µl before being analyzed using a BD FACSCanto cytometer (BD Biosciences, San Jose, CA). Gating of populations analyzed by flow cytometry was done using FlowJo 10.5.0 software (FlowJo LLC, Ashland, OR).

Statistical Analysis

Data were analyzed using PROC MIXED (comparisons of all dietary treatments) on SAS version 9.4 (Cary, NC). The performance data were analyzed with the fixed effect of dietary treatment, and contrast statements were performed to obtain the effects of probiotic type, inclusion level, and the probiotic type × inclusion level interaction. FITC-d fluorescence data were analyzed with the fixed effects of dietary treatment, feed restriction, and the dietary treatment × feed restriction interaction.

Flow Cytometry

Due to the addition of a control group without probiotic supplementation, the treatment design of this study was characterized as a 2 × 2 + 1 factorial (2 probiotic types, 2 inclusion levels, plus a "no additive" control). Contrast statements in the MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC) were used to obtain the fixed effects of probiotic type, inclusion, and their interaction with the following statistical model:

$$y_{(i)jkl} = \mu + C_i + P_{(i)j} + I_{(i)k} + (P \times I)_{(i)jk} + e_{(i)jkl}$$

In this model, y_{ijk} is the dependent variable (cell population) of the l^{th} replicate from the j^{th} level of probiotic type and k^{th} level of inclusion nested within the i^{th} level of control, μ is the overall mean, C_i is the control effect at the i^{th} level (i=2), $P_{(i)j}$ is the fixed effect of probiotic type at the j^{th} level (LactoCare or LactoPlan; j=2) nested within the control, $I_{(i)k}$ is the fixed effect of inclusion at the k^{th} level (0.05 or 0.10%; k=2) nested within the control, $(P \times I)_{(i)jk}$ is the interaction effect between probiotic type at the j^{th} level and inclusion at the k^{th} level nested within control, and $e_{(i)jkl}$ is the random error associated with $y_{(i)jkl}$.

RESULTS

Performance

Performance parameters for each growth period and overall (d0-42) are presented in Table 2. Feed intake, weight gain, and Gain:Feed were not affected by dietary treatment ($P > 0.05$).

FITC-d Serum Fluorescence

The FITC-d relative fluorescence serum data indicated that 12-h FR was successful in stressing gut integrity, as a 70% FITC-d increase was found in the serum of FR vs. NFR birds, irrespective of dietary treatment ($P < 0.0001$, Fig. 1A). The main effect of diet was also

significant (P=0.0191, Fig. 1B); 0.05% LactoPlan resulted in greater FITC-d crossing into circulation compared to all other diets, with a 33% increased absorbance compared to the control. The diet × FR interaction was significant (P < 0.0001, Fig. 1C). No treatments showed differences in FITC-d absorbance before FR (P ≥ 0.05), however, after FR, 0.05% LactoPlan bird serum had 57% increased relative fluorescence compared to the control (P=0.0001). There were no differences detected in either inclusion level of the LactoCare dietary treatments or 0.10% LactoPlan after FR compared to the control. Notably, 0.05% LactoCare and 0.10% LactoPlan did not show a difference in FITC-d absorption with or without feed restriction (P > 0.05), indicating maintenance of epithelial cell integrity.

Flow Cytometry

***Probiotic Composition*.** Changes to splenic immune cell profiles differed between compositionally different LactoPlan and LactoCare. Examination of innate immune cell types showed that broilers fed diets supplemented with LactoPlan had a 52.4% reduction in splenic monocytes/macrophages compared to diets supplemented with LactoCare, and an approximately 40.7% reduction in these cells compared to the no additive control ($P < 0.0001$; SEM = 0.7). Similarly, LactoPlan-supplemented diets showed a 16 and 20.3% reduction in lipid antigen-presenting $CD1.1^+$ cells compared to the control and LactoCare diets, respectively ($P < 0.0001$; SEM = 0.9), which translated to similar reductions in the percentage of $CD1.1^+CD8\alpha^+$ cells within this population (23.1%) relative to LactoCare diets ($P = 0.02$; SEM = 0.3; Fig. 2A).

While feeding LactoPlan altered innate immune cells in the broiler spleen, LactoCare had greater impacts on adaptive immune cell profiles. LactoCare diets increased overall leukocyte ($CD45^+$) populations by 17.7 and 11.3% compared to the control and LactoPlan diets ($P = 0.007$;

SEM = 4.0). Within measured $CD45^+$ subpopulations, LactoCare also increased populations of $CD45^+CD4^+$ T_H cells by 31.9 and 21.4% relative to the control and LactoPlan diets ($P < 0.0001$; SEM = 1.3). Notably, a differing effect of probiotic type was observed in $CD45^+Bu\text{-}1^+$ B-cells, with LactoPlan diets having 33.2 and 27.0% fewer B-cells compared to the no additive control and LactoCare diets, respectively ($P < 0.0001$; SEM = 2.4; Fig. 3A).

In addition to increasing overall leukocyte populations, LactoCare increased overall $CD3^+$ T-cells in the broiler spleen by 38.4 and 38.8% compared to the control and LactoPlan diets ($P < 0.0001$; SEM= 4.4). Within measured T-cell populations, LactoCare diets increased percentages of $CD3^+CD4^+$ T_H cells by 35.0 and 41.0% compared to the control and LactoPlan diets, respectively ($P < 0.0001$; SEM = 2.1). Feeding LactoCare also increased the percentage of conventional αβ T-cells ($CD3^+TCR\alpha\beta^+$) by 28.5 and 39.3% compared to the control and LactoPlan diets ($P < 0.001$; SEM = 1.6). In contrast to general patterns of LactoCare having greater impacts on overall T-cells and underlying subpopulations, birds fed LactoPlan had 10.1% greater percentages of $CD3^+CD8\alpha^+$ T_C cells compared to LactoCare diets ($P = 0.007$; SEM = 2.9; Fig. 4A).

To gain preliminary insight into the activity of measured T-cells, CD28 was used as a marker of T-cell activation. Probiotic type altered overall populations of activated T-cells, with LactoCare increasing the overall percentage of $CD28^+$ cells by 33.9% compared to LactoPlan ($P = 0.007$; SEM = 2.2). While LactoCare altered overall $CD28^+$ cells, LactoPlan diets impacted underlying cell populations by reducing $CD28^+CD8\alpha^+$ activated T_C cells by 17.0 and 22.0% compared to the control and LactoCare diets, respectively ($P = 0.0009$; SEM = 1.8; Fig. 5A).

Probiotic Inclusion Rate. Inclusion rate as a main effect impacted T cell populations and CD28 activation markers within the splenic immune cell profiles in healthy broilers. Within the measured T-cell subpopulations, incorporating either probiotic at 0.10% of the diet increased the percentage of $CD3^+CD8\alpha^+$ T_C cells by 7.8% compared to lower 0.05% inclusions ($P = 0.04$; Fig. 4B). Higher inclusion rates increased T-cell activation with 0.10% inclusion increasing overall $CD28^+$ cells by 28.7% compared to 0.05% ($P = 0.03$). Within the measured subpopulations of activated T-cells, 0.10% probiotic inclusion increased the percentage of $CD28^+CD4^+$ T_H cells by 17.9 and 15.8% compared to the control and 0.05% inclusions, respectively ($P < 0.0001$; SEM = 3.49; Fig. 5B).

Interactions Between Probiotic Composition and Inclusion. The effect of the interaction between probiotic composition and inclusion level was primarily observed at higher inclusions of LactoCare. Changes to overall leukocyte populations due to LactoCare supplementation were greater at 0.10% inclusion, which resulted in 28.2, 25.2, 16.4, and 28.5% increases in this cell type compared to the control, 0.05% LactoCare, 0.05% LactoPlan, and 0.10% LactoPlan diets, respectively ($P < 0.0001$). Similarly, feeding higher inclusion levels of LactoCare resulted in increases to underlying $CD45^+CD4^+$ T-helper (T_H) populations, with the spleens of broilers fed 0.10% LactoCare having 39.3, 21.4, 25.9, and 33.6% greater percentages of these cells within the $CD45^+$ gate compared to the control, 0.05% LactoCare, 0.05% LactoPlan, and 0.10% LactoPlan diets, respectively ($P = 0.001$; SEM = 1.3; Fig. 3C).

Similar to overall leukocyte populations, higher inclusions of LactoCare resulted in increased $CD3^+$ T-cells within the broiler spleen compared to other probiotic treatments. Birds fed these diets had 47.9, 31.0, 37.6, and 59.0% more T-cells than birds fed the no additive control, 0.05% LactoCare, 0.05% LactoPlan, and 0.10% LactoPlan diets, respectively ($P <$

0.0001; SEM = 4.4). In contrast to the generally observed pattern, lower inclusions of LactoCare at 0.05% increased the percentage of conventional αβ T-cells compared to the control, 0.05% LactoPlan, and 0.10% LactoPlan diets by 34.5, 49.0, and 39.7%, respectively, while the higher inclusion of LactoCare differed only from the 0.05% and 0.10% LactoPlan diets by 38.9 and 27.8%, respectively ($P = 0.03$; Fig. 4C).

Higher inclusions of LactoCare also changed percentages of T-cell activation. Diets supplemented with LactoCare at 0.10% had 49.0, 58.8, 43.7, and 63.1% greater percentages of $CD28^+$ cells compared to the control, 0.05% LactoCare, 0.05% LactoPlan, and 0.10% LactoPlan diet, respectively ($P < 0.0001$; Fig. 5C). In contrast to the noted effects of high inclusion levels of LactoCare, 0.10% inclusion of LactoPlan reduced activated T_C cells by 27.6 and 33.8% compared to 0.05 and 0.10% LactoCare diets, respectively ($P = 0.04$; Fig. 5C).

DISCUSSION

Performance and Gut Integrity

The lack of probiotic treatment effect on performance outcomes is not uncommon in published literature (Willis and Reid, 2008; Sharifi, et al., 2012; Bai, et al., 2013), but is in contrast to several studies feeding *Lactobacillus* products to broilers (Liu, et al., 2007; Mountzouris, et al., 2007; Yu, et al., 2007; Peng, et al., 2016; Forte, et al., 2017). The lack of a difference observed here may have been due to a cleaner research environment compared to commercial barns. Although the litter had been re-used 4 times to better reflect a production environment in the U.S., the research setting does not contain the number of birds, pathogens, and volume of manure and recycling typical in a commercial setting.

The intestinal epithelium integrity and microbiome are crucial to host health; the selectively permeable epithelial cell barrier allows nutrient absorption while preventing pathogen passage into the bloodstream (Buckley and Turner, 2018). Probiotics have shown the ability to prevent translocation of pathogenic bacteria and benefit the GI tract (Madsen, et al., 2001; Zareie, et al., 2006), likely through the process of competitive exclusion, or reduction of the growth of harmful species (Edens, et al., 1997). The increased FITC-d translocation into the serum following FR in this study agrees with other work using this model to induce stress and gut permeability (Baxter, et al., 2017; Kuttappan, et al., 2015; Vicuña, et al., 2015).

Under our research conditions, the FITC-d translocation before FR did not differ between dietary treatments (Fig. 1C). Following 12h FR, the serum fluorescence was increased in the control, 0.10% LactoCare, and 0.05% LactoPlan® dietary treatments, indicating increased FITC-d translocation following FR stress. However, in the 0.05% LactoCare treatment, as well as the 0.10% LactoPlan treatment, FITC-d absorption did not differ before and after FR. This result indicates a maintenance of barrier function due to probiotic diet, a protective effect that has been previously observed in *Lactobacillus reuteri* and *Lactobacillus plantarum* probiotics through suppression of certain opportunistic or pathogenic bacterial species (Nakphaichit, et al., 2011; Wang, et al., 2017).

Splenic Immune Cell Populations

Percentages of splenic immune cells in healthy broilers were primarily impacted by probiotic composition, and inclusion rate played a secondary role in further changes to these populations. The overall observed trend was a reduction in all analyzed innate immune cell populations by LactoPlan in addition to reductions to B-cell populations while maintaining T-cell

populations at levels similar to the control. The lack of changes to overall T-cell populations in the spleen as a result of LactoPlan supplementation are in agreement with published results by Wang et al., who found no changes to T lymphocytes in the spleen of broilers given the same probiotic species as LactoPlan, *L. plantarum* (Wang, et al., 2015). Notably, Wang et al., observed increased $CD3^+$ T-cells, IgA^+ B-cells, and an increased number of Bu-1 transcripts in the broiler jejunum, suggesting that changes to lymphocyte populations by *L. plantarum* are more likely to occur at local sites of colonization with minimal systemic effects (Wang, et al., 2015). Alternatively, the reduction in Bu-1^+ B-cells in the spleen of LactoPlan-supplemented birds observed in this study, combined with increased jejunal B-cell presence noted in other studies, could suggest that *L. plantarum* increased B-cell recruitment from the spleen to peripheral tissues; however, future studies to examine this relationship are needed.

In contrast, LactoCare maintained populations of innate immune cells and B-cells while significantly increasing overall leukocyte and T-cell populations. Notably, these effects were increased at higher inclusions of LactoCare while inclusion level seemed to have little effect on the overall immune cell populations affected by LactoPlan. Changes to overall leukocyte populations at the systemic level are consistent with published increases in overall white blood cell counts in the peripheral blood of birds given *L. reuteri*, the same probiotic species as LactoCare (Salim, et al., 2013). While changes to leukocytes were similar to those reported by Salim et al., increased $CD3^+$ T-cells and maintained innate immune cell populations in LactoCare-supplemented birds are in contrast to this published report, which found no changes to lymphocytes and increased peripheral monocytes (Salim, et al., 2013). It is important to note that differences in methods (blood cell counts vs. flow cytometry) and examined tissue (peripheral blood vs. spleen) may be underlying these discrepancies.

In looking at underlying cell populations in this study, there were notable changes in T-cell profiles between the two different probiotic compositions. Diets with LactoCare displayed shifts in splenic T-cell populations that favored T_H cells while LactoPlan shifted T-cell populations in favor of T_C cells. While changes to T-cell subpopulations are not fully understood for supplementation with *L. reuteri*, Wang et al., found no changes to splenic T_C cells as a result of *L. plantarum* supplementation (Wang, et al., 2015). Both LactoPlan and LactoCare are proprietary strains of their respective probiotic species and discrepancies between the results reported here and other published studies may be due to differences between strains within the same species. Of note, while $CD8\alpha^+$ T-cells were the predominant T-cell population in the broiler spleen, T_H cells comprised the majority of activated T-cells (Fig. 4 and 5). Activated populations of T_H cells were increased with inclusion rate, regardless of probiotic type, suggesting that probiotic composition may cause shifts in general T_H cell populations, but higher probiotic inclusions are responsible for increasing markers of T_H cell activation. Also noted was the decrease in activated T_C cells in LactoPlan diets, despite an observed shift in T-cell populations that favored T_C subpopulations, suggesting that increases in T_C populations due to LactoPlan supplementation were primarily in naïve cell populations. Overall, these results emphasize differential shifts in immune cell profiles in response to probiotic composition in healthy broilers.

The broilers in this study were housed in a barn with 4× re-used bedding to mimic the reused litter conditions commonly practiced in U.S. production settings. While this allowed for the observation of changes to immune cell profiles in an environment with mild, non-specific health challenges commonly associated with commercial broiler production, future studies with a specific pathogen challenge can elucidate the effect of probiotic-induced changes to immune cell

profiles. Flow cytometry is a useful tool for determining the presence of different immune cell populations, but additional methods (i.e. cell killing assays, proliferation assays, etc.) can provide better functional insights. While these assays were not implemented in the current study, examining CD28 expression profiles provided some preliminary insight into T-cell activation in response to probiotic supplementation as this is an extracellular marker on T-cells associated with the reception of costimulatory signals (Young, et al., 1994).

Overall, the results of this study demonstrate that two probiotics fed to Ross 708 broilers at 0.05% and 0.10% each did not affect feed conversion nor weight gain but showed the capacity to maintain gut integrity following a 12-h FR challenge in the 0.10% inclusion treatment groups of both additives (LactoCare and LactoPlan), and the 0.05% inclusion rate of LactoCare (*Lactobacillus reuteri*). Probiotic composition had a significant impact on baseline immune cell populations in the spleen, with inclusion levels impacting some measures of T-cell activation. Future work should involve validation of the gut integrity outcomes following FR identified here as well as studying specific pathogen challenges to provide insight into mechanisms of probiotics to reduce gut leakiness and examine the responsiveness of altered immune cell profiles.

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Figure 1. Main effects of (**A**) feed restriction, (**B**) diet, and (**C**) the interaction. Values are expressed as mean fluorescence (ng/mL) of serum fluorescein isothiocyanate dextran (FITC-d) using a 12-hour feed restriction model with different probiotic-supplemented dietary treatments fed to Ross 708 broiler chickens. Bars with different superscripts denote means that are significantly different ($P \leq 0.05$).

Figure 2. Percentages of innate immune cell types within the spleen of healthy Ross 708 broilers (8 per dietary treatment) fed compositionally different probiotics at two inclusion rates. The main effects of (**A**) probiotic type and (**B**) inclusion are shown in addition to the (**C**) interaction. Data represent the average percentage of each cell population ± SEM. Bars with different superscripts are significantly different at $P \leq 0.05$.

Figure 3. Percentages of splenic $CD45^+$ leukocytes and underlying subpopulations in healthy Ross 708 broilers (8 per dietary treatment) fed compositionally different probiotics at two inclusion rates. The main effects of (**A**) probiotic type and (**B**) inclusion are shown in addition to the (**C**) interaction. Data represent the average percentage of each cell population ± SEM. Bars with different superscripts are significantly different at $P \leq 0.05$.

Figure 4. Percentages of splenic $CD3^+$ T-cells and underlying subpopulations in healthy Ross 708 broilers (8 per dietary treatment) fed compositionally different probiotics at two inclusion rates. The main effects of (**A**) probiotic type and (**B**) inclusion are shown in addition to the (**C**) interaction. Data represent the average percentage of each cell population ± SEM. Bars with different superscripts are significantly different at $P \leq 0.05$.

Figure 5. Percentages of splenic $CD28^+$ activated T-cells and underlying subpopulations in healthy Ross 708 broilers (8 per dietary treatment) fed compositionally different probiotics at two inclusion rates. The main effects of (**A**) probiotic type and (**B**) inclusion are shown in addition to the (**C**) interaction. Data represent the average percentage of each cell population ± SEM. Bars with different superscripts are significantly different at $P \leq 0.05$.

Table 1. Starter, grower, and finisher diets provided ad libitum to Ross 708 broilers.

Ingredients,%[1]	**Starter**	**Grower**	**Finisher**
Corn	55.32	58.69	62.78
Soybean Meal 48	37.15	33.40	28.59
Soy Oil	2.02	2.98	3.97
Salt	0.40	0.40	0.40
DL Methionine	0.33	0.30	0.27
Lysine HCl	0.25	0.23	0.21
Threonine	0.15	0.15	0.15
Limestone	1.30	1.01	1.00
Dicalcium Phosphate	2.05	1.81	1.60
Choline Chloride 60	0.40	0.40	0.40
Vitamin Premix[2]	0.63	0.63	0.63
Calculated Values			
Fat, %	4.59	5.59	6.64
Crude Protein, %	23.05	21.50	19.50
ME, kcal/kg	3000	3100	3200
Digestible Lysine, %	1.30	1.19	1.06
Digestible Arginine, %	1.39	1.28	1.14
Digestible Threonine, %	0.92	0.87	0.80
Analyzed Values (As fed)[3]			
Dry Matter, %	89.88	90.30	90.58
Crude Fat, %	5.84	6.83	7.93
Crude Protein, %	21.50	20.38	18.50
Gross Energy, kcal/kg	3971.0	4062.8	4142.6

[1]All diets were formulated and mixed using the same basal diet described with feed additive LactoCare included at 0.05%, 0.10%, and feed additive LactoPlan included at 0.05% and 0.10%

[2]Vitamin and mineral premix provided per kg of diet: Selenium 200 µg; Vitamin A 6,600 IU; Vitamin D_3 2,200 IU; Vitamin E 14.3 IU; Menadione 880 µg; Vitamin B_{12} 9.4 µg; Biotin 33 µg; Choline 358 mg; Folic acid 1.1mg; Niacin 33 mg; Pantothenic acid 8.8 mg; Pyridoxine 880 µg; Riboflavin 4.4 mg; Thiamine 1.1 mg; Iron 226 mg; Magnesium 100 mg; Manganese 220 mg; Zinc 220 mg; Copper 22 mg; Iodine 675 µg

[3]Analyzed values presented are the mean proximate analyses for all dietary treatments.

[4]Calculated according to NRC (1994)

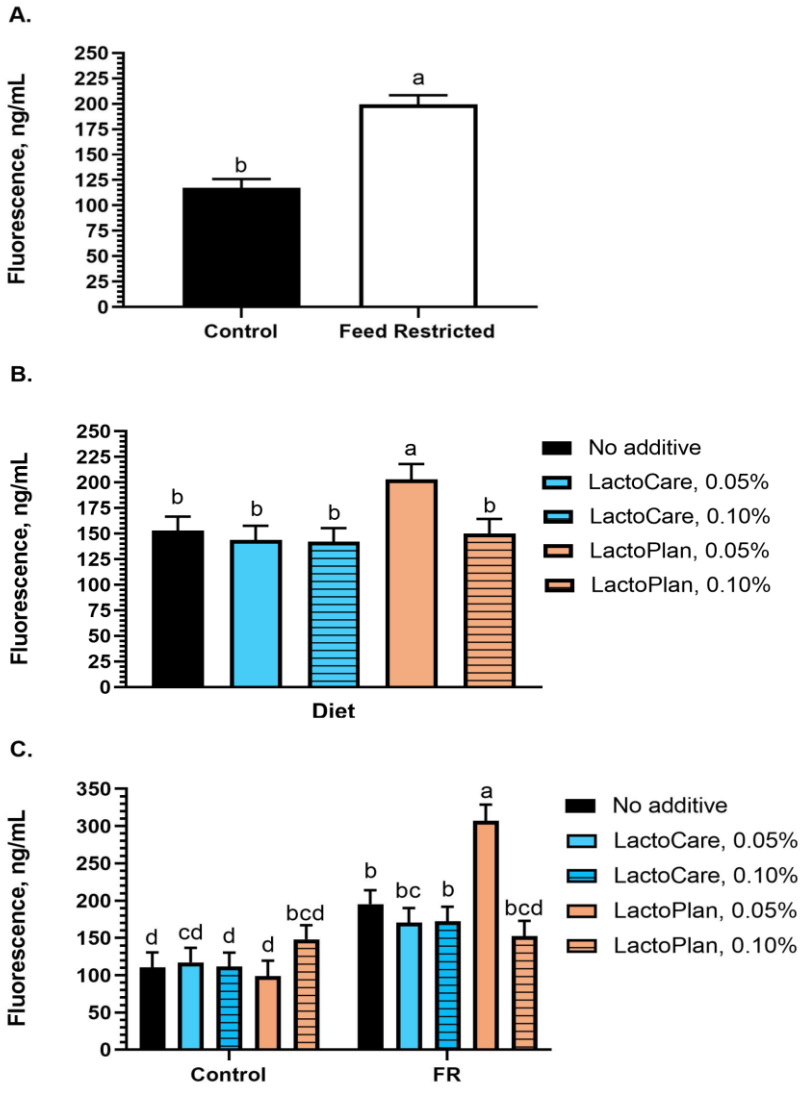
Table 2. Ross 708 straight run broiler performance outcomes[1] including feed intake, weight gain, and Gain:Feed by each 2-wk performance period and overall

Performance Measure	Control	0.05% LactoCare	0.1% LactoCare	0.05% LactoPlan	0.1% LactoPlan	Pooled SEM	Trt P-value	Contrast P-Value		
								Probiotic	Inclusion	Probiotic*Inclusion
Feed intake, g										
Starter	400	410	400	400	390	8.0	0.762	0.480	0.417	0.939
Grower	1290	1230	1250	1280	1280	26.0	0.520	0.184	0.705	0.691
Finisher	2270	2220	2230	2260	2310	56.0	0.794	0.310	0.519	0.703
Overall	3950	3860	3890	3930	3980	79.0	0.801	0.281	0.621	0.895
Weight gain, g										
Starter	280	290	280	290	290	6.0	0.251	0.991	0.158	0.257
Grower	820	830	820	850	820	13.0	0.414	0.274	0.127	0.577
Finisher	1310	1320	1320	1320	1410	35.0	0.287	0.193	0.258	0.237
Overall	2410	2440	2410	2460	2510	45.0	0.492	0.190	0.785	0.367

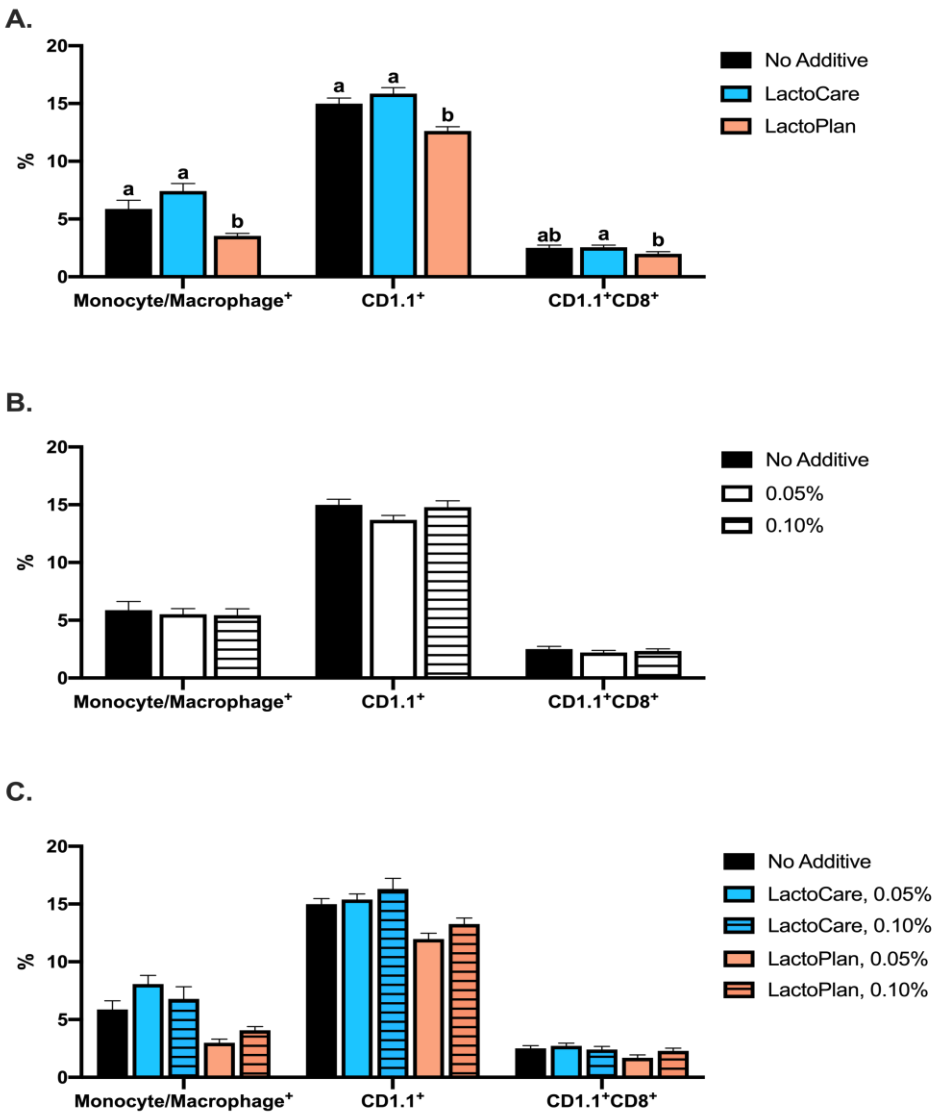
Gain:Feed										
Starter	0.708	0.716	0.700	0.710	0.726	0.011	0.521	0.334	0.993	0.147
Grower	0.644	0.670	0.657	0.663	0.652	0.009	0.362	0.519	0.216	0.953
Finisher	0.578	0.595	0.591	0.587	0.611	0.010	0.241	0.563	0.327	0.174
Overall	0.612	0.632	0.624	0.625	0.635	0.007	0.208	0.766	0.895	0.207

[1]Starter period indicates wk 0-2, grower wk 2-4, and finisher wk 4-6. Values presented are LSMeans (pooled SEM) averaged per bird with the main effect of treatment

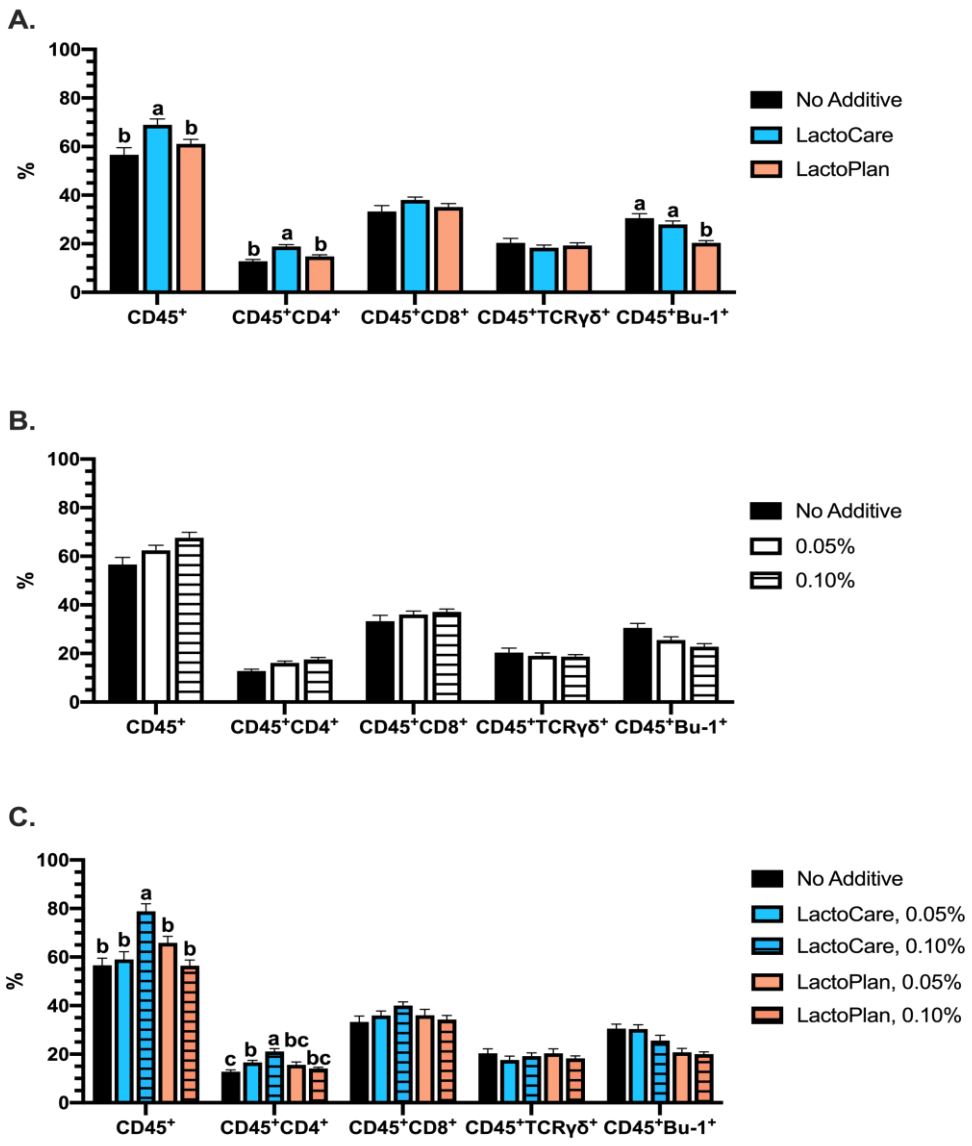
NutrafermaFinalFigure1



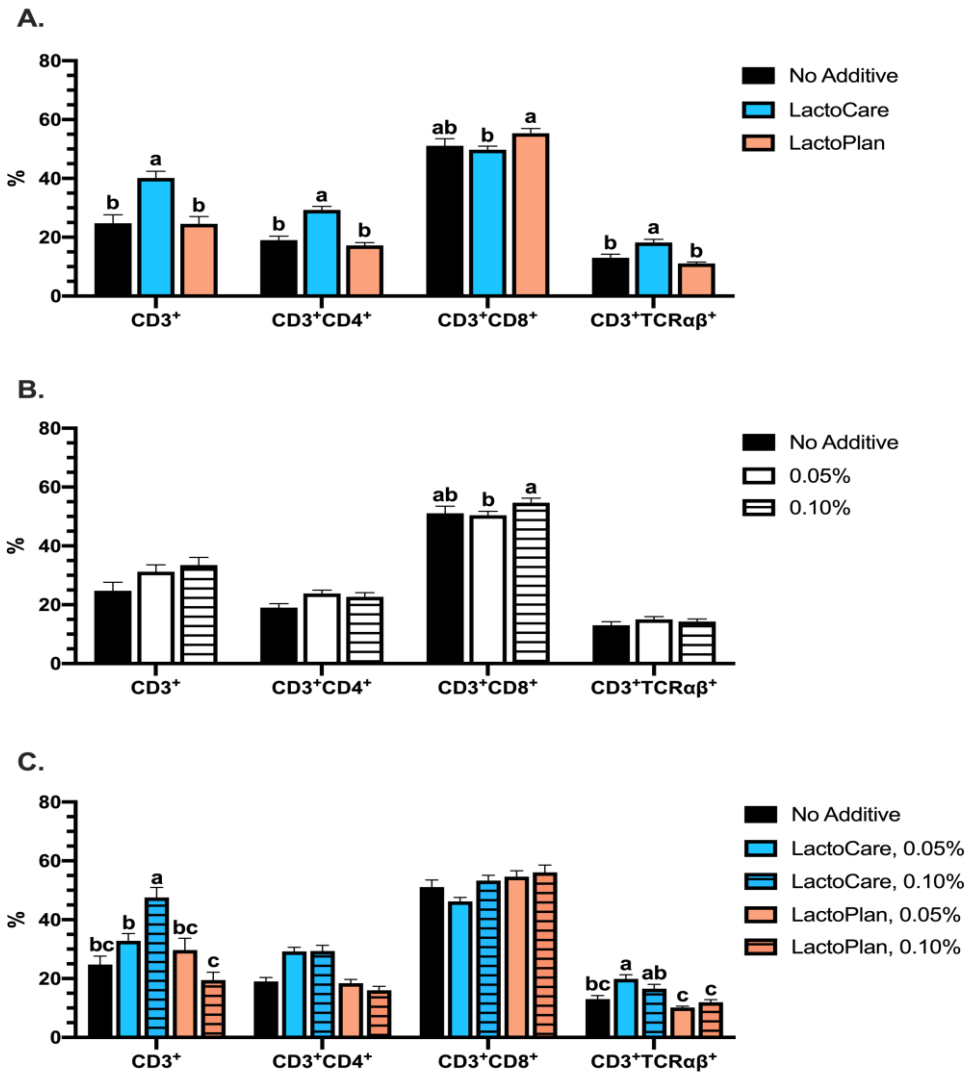
NutrafermaFinalFigure 2



Nutraferma Final Figure 3



NutrafermaFinalFigure 4



NutrafermaFinalFigure5