UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

TÍTULO: Effects of *Lactobacillus fermentum* administration on caecal cytokine expression and serum immunoglobulin concentration in *Salmonella* Infantis-challenged chickens

Trabajo de integración curricular presentado como requisito para la obtención del título de Biólogo

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Urcuquí, diciembre 2022.

Urcuquí, 14 de diciembre de 2022

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CARRERA DE BIOLOGÍA
ACTA DE DEFENSA No. UITEY-BIO-2022-00035-AD

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A los Zavala.

AGRADECIMIENTOS

A mis mentores: Dr. Marco Larrea-Álvarez, Dra. Miroslava Šefcová y Dr. David Ortega por su constante supervisión y guía. De igual manera a mi tutor, Dr. Santiago Ballaz García, por su acompañamiento durante la realización de este trabajo de investigación.

A los miembros de la Unidad de Investigación de Enfermedades Transmitidas por Alimentos y Resistencia a los Antimicrobianos (UNIETAR) y al Team CEDIA por su recibimiento y disposición durante la etapa experimental del proyecto.

A los profesores de la Escuela de Ciencias Biológicas e Ingeniería y Yachay Tech, en general, por sus esfuerzos en pro de la formación de la nueva generación de científicos del Ecuador.

A mi familia por ser mi refugio incondicional.

A los amigos que hice en Yachay Tech por su camaradería. Especialmente a mi colega, Melissa Chávez, por brindarme su apoyo y confianza a lo largo de la carrera.

- Ivan Ronnaldo Mina Zavala

RESUMEN

Salmonella Infantis es una de las serovariedades más comunes de *S. enterica* en la industria avícola del Ecuador, donde se reportan diversas cepas resistentes a múltiples antibióticos. Sin embargo, se desconocen varios aspectos inmunológicos relacionados a este patógeno. El uso de probióticos es una estrategia emergente para contrarrestar la colonización intestinal de bacterias patógenas en pollos, regulando la actividad de su sistema inmune. Este estudio evalúa la influencia de la infección por *S.* Infantis en la expresión de genes relacionados al sistema inmune (Interleucina-1β (*IL1B*), *IL13*, *IL15*, *IL17*, y *MYD88*) y en los niveles de producción de inmunoglobulinas (IgA e IgM) en pollos de engorde. Además, estudia los cambios en estos mismos indicadores durante la administración de *Lactobacillus fermentum* como probiótico para determinar su actividad inmunomoduladora. Doscientos pollos de engorde de un día de edad fueron asignados aleatoriamente a uno de cuatro grupos experimentales: (i) C, grupo control; (ii) Lf, inoculados con *L. fermentum*; (iii) Se, infectados con *S.* Infantis; y (iv) LfSe, inoculado con ambas bacterias. El periodo experimental se extendió por 15 días. Por cada grupo experimental, se obtuvieron 8 muestras de suero y 10 de tejido cecal. La expresión de genes se analizó por medio de RT-qPCR, mientras que la concentración de inmunoglobulinas se determinó mediante pruebas de ELISA. La expresión relativa de estos genes no se vio influenciada por ninguna de las condiciones experimentales, al igual que la producción de IgA. Por otro lado, los niveles de IgM aumentaron cuando se administró *L. fermentum* previo a la infección con *S*. Infantis. Aun cuando los mecanismos detrás de estos efectos no han sido descritos a la fecha, el aumento en la producción de anticuerpos sostiene que el tratamiento probiótico usando *L. fermentum* en pollos de engorde infectados con *S.* Infantis favorece la respuesta inmune intestinal. Se espera que futuros estudios contribuyan a la explicación de los procesos detrás de estas dinámicas entre el probiótico y el sistema inmune en pollos.

Palabras clave: expresión de genes de citoquinas, inmunomodulación, *Lactobacillus fermentum*, niveles de anticuerpos, pollos de engorde, probióticos, *Salmonella* Infantis.

ABSTRACT

Salmonella Infantis is one of the most predominant serovars of *S. enterica* in the poultry industry of Ecuador, where numerous multidrug resistant strains have been reported. Nevertheless, many immunological features related to this pathogen remain unknown. The use of probiotics rises as an emerging approach to mitigate gut colonization by pathogenic bacteria in chickens. This research aimed to assess the influences of *S*. Infantis infection on the expression of immune related genes (Interleukin-1β (*IL1B*), *IL13, IL15, IL17,* and *MYD88*) and on the serum immunoglobulin (IgA, IgM) levels in broiler chickens. Additionally, the same criteria were evaluated during the administration of *Lactobacillus fermentum* as a probiotic to evaluate its immunomodulatory properties. Two-hundred 1-day old broiler chickens were randomly assorted into four experimental groups as follows: (i) Control, with untreated chicken; (ii) Lf, treated with *L. fermentum*; (iii) Se, infected with *S.* Infantis; and (iv) LfSe, inoculated with both bacteria. Experiments were conducted for 15 days. Per group, serum and caecal samples were collected from eight and ten animals, respectively. Gene expression analysis was performed using RT-qPCR, while immunoglobulin levels were measured with ELISA. Results revealed that relative expression of these genes was not affected by any of the experimental treatments, as were the levels of IgA. The concentration of IgM, on the other hand, was augmented when animals were exposed to the probiotic before infection with *S.* Infantis. Although the mechanisms behind the effects remain unclear, the increased antibody production ascertains that the probiotic treatment with *L. fermentum* in *S.* Infantis infection favors the immune response in the gut of broiler chicken. Further research might elucidate the complex dynamics involved.

Keywords: broiler chickens, cytokine gene expression, immunoglobulin levels, immunomodulation, *Lactobacillus fermentum*, probiotics, *Salmonella* Infantis.

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1. INTRODUCTION

1.1. *Salmonella enterica* subsp. *enterica* serovar Infantis

Salmonella enterica subspecies *enterica* (*S. enterica*) is one of the most important, ubiquitous gastrointestinal pathogens affecting farm animals and humans (1). These Gram-negative, facultative anaerobe bacilli belong to the Enterobacteriaceae family and are characterized by their extremely high serological diversity, comprising over 2600 serological variations (2,3). The serovars of *S. enterica* are traditionally grouped into two classes based on the nature of the disease, host range, and specificity (4). The first class comprises serovars in the host-specific group, which cause aggressive typhoid-like disease known as typhoid or enteric fever, and rarely cause complications in organisms different than their own host (2). Some of the serovars included in this classification are *S.* Typhi and *S*. Paratyphi A, B, and C in humans, and *S*. Pullorum and *S*. Gallinarum in poultry. The rest of serovars cause non-typhoid salmonellosis (NTS) in a broad range of hosts, including humans (5,6).

The NTS serovars do not cause disease in normal healthy adult birds, but are adept to colonize the gut, and other systemic organs to some extent (7). Thus, chickens can be healthy carriers of *Salmonella*. For instance, these serovars are able to grow in the gastrointestinal tract of poultry with no symptoms of disease, with the exception of newly hatched chickens, which eases the farm-to-fork contamination due to the infection going under the radar (8). Additionally, chicken meat for human consumption can be infected with *Salmonella* after contact with intestinal contents or fecal materials, and from cross-contamination during slaughtering (9). Some serovars of NTS can also colonize the linings of the reproductive tract of chicken, contaminating eggs too (4). Although *Salmonella* is also present in vegetable and fruits, salmonellosis often develops in humans after ingestion of animal-derived products, such as eggs, dairy, or meat (9). Salmonellosis is one of the most common foodborne infections affecting humans around the world, adding up to 1.3 billion infections and 155.000 deaths annually (10,11). In most cases salmonellosis evolves into a self-limiting gastroenteritis with symptoms like abdominal cramps, diarrhea, and vomiting, which develops between 12 and 72 hours after infection. However, there has been reports of *Salmonella* infections being responsible for mortal cases related to bacteremia and dehydration. Fatal cases of the disease often develop in child, elderly, and immunocompromised populations (12).

Unlike the host-specific serovars of *Salmonella*, which are limited to developing countries and regions, the NTS serovars are distributed worldwide (1). These regional variations account for different levels of control systems, use of antibiotics and vaccines, environmental

contamination, slaughtering, and sampling methods. However, globalization and increased trade of raw meat (poultry and pork specifically) are considered to contribute for the spreading of serovars to novel areas (13). Among the more widely distributed, *S.* Typhimurium and *S.* Enteritidis are responsible for the majority of cases involving gastroenteritis in humans both in the US and the European Union (14). These serological variations have also notable prevalence in other regions such as Asia, Africa and Latin America (15). The prevalence of other serovars seems to be specific to certain areas. For example, *S*. Kentucky in North America, *S*. Sofia in Oceania, and *S*. Weltevreden in the southern regions of Asia (15). While some diversity is observed regarding serovars present in animal derived products of the Andean region, *S*. Infantis has been reported as a predominant serological variation in Ecuador (16). High prevalence of *S.* Infantis has also been reported in more distant areas of the world, including India and Japan (9,17). Increasing occurrences of *S.* Infantis in the European Union and Asia associated with poultry meat consumption have been reported as well (11,13).

Local studies testing the presence of *Salmonella* in chickens from caecal samples, meat as well as skin, feed from farms, and slaughterhouse equipment report that contamination levels ranged from 15.9% to 69.1%; the prevalence of *S*. Infantis has been determined to be around 83.9% up to 100% (18–21). *S*. Infantis is confirmed to infect a wide range of hosts, as it has been isolated from poultry, turkey, beef, veal, lamb, pork, and clinical samples from humans (21). According to the Ministerio de Salud Pública del Ecuador (MSP), there were 1082 reported cases of gastroenteritis due to *Salmonella* infection in the country in 2020 (22). Considering that there are around 30 estimated unconfirmed cases of salmonellosis for each confirmed case, as stated by the CDC (23), the incidence of salmonellosis in our country could be considered a threat to public health.

1.2. Antibiotic Administration in Broiler Chicken and Drug Resistance

In order to promote higher growth rates in broiler chickens, antibiotics are incorporated into their diet (24). These are known as Antibiotic Growth Promoters (AGPs), and refer to any drug capable of eliminating or inhibiting bacterial growth in the intestinal tract of the birds (9,25). Even though the use of AGPs is positive from an economic point of view, its extensive and improper use in the industry is often related to antimicrobial resistance in pathogenic bacteria, as they act as a selective force favoring resistant strains (9,26). Thus, the use of AGPs in broilers has been banned in the European Union, linked to a reduced prevalence of multidrug resistant strains (13,27).

However, in Latin America this is still a common practice among producers. Correspondingly, there is a high prevalence of resistant strains of *Salmonella* in the region (16). In Ecuador,

studies report that *S*. Infantis encloses more resistant strains compared to other local serological variations of *Salmonella* (18,19,21,27), in addition to broader drug resistance patterns (16,28). Some drug resistance patterns are shared among non-related serovars in the Ecuadorian meat industry, implying a role of horizontal gene transfer in the development of resistant strains (21,29).

Some strategies have been implemented to achieve similar results as AGPs with regard to growth performance, including administration of plant essential oils, polyphenolic extracts, organic acids, enzymes, prebiotics, and probiotics (25,30,31).

1.3. Probiotic Administration as an Alternative to AGPs

Probiotics are live microorganisms that promote gastrointestinal health in the host (32). Some of the beneficial effects linked to probiotic administration include pathogen inhibition, gut microbiota regulation, immunomodulation, improved growth performance and meat quality parameters in broiler chickens (30). There are three main mechanisms behind the effects of probiotics in the host gut: (i) competitive exclusion, by inhabiting the gut linings and preventing adhesion of pathogens, (ii) immune system stimulation, their presence elicits immune responses without causing disease, and (iii) bacterial antagonism, in which their metabolism produces organic and fatty acids that lower the pH of the gut hindering pathogen growth (30,33). All these features make the use of probiotics as diet supplement in poultry an important topic for research.

Lactic acid producing bacteria are widely used as probiotics in animal feed, especially those from the genera *Lactobacillus* and *Bifidobacterium* (34). The immunomodulatory properties of these bacteria are often attributed to the induction of the signaling activity of the receptors in the gut epithelium of birds, which gives rise to the initiation of intricate immune responses in the intestine (35). The downregulated expression of inflammatory cytokines, together with the increased levels of anti-inflammatory cytokines (e.g., interleukin-10), alleviates the manifestation of any disease-related sign during this process (36,37). Moreover, during *Salmonella* infection, the presence of the pathogen in the caeca appears to be reduced when challenged chicken are treated with lactic acid bacteria (36).

The use of *Lactobacillus* spp. as probiotics influences the immune activity in the gastrointestinal tract of broiler chickens without compromising their growth performance (32,33). *Lactobacillus*-based probiotic culture administration is known to improve the development of the lymphoid tissue in the gut of newly hatched chickens (38), increase the presence of intraepithelial lymphocyte subpopulations (39), and reduce the infection rates of *Salmonella* (40). Also, inhibitory effects on the invasion and colonization during *Salmonella*

infection have been described as a result of the supplementation of *Lactobacillus* spp. as probiotics: spleen and liver colonization was prevented by some species of *Lactobacillus* (41), while caecal counts of *Salmonella* were significantly reduced (37,41,42). As a consequence, its use stands as a promising alternative to the administration of AGPs in poultry.

Lactobacillus fermentum has been shown to elicit beneficial physiological effects in birds. As a diet supplement, *L. fermentum* demonstrated great competence in promoting growth in broiler chickens when compared to commercial AGPs. It also resulted in an increase of villus height and crypt depth in the intestinal mucosa of the birds, which is related to a better absorption of nutrients (35,43). Other studies have reported that *L. fermentum* administration improves the immune response during infection with pathogenic bacteria by regulating cytokine expression, alleviating disease signs, and reducing inflammation (35,44–46). Additional immunomodulatory effects of *L. fermentum* include the increased activity of immunologic receptors in the gut, along with proliferated subpopulations of T lymphocytes (CD4+, CD8+, and CD3+), when supplemented in the diet of broilers together with *Saccharomyces cerevisiae* (35).

1.4. Immune Response in *Salmonella*-challenged chicken

After entrance into the organism, via fecal-oral contamination, the bacteria encounter a number of defense mechanism from the organism. The first line of defense against infection consists of physical barriers. Tight junctions in the gut epithelium as well as antimicrobial molecules are able to limit infections from spreading. Some broad-spectrum antimicrobial peptides found in gut epithelial barriers include cathelicidins, S100 proteins, and gallinacins, which are embedded in the mucosal layers of gel-forming mucins produced by the goblet cells (47,48). These molecules trap and aid in the clearance of the pathogenic bacteria before invasion. However, some bacteria are equipped with mechanisms that surpass these barriers and invade the gut of birds and promote colonization. The proteins involved in such mechanisms are encoded in genes located in the so-called *Salmonella* Pathogenicity Islands (SPIs) (9). Although the main site of colonization is the caecum, large-scale disseminations can cause systemic infections (47).

Recognition of pathogen-associated molecular patterns (PAMP) is key for the activation of the innate immune system in birds. This process is mediated by Toll-like receptors (TLR) in the membranes of the enterocytes. There are at least 11 different TLRs with specificity to recognize an array of PAMPs, such as flagellin, lipopolysaccharide, peptidoglycan, among others. (48,49). The role of TLR5 involved in flagellin recognition appears to be critical for eliciting a response, as non-flagellated or mutant strains of *Salmonella* are able to invade the

epithelial cells faster and with reduced inflammation (47). Other receptors that participate in PAMP recognition are nucleotide-binding oligomerization domain-like receptors (NLR) (48).

Upon binding of ligands to the receptors, there is an activation in the signaling pathway mediated by adaptor proteins (MyD88, TRIF), and their associated transcription factors (NF-κB), leading to the expression of a wide array of cytokines and chemokines (48). The initial activation of the innate immune responses involves the recruitment of macrophages and heterophils from gut-associated lymphoid tissue (GALT) into the sites of infection, leading to inflammation. Heterophils play a phagocytic role and produce antimicrobial peptides to eliminate pathogens; while macrophages participate in phagocytosis, antigen presentation, and are involved in the regulation of immune system (49). Also, some *Salmonella* strains are able to invade and thrive inside macrophages and dendritic cells, so the activation of intracellular antimicrobial action is important to avoid its propagation (47). Various studies propose that some strains of *Salmonella* utilize these cells as a way of transportation from the site of infection towards other organs, including the spleen and liver (50). The proinflammatory cytokines involved in this step are interleukin-1β (IL-1β), IL-6, and the tumor necrosis factor alpha (TNF-α) among others (48). The chemokines CXCLi1 and CXCLi2 also favor inflammation and phagocyte recruitment (47). This deployment is usually capable to stop dissemination of the infection. However, excessive expression of inflammatory effector molecules (i.e., cytokines) is often related to acute disease. Cells from the innate immune system also produce other several cytokines and chemokines that lead to the activation and maturation of leukocytes.

Adaptive immunity is comprised by the action of lymphocytes B and T, which are antigen-specific upon their interactions with antigen-presenting cells (APC) or any infected nucleated cell (48). The mechanisms behind antigen presentation can occur as follows:

- After phagocytosis, the bacteria are processed by proteases in the lysosomes of the APCs. The resulting contents are trafficked and exteriorized to their membranes within the major histocompatibility complex class II (MHC-II). MHC-II is exclusive for APCs (48,51).

- Upon effects of oxidative stress molecules (i.e., NO, ROS) in infected cells, some defective cytosolic molecules are taken up and externalized with the MHC class I. MHC-I interacts with CD8+ cytotoxic T (Tc) cells, leading to the apoptosis of the infected cells (48,51).

Naïve helper T (Th) cells develop in the thymus and migrate towards secondary lymphoid organs, where they encounter APCs. This interaction involves the contact of their

corresponding membrane molecules (CD4 and MHC-II) together with other co-stimulatory molecules, resulting in their activation. The denominated CD4+ Th cells produce IL-2 and IL-15 for their own proliferation (52). These interleukins also promote the proliferation of natural killer (NK) cells from the innate immune response (53). Based on the cytokines present in their environment, mature Th cells can differentiate into Th1, Th2, Th17, and T regulatory (Treg) cells. Polarization into Th1 cells is driven by the presence of IL-12 and interferon γ (IFN-γ), and their activity stimulates the cytotoxicity of Tc cells and phagocytosis in innate immune cells by producing IL-12, IFN- γ, and IL-18. IL-4 aids towards differentiation into Th2 cells, which in return produce IL-4, IL-5, and IL-13. Th2 activity promotes B cell activation, which leads to their differentiation and production of immunoglobulins that are later secreted (48). Chickens bear three types of immunoglobulins: Immunoglobulin M (IgM), IgY (homologue to the mammalian IgG), and IgA. IgM is predominant during the initial exposure to an unknown antigen, while IgY presence is characteristic of secondary humoral responses (54). Their presence in serum is still detected by the time of clearance from infection (2-3 weeks after infection) (49). Secretory IgA is present in the mucosal layers of the gut and conjoin with antimicrobial peptides as a first line of defense against pathogens (48).

Transforming growth factor β (TGF- β) plays a role in Treg cell differentiation, and Th17 polarization along with IL-6. Treg produce TGF- β and IL-10 and participate in immune response regulation. Th17 produce IL-17 and IL-22, and it is involved pathogen extracellular attack (52). Other T cells lineages, such as Th22, Th9, and follicular T helper cells (TFh), have not been functionally and phenotypically described in chickens, although related components have been identified (55). The intricate interactions between immune cells and their secreted molecules entails a specific response toward targeted pathogens (Figure 1).

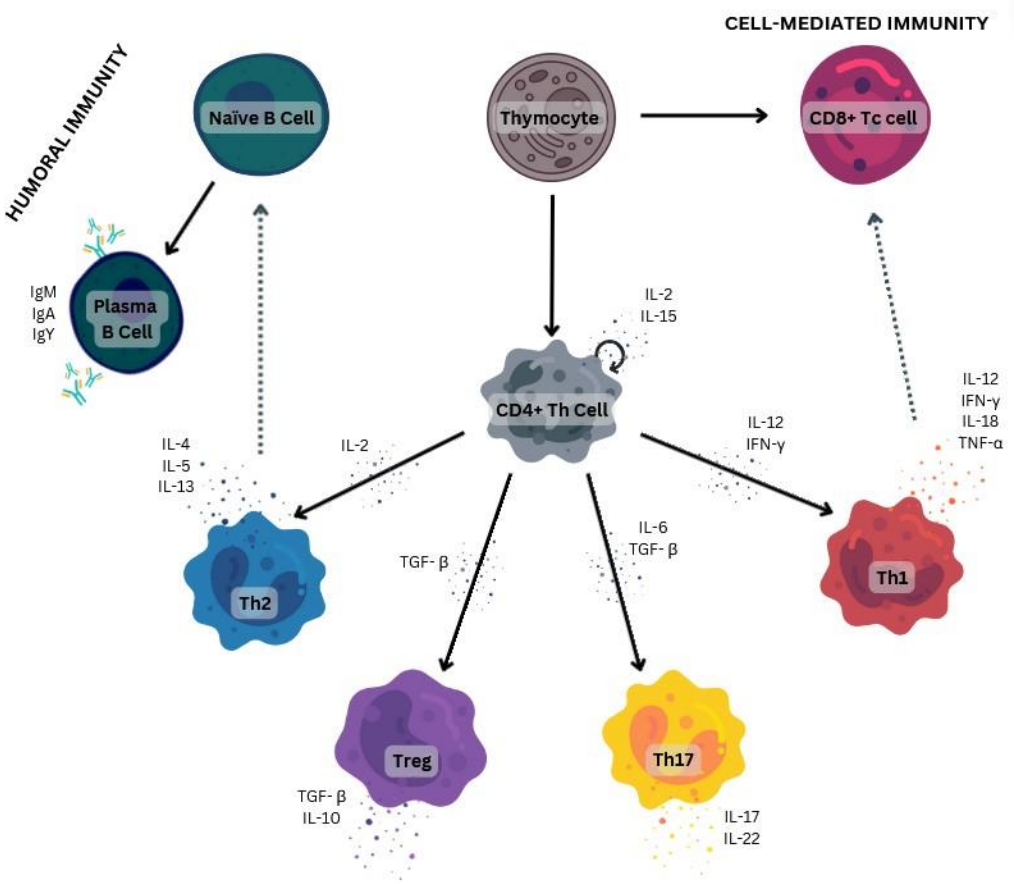

Figure 1. Development of adaptive immune responses. The deployment of the humoral and cell-mediated responses of the adaptive immune system is regulated by the activation and differentiation of lymphocytes. Cytokines are crucial molecules during differentiation of the T helper (Th) lineages: Th1, Th2, Th17, and T regulatory (Treg) cells. Differentiated lineages are capable of producing their own effector molecules and elicit a humoral response in B cells and the cytotoxic activity of T Cytotoxic (Tc) cells. Adapted from Bean and Lowenthal, 2022 (52).

The innate and adaptive immune responses can unfold into three possible outcomes, detailed in Table 1.

Table 1. Consequences of the immune system responses in *Salmonella* infection. The deployment of the humoral and cell mediated activity can lead to three different outcomes. Adapted from Wigley, 2014 (47).

Outcome	
Clearance	• After 2-4 weeks of infection, cellular and antibody responses achieve systemic clearance. • Th1 activity leads to intestinal clearance at around 3-12 weeks post infection.
Persistence	• Adapted serovars are able to survive intracellularly. • Immunosuppression in birds cause occasional recrudescence of infection.

Death	• Replication inside macrophages cannot be controlled, resulting in bacteriaemia.

The presence and composition of the gut microbiota in young birds affects morphological features of their gastrointestinal tract, along with its functionality (48,56). It has been reported that in germ-free reared chickens, there is a reduction in lymphoid tissue concerning the intestinal tract, demonstrating that microbial exposure is required for a correct development and maturation of the immune system. Similarly, other factors of the innate and adaptive response were affected by this condition: lowered mucin production with altered chemical composition, reduced presence of antimicrobial peptides in the gut, absence of IgA in the gut and serum, and the absence of T cells in GALT (48). These effects have been reversed by performing fecal microbial transplants from healthy adult birds into newly hatched chickens (57), supporting the importance of the gut microbiota for the proper development of the immune system.

2. PROBLEM STATEMENT

Salmonella is one of the key causes of foodborne diseases around the world, adding up to billions of infections and more than one hundred thousand deaths each year (11). *Salmonella* Infantis is the most predominant serological variation in our country, plus noteworthy antimicrobial resistance patterns are associated to this serovar (16,21). However, there is not plenty of information regarding this serovar compared to other *Salmonella* variations that are more widely distributed around the world and affect developed nations (*S.* Enteritidis, *S.* Typhimurium). Especially regarding immunological aspects associated to the infection, such as receptor activity and signaling, cytokine expression, immunoglobulin production, etc.

Administration of lactic acid bacteria (*Lactobacillus, Enterococcus, Bifidobacterium*) as probiotics in the diet of broiler chickens is a current approach in the poultry industry (33). One of the main reasons for probiotic use is their immunomodulatory properties, including the enhancement of immune responses in the gut (41).

We hypothesized that the infection with *S*. Infantis is capable of altering the cytokine expression and immunoglobulin levels in broiler chickens. In addition, we speculated that the immunomodulatory effects of *Lactobacillus fermentum* are capable of regulating the same parameters when supplemented as probiotics in the diet. Consequently, mRNA abundance of caecal *IL1B*, *IL13*, *IL15*, *IL17*, and *MYD88*, along with IgA and IgM levels in the serum were assessed in 14-day old broiler chickens. Assessment of gene expression would provide information regarding different lines of action (namely, signaling pathway, initial inflammation, Th17 and Th2 pathways, etc.) of the immune system in the birds and how they are influenced during salmonellosis and by the presence of probiotics.

3. OBJECTIVES

3.1. General Objective

To evaluate the influences of *Salmonella* Infantis infection and of the probiotic supplementation of *Lactobacillus fermentum* on the immunological responses in the gastrointestinal tract of broiler chickens.

3.2. Specific Objectives

- To assess and compare the expression levels of immune-related genes (*IL1B*, *IL13*, *IL15*, *IL17*, and *MYD88*) in caecal tissue.
- To determine the concentration of immunoglobulins (IgA and IgM) in the serum of the birds.

4. METHODOLOGY

4.1. Ethics Statement

All experimental procedures were performed following the guidelines for animal management specified by the Agencia de Regulación y Control Fito y Zoosanitario (AGROCALIDAD, technical resolution n. 0017). The study was approved by the Comité de Ética en el Uso de Animales en Investigación y Docencia of the Universidad San Francisco de Quito (USFQ) (reference number: 2020-008).

4.2. Bacterial Strains and Determination of Colony Forming Units (CFU)

Lyophilized *L. fermentum* strain CCM7514 (supplied by the Czech Culture of Microorganisms, Brno, Czech Republic), from the intestine of domestic chickens, was resuspended in 1 mL saline solution, and grown at 37 °C for 48 h in De Mann–Rogosa–Sharpe (MRS) agar (Merck, Germany) at pH 5.65 inside an anaerostate (BBL GasPak Plus, Albany, NY, USA). After incubation, positive colonies presented a white coloration (Figure 2). Six colonies from the plate were selected and inoculated in 50 mL of MRS broth, which were incubated at 37 °C for 24 h. Subsequently, 450 mL of MRS broth were added to the culture repeating the incubation step. Afterwards, centrifugation of the culture was performed at 4 °C, 2268 × g for 45 min. Resuspension of the sediment was carried out in 50 mL of saline solution and determination of the number of bacteria was managed by preparing decimal dilutions. Culture and recovery protocols were conducted as previously described by Šefcová et al. (45).

Figure 2. Positive growth of *Lactobacillus fermentum* in MRS agar.

Strain U1068s of *Salmonella enterica* subsp. *enterica* serovar Infantis (*S.* Infantis), isolated from chicken caecal content, was obtained from the culture collection of Unidad de Investigación de Enfermedades Transmitidas por Alimentos y Resistencia a los

Antimicrobianos (UNIETAR). Reconstitution was carried out on Xylose, Lysine, Deoxycholate (XLD) differential selective medium at 37 °C for 24 h. Positive selection produced colonies with a black center with slim clear borders (Figure 3). For generation of biomass, a typical colony was incubated in buffered-peptone water (as liquid culture) at 37 °C for 18-24 h under constant agitation. Recovered biomass was centrifuged at 500 × g for 45 min for concentration. Resuspension of the sediment was carried out in saline solution (NaCl, 5%) until obtaining an OD600 of 1.0. Culture and recovery protocols were conducted as previously described by Corrales-Martinez et al. (58).

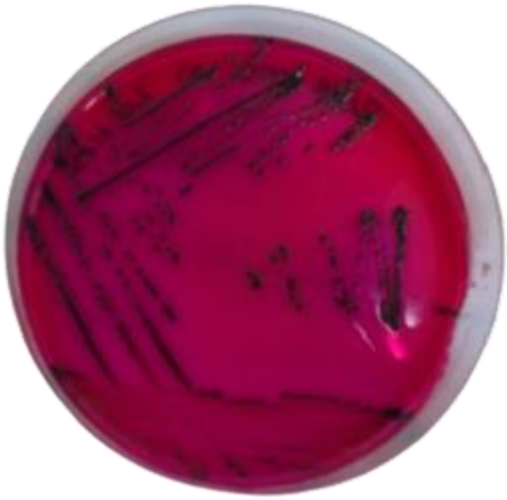

Figure 3. Positive growth of *S.* Infantis in XLD agar.

4.3. Experimental groups and housing

A total of 200 1-day-old broiler cock chickens (COBB 500) were obtained from Procesadora Nacional de Alimentos C.A. (PRONACA). Upon arrival, chickens were weighed and randomly distributed into four experimental groups, making sure that weight was similar in all of them. The birds were classified into groups as follows: control group, where birds were left untreated (C); probiotic group, where chickens were treated with *L. fermentum* (Lf); challenged group, where chickens were infected with *S.* Infantis (Se); and a mixed group in which birds were treated with both bacteria (LfSe). A suspension of the probiotic (10^9 CFU per 0.2 mL) was administered to the birds in groups Lf and LfSe during the first seven days of the experimental period. A suspension of *S.* Infantis (10^7 CFU per 0.1 mL) was orally administered to the chickens in groups Se and LfSe on day 4. Saline solution (0.2 mL) was applied orally to chickens in the control group at each stage of the treatment. The experiment was conducted for a total of 15 days in the Centro Experimental de Investigación Animal of the Facultad de Medicina Veterinaria y Zootecnia of the Universidad Central del Ecuador, located in the parish of Uyumbicho (23 km southeast of Quito, Pichincha).

Each experimental group was assigned to a separate pen of 3 m × 3 m and divided into five subgroups (denominated as I, II, III, IV and V) of ten chickens each (1.5 m × 1 m). Hardwood shavings were used to cover the floor of the pens. For molecular analysis, caecal samples were collected from 10 animals, two per subgroup (n=10); for serological analysis, blood was collected from 8 animals, two selected from subgroups I, II, and III and one selected from subgroups IV and V (n=8). The animal was considered the experimental unit (EU) as individuals were independently assigned to treatment conditions and experimental interventions; furthermore, EU could not alter the measured outcomes of each other (59).

Chickens were provided with commercial feed, both starter (from day 0 to 8) and grower diets (from day 9 to 18), with no antibiotics, probiotics, or coccidiostats (60). Diet components are detailed in Table 2. Birds had constant access to feed and water *ad libitum* for the entire experimental period. Relative humidity was kept around 50 to 70%. A regime of continuous light (intensity 30–40 Lux) was implemented for the first 24 hours of the experiment. From day 2, until chickens weighed between 130 and 180 g, the light regime shifted to 23 hours of light and 1 hour of dark. Afterwards, a regime of 18 hours of light (intensity 5–10 Lux) followed by 6 hours of dark was maintained until the end of the experiment. Temperature was kept between 30 to 32 °C for the first week; from day 7, it was decreased by 2 °C per week. At day 7, it was kept at around 28–30 °C and at day 14 at around 25–27 °C. Housing conditions were followed as recommended by the COBB 500 Management Guide (61). On day 15 (11 days post-infection, dpi) birds were sampled for blood and then electrically stunned and euthanized by bleeding followed by caecal collection.

Table 2. Components of COBB 500 feed for starter (from days 0 to 8) and grower (from days 9 to 18) diets of broiler chickens.

Components (%)	Diet	
	Starter	**Grower**
Antimycotic	0.10	0.08
Antioxidant	0.02	0.02
Calcium carbonate	1.52	1.49
Crude vegetal fat	7.4	7.71
Ground corn	52.99	57.23
Monocalcium phosphate	1.07	0.82
Mycotoxin Sequestrant	0.05	0.05
Phytase	0.01	0.01
Sodium chloride	0.31	0.26
Soybean meal	36.3	32.07
Vitamin and mineral premix*	0.23	0.26

* Per kg of basal diet: Vitamin A 1 0,000 IU; vitamin D3 5000 IU; vitamin E 80 IU (starter), 50 IU (grower); vitamin K 3 mg; vitamin B1 3 mg (starter), 2 mg (grower); vitamin B2 9 mg (starter), 8 mg (grower); vitamin B6 4 mg (starter), 3 mg (grower); vitamin B12 0.02 mg (starter), 0.015 mg (grower); Biotin 0.15 mg (starter), 0.12 mg (grower); Pantothenic acid 15 mg (starter), 12 mg (grower); Folic acid 2 mg; Mn, 100 mg; Zn, 100 mg; Fe, 40 mg; Cu, 15 mg; I, 1 mg; Se, 0.35 mg.		
Nutrient specifications		
Metabolizable energy Kcal/kg diet	2975	3025
Available phosphorus (%)	0.45	0.42
Calcium (%)	0.90	0.84
Chlorine (%)	0.22	0.19
Choline (mg/kg)	500	400
Crude protein (%)	21.50	20.00
Digestible Arginine (%)	1.28	1.18
Digestible Isoleucine (%)	0.77	0.72
Digestible Lysine (%)	1.22	1.12
Digestible Methionine (%)	0.46	0.45
Digestible Methionine + Cysteine (%)	0.91	0.85
Digestible Threonine (%)	0.83	0.73
Digestible Tryptophan (%)	0.20	0.18
Digestible Valine (%)	0.89	0.85
Linoleic acid (%)	1.00	1.00
Potassium (%)	0.95	0.72
Sodium (%)	0.23	0.16

4.4. Serological analysis

Blood samples (2 ml) were obtained from the brachial vein into 4 mL vacuum tubes for a maximum of two minutes, they were maintained at room temperature for 120 minutes, and later stored overnight at 4 °C. Centrifugation for serum separation was performed at 2500 × g at 4 °C for 10 minutes, which was then stored at -80 °C (62).

Detection of immunoglobulin A (IgA) titers in serum samples was performed by ELISA (Enzyme-Linked Immunosorbent Assay) following the instructions provided by manufacturer (IgA Chicken ELISA kit, Abcam, Cambridge, UK). First, the 96 wells of the microplate were designated for standards, controls, and samples: all of these in duplicates. Samples were thawed at room temperature, and then properly diluted (1:5000) with a previously prepared diluent solution (DS). 100 µL of each dilution were added to the respective wells. A standard curve was used to quantify the concentrations of IgA in samples. An IgA standard from the kit was serially diluted in DS to obtain the following concentrations: 400, 200, 100, 50, 25 and 12.5 ng/mL; DS alone was used as a blank control. The plate was incubated for 20 minutes at

room temperature. Following incubation, four washing step were carried out using 1X Wash Buffer. 100 µL of the Enzyme-Antibody Conjugate were added into the wells and incubation was performed for 20 minutes in the dark. Then, the washing steps were repeated, and 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) Substrate Solution was administered into each well. The microplate was incubated for ten minutes. Subsequently, 100 µL of Stop Solution was added. Absorbance was determined at 450 nm in a Multiskan EX microplate reader (Thermo Scientific, Waltham, MA, USA).

Similarly, detection of IgM titers was performed, in duplicates, by ELISA following the provided protocol (IgM Chicken ELISA kit, Abcam, Cambridge, UK). First, the 96 wells in the microplate were designated for standards, controls, and samples. Samples were thawed at room temperature, and diluted (1:2000) as described for the IgA detection. A standard curve was used to quantify the concentrations of IgM in samples; a chicken IgM standard was serially diluted in 1xDS to obtain the following concentrations: 400, 200, 100, 50, 25, 12.5 and 6.25 ng/mL. 1xDS was used as the blank control. Standard dilutions were added to the respective wells (100 µL/well). The microplate was incubated for 30 minutes at room temperature. Four consecutive washes were conducted by treating the wells with 1X Wash Buffer. The Enzyme-Antibody Conjugate was added to the wells (100 µL), followed by a 30-minute incubation period in the dark. The washing steps was repeated. Then, 100 µL of TMB Substrate Solution was added to the wells and the microplate was incubated for ten minutes. After incubation, 100 µL of Stop Solution was added, and absorbance was assessed in a microplate reader at 450 nm (Thermo Scientific, Waltham, MA, USA). Duplicate values for each standard, sample, and control were averaged. Subsequently, the control was subtracted from all readings. The concentration of both IgA and IgM was calculated using a standard curve developed with GraphPad Prism 9 Software (San Diego, CA, USA) (see Appendix A for details).

4.5. RNA extraction and cDNA synthesis

Samples from the caecum were taken from selected birds (n = 10) and stored in RNA-later (Thermo Scientific, Waltham, MA, USA) at -80 °C. For RNA extraction, tissues were thawed and homogenized by manual grinding in 1 mL of TRIzol (Invitrogen, Waltham, MA, USA) for around 10 minutes. The extracts were stored for 10 minutes at -20 °C. Later, 50 µL of 4-bromoanisole (Molecular Research Center, Cincinnati, OH, USA) were added to the tubes; shaking is required for appropriate mixing. Then, centrifugation was carried out at 12000 rpm for 15 minutes. Extracted RNA was precipitated and purified using the AccuPrep Universal RNA Extraction Kit (BioNeer Corporation, Daejeon, Republic of Korea) following the instructions supplied by the manufacturer. The concentration and quality of RNA samples were measured in a NanoDrop One spectrophotometer (Thermo Scientific, Waltham, MA, USA),

and later diluted to obtain a standard concentration of 25 ng/µL. The RNA samples were stored at -80 °C until use.

The synthesis of cDNA was carried out using the OneScript Plus cDNA Synthesis Kit (Applied Biological Materials Inc., Vancouver, Canada), following the instructions provided by the manufacturer. The synthesis reactions were performed by incubating the mix at 55 °C for 15 minutes, and later stopped by heating at 85 °C for 5 minutes and kept on hold at 4 °C. All reactions were performed in a MultiGene™ OptiMax Thermal Cycler (Labnet International Inc., Edison, NJ, USA). The obtained cDNA was maintained at -20 °C.

4.6. Real-time reverse transcriptase polymerase chain reaction (RT-qPCR)

Expression levels of cytokines *IL1B, IL13, IL15*, and *IL17*, along with an innate immune signal transduction adaptor (*MYD88*), were assessed by RT-qPCR, using a Forget-Me-Not EvaGreen qPCR Master Mix (Biotium Inc., Fremont, CA, USA). The expression of *GAPDH*, a housekeeping gene encoding for the glyceraldehyde-3-phosphate dehydrogenase enzyme, was also measured. Primer sequences are detailed in Table 3.

Cycling conditions included an initial denaturation step at 95 °C for 15 minutes, followed by 45 amplification cycles consisting of 20 seconds of denaturation at 95 °C, 30 seconds of annealing (in a range from 60 to 63 °C, see Table 3), and elongation at 72 °C for 30 seconds. A melting curve ranging from 55 °C to 95 °C with readings at every 0.5 °C increment was developed. Amplification and quantification were performed using the Eco™ Real-Time PCR System and Eco Study Software (Illumina, San Diego, CA, USA). Amplification efficiencies for all primer sets were assumed as equal and at 100% by the software (63). Each sample was subjected to qPCR in duplicates and values were averaged for further analyses. The Ct values of the studied genes were normalized to the computed Ct values of the housekeeping gen and expressed as $2^{-\Delta\Delta Ct}$ (see Appendix B for details) (64).

Table 3. Primer sequences used for RT-qPCR.

Gene	Primer sequence (5'–3')	Annealing Temperature (°C)	Reference
GAPDH	F: CCTGCATCTGCCCATTT	60	(65)
	R: GGCACGCCATCACTATC		
IL1B	F: GAAGTGCTTCGTGCTGGAGT	60	(66)
	R: ACTGGCATCTGCCCAGTTC		
IL13	F: ACTTGTCCAAGCTGAAGCTGTC	60	(67)
	R: TCTTGCAGTCGGTCATGTTGTC		

IL15	F: TGGAGCTGATCAAGACATCTG	60	(68)
	R: CATTACAGGTTCCTGGCATTC		
IL17	F: TATCAGCAAACGCTCACTGG	60	(66)
	R: AGTTCACGCACCTGGAATG		
MYD88	F: TGAAGCAGCAGCAGGAGGCA	63	(69)
	R: TCGCTGGGGCAGTAGCAGATGA		

4.7. Statistical analysis

Statistical analyses were carried out using MATLAB, version 9.9.9341360 (MathWorks, Natick, MA, USA) (R2016a). Assessment of normality was performed with the Shapiro-Wilk's test, and homogeneity of variance was determined with Levene's test. For homoscedastic and normally distributed data, a one-way ANOVA along with a Tukey post hoc test was performed to determine differences between groups. Welch's analysis of variance and Welch's t-test were utilized for heteroscedastic and normally distributed data. When homoscedastic data followed a non-normal distribution, the Kruskal-Wallis test and the Mann-Whitney U test (Wilcoxon rank sum test) were employed. In the latter cases, medians were used to the depict the center of distribution. Significance was set at $P < 0.05$.

5. RESULTS

5.1. Gene expression in caecum

The expression levels of immune-related genes (*IL1B, IL13, IL15, IL17,* and *MYD88*) in caecal tissue of broiler chickens inoculated with *Lactobacillus fermentum* (Lf) and *Salmonella* Infantis (Se) separately, and with both bacterial species (LfSe), at 11 days post-infection were determined by RT-qPCR. No significant changes were detected in the expression levels of the genes of interest in response to any of the treatments applied in this study in comparison with the untreated controls ($P > 0.05$). Relative expressions of the genes of interest are presented in Figure 4.

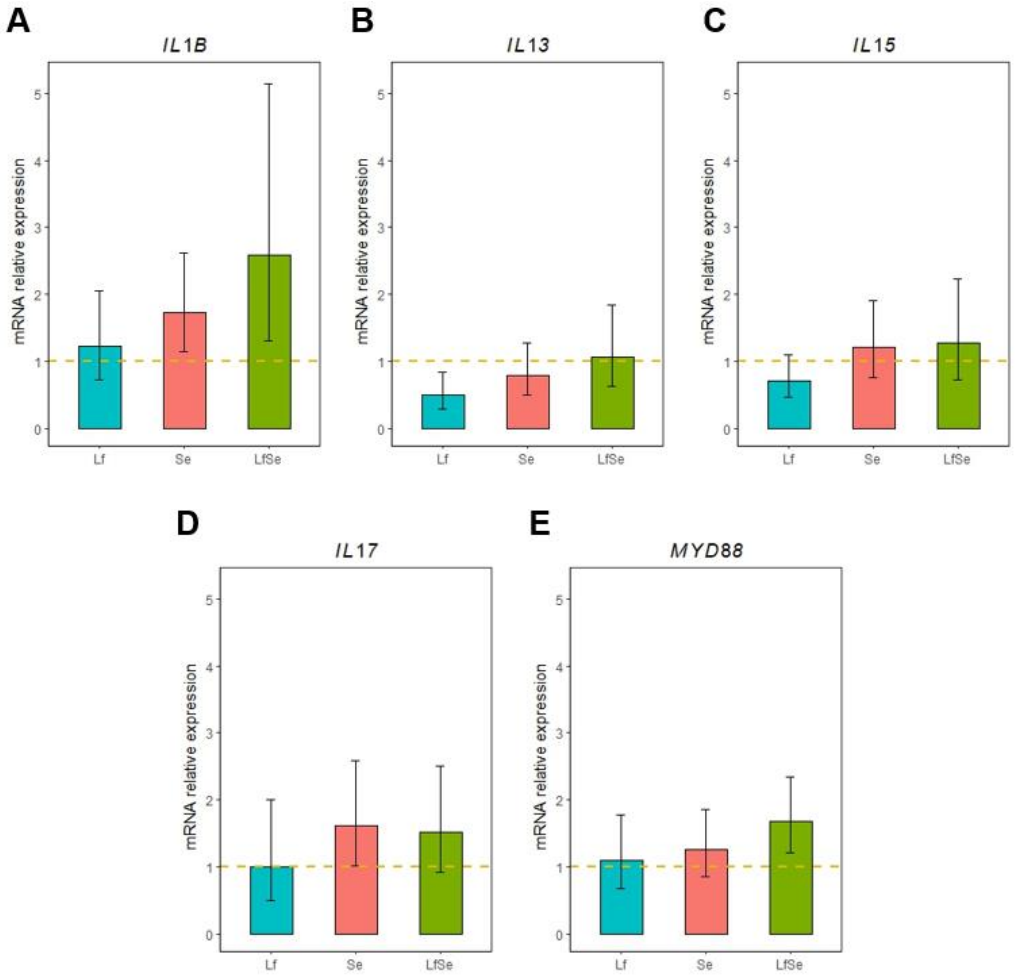

Figure 4. Interleukins and adaptor protein gene expression in caecum of broiler chickens at 11 dpi. Data are presented as *n*-fold changes in transcription levels of the studied genes normalized to a reference gene in inoculated birds relative to untreated controls. Relative expression was calculated and expressed as $2^{-\Delta\Delta Ct}$, error bars present SE (n = 10). Dashed lines represent the reference value from controls. Lf, probiotic group; Se, challenged group; LfSe, mix group; dpi, days post-infection; SE, standard error.

Expression of *IL1B* (Figure 4A) reported an upregulation of 2.59-fold when both *L. fermentum* and *S.* Infantis were inoculated compared to untreated birds, while the inoculation of the bacteria separately reported slighter increases of expression levels (1.23- and 1.73-fold changes for the Lf and Se groups, respectively).

In the same fashion, relative expression of both *IL17* (Figure 4D) and *MYD88* (Figure 4E) genes presented slight, non-significative changes compared to controls. Expression of *IL17* is somewhat higher in the Se group (1.62-fold change), than in the co-exposure group LfSe (1.52-fold change). The fold changes for the expression of MYD88 were 1.25 for the challenged chickens (Se), and 1.68 for chickens inoculated with both bacteria (LfSe). Interestingly, for both of these genes the computed $2^{-\Delta\Delta Ct}$ values were as close to the reference values from the untreated control (1.00 for *IL17*, and 1.07 for *MYD88*) in the Lf groups, where *L. fermentum* was administered alone.

In the case of the expression levels of *IL13* (Figure 4B), downregulations were reported for both the Lf (0.49-fold) and Se (0.79-fold) groups; while the expression levels were brought back to normal values compared to controls in the co-exposure group, LfSe (1.07-fold).

There is a downregulation in the relative gene expression of *IL15* (Figure 4C) when *L. fermentum* is supplemented as a probiotic by itself (0.71-fold change). Contrastingly, when broiler chickens are inoculated with *S.* Infantis, both alone (Se) and together with the probiotic (LfSe), the relative gene expression in caecal tissue reveals a slight similar upregulation for both treatment groups (1.20- and 1.28-fold changes, respectively).

5.2. Antibody-mediated immune responses

The antibody-mediated immune responses after the treatments (Control, Lf, Se, LfSe) conducted on broiler chickens were assessed by conducting ELISA on serum samples collected at 11 days post-inoculation. Antibody concentrations are presented in Figure 5. No differences were found among the experimental groups regarding the IgA concentrations in sera (Figure 5A). However, in the co-exposure group (LfSe) the concentration of IgM increased when compared to the control, the probiotic group (Lf), and *Salmonella*-challenged (Se) group ($P < 0.05$) (Figure 5B).

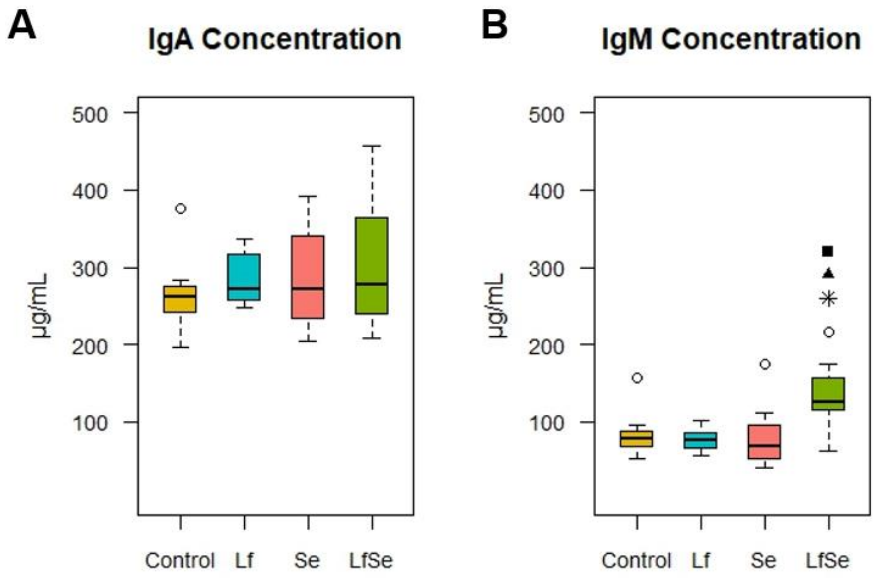

Figure 5. Concentration of chicken immunoglobulins detected in serum at 11 dpi. Serum levels of IgA (A) and IgM (B) among experimental groups. Values are medians plus their corresponding interquartile range (IQR) (n = 8). * indicates significant differences with the control group; ■ with the Lf group; ▲ with the Se group. Lf, probiotic group; Se, *S.* Infantis-challenged group; LfSe, mix group; dpi, days post-infection. Circles denote the outliers.

6. DISCUSSION

Most cases of gastroenteritis in humans are caused by infection of a broad range of *S. enterica* serotypes, commonly known as non-typhoidal or generalist serovars. This foodborne disease is often related to consumption of contaminated poultry products (e.g., chicken meat, eggs). These serovars can cause a systemic disease in chicken with minor to no symptoms, therefore inhabiting their gastrointestinal tract without alert (70). In this way, these apparently normal, healthy birds are allowed to enter the human food chain leading to cases of food poisoning.

While there are many studies involving the analyses of gene expression after *Salmonella* infections in chicken, these are often focused on certain serovars with higher distributions in developed countries, such as *S.* Enteritidis and *S.* Typhimurium (8,71). Other serotypes in the same group of non-typhoidal strains of *Salmonella* include *S.* Hadar and *S.* Infantis, which are less virulent than the previously mentioned serovars but superior regarding gut colonization (50). *S.* Infantis represent a public health risk for the Andean region, especially due to its high presence in the local poultry industry. For instance, the prevalence of *S.* Infantis in Ecuador at a farm level has been determined to be around 80% (16), with the presence of resistant traits to various antimicrobials (19,20). Currently, no information is available regarding caecal inflammatory cytokine expression in response to a *S.* Infantis infection. Similarly, no studies have reported the levels of IgA and IgM in response to this serovar. Moreover, no data have been revealed concerning the use of probiotics to modulate the physiological effects of *S.* Infantis.

Upon interaction with *Salmonella*, epithelial cells from the gut initiate an immune response by producing an array of cytokines and chemokines that activate macrophages, dendritic cells, and granulocytes into the site of infection (48). This leads to the activation of other specific immune cells and antibody production, which induce a stronger reaction to eliminate pathogens (8). Although all the generalist serovars causing non-typhoidal salmonellosis (NTS) deploy similar pathogenesis in chicken, the intensity of the mechanisms involved in the immune responses elicited within the gut-associated lymphoid tissues (GALT) varies depending on the serovars (50); since they involve different biological and pathological features in interaction with the host (70). Gene expression analyses are employed to assess the evolution of diseases and host-pathogen interactions during salmonellosis. These analyses include expression of cytokines (e.g *IL1B, IL6, IL15, IL17*), interferons (*IFN*), lipopolysaccharide-induced tumor necrosis factor α (*LITAF*), transforming growth factors (*TGF*), chemokines (*IL8L1, IL8L2*), along with factors involved in inflammatory processes, including nitric oxide synthase (*iNOS*) (66,68,70).

The present study aimed to understand the fluctuations of gene expression after *S.* Infantis infection by evaluating the transcription abundance of four cytokines: IL-1β an important pro-inflammatory cytokine, IL-13 a Th2 cytokine implicated in antibody production, IL-15 involved in the maturation of T lymphocytes and natural killer (NK) cells, and IL-17 a key molecule for the Th17 immune response involving monocytes and heterophils, and the myeloid differentiation primary response (MYD88) gene, which encodes for a signal transduction adaptor in the innate immune response (72,73). Previous studies have reported that administration of *Lactobacillus* strains in chickens has elicited positive immunomodulatory effects during pathogenic bacteria infection (74), thus we tested the influence of *L. fermentum* administration on gene expression in *S*. Infantis challenged chickens. In particular, *L. fermentum* has demonstrated to be useful in reducing the negative effects triggered by *Campylobacter jejuni* and *C. coli* in broiler chickens (44,45). The probiotics have improved the inflammatory reactions along with stimulating intestinal architecture. By mediating the expression of modulatory cytokines, physiological responses to *C. jejuni* infection such as body weight loss were counteracted (44,75). The administration of the probiotic also influenced the morphometry of the intestine of the chickens, as the villi height and crypt depth increased, albeit these parameters are often reduced in *C. jejuni* infected chickens (75). In *C. coli*-challenged birds treated with *L. fermentum*, the presence of intraepithelial CD8 lymphocytes increased in the lamina propria even with the downregulation of pro-inflammatory cytokines, interestingly; along with the increased population of plasma B cells (IgM and IgA), which also increased in the epithelium (45).

The results obtained herein reveal that the administration of *L. fermentum* did not alter expression of the studied genes in *S.* Infantis-challenged chickens. This suggests that the influence of *L. fermentum* were not sufficient to modify the host-pathogen interaction and influence inflammation. The probiotic did not alter the expression of these genes when administered in isolation (Lf group). At present, there are no studies reporting gene expression in broiler chicken infected with *S.* Infantis alone or in combination with probiotics. However, various studies have shown the utility of probiotics in the context of a *Salmonella* infection. For example, administration of a mix of *Lactobacillus* spp., containing *L. fermentum*, in *S.* Enteritidis-challenged birds influenced the innate immune reaction by decreasing inflammation and preventing the progression of pathogen colonization in the gut, which is accompanied by a downregulation of pro-inflammatory cytokine genes such as *IL1B*, *IL6*, and *LITAF* (76). Similar conditions revealed that the ability of the pathogenic bacteria to adhere to chicken intestinal mucosa was considerably reduced by *Lactobacillus* spp., including *L. fermentum*, in cases of infection with *S.* Enteritidis and *S.* Infantis (77,78). Nonetheless, the expression on *IL1B* was not altered when *L. fermentum* was administered during the infection of chicken

peripheral mononuclear blood cells (PMBC) with *S.* Enteritidis (79). In infection with *S.* Typhimurium, the administration of four species of *Lactobacillus* (*L. fermentum* included) downregulated the expression of *IL1B* and decreased the presence of *Salmonella* in the liver, spleen, and caecum (41). Incidentally, when compared to other *Lactobacillus* species, the immunomodulatory effects of *L. fermentum* during infection seem to have milder effects (77).

The effects of this probiotic have also been tested in the context of other pathogenic infections. *L. fermentum* inoculation led to a reduction of inflammation while also improving the immune responses in birds challenged with *C. coli* and *C. jejuni* (44,45,75). For both *Campylobacter* species, the expression of *IL15* increased in infected chicken but was reduced when combined with *L. fermentum*. In *C. coli* infection, an increased expression of *IL13* was reported both for challenged chickens and infected chickens inoculated with the probiotic, where the increment was more evident. In contrast, when *L. fermentum* is administered as a probiotic in *C. jejuni*-infected chickens, the expression of *IL17* is downregulated. In terms of physiological parameters, the administration of probiotics also counterbalanced the loss of weight caused by these pathogenic bacteria (44,45,75). In *Clostridium perfringens*-challenged broiler chickens, *L. fermentum* reduced expression of *IL1B* in the jejunal mucosa, also it inhibited gut colonization and reduced intestinal injury during the acute phase of infection. No significant changes were reported for *IL13*. Similarly, no changes have been reported for *IL17* during the first seven days post infection, then their expression increased along with the abundance of *IL1B* expression, when compared to control conditions (46). Downregulation of other pro-inflammatory cytokines, such as *IFNG*, in similar experimental conditions has been also reported (80).

Evidently, the use of *L. fermentum* as a probiotic in challenged birds do not always influence gene expression similarly. For instance, in the case of pro-inflammatory cytokines, some are upregulated, *IL1B*, while others are downregulated, *IFNG*, in animals infected with *C. perfringens* and *Salmonella* (75,80). Some cytokines (IL-1β) have been observed to remain unaltered right after exposure to *Campylobacter* species, but downregulated as infection progresses (44,75). This suggests that the immunomodulatory effects are dependent on the progress of the disease and, more importantly, on the biological features of the pathogen. Thus, it is also important to review the immune dynamics occurring in broiler chickens following *S.* Infantis infection alone.

No changes regarding gene expression were observed in *S.* Infantis-challenged animals either. Clearly, *S*. Infantis does not seem to alter the expression of immune related genes in chickens. An in vitro study followed the immune responses in cell cultures of chicken kidney epithelial cells (CKC) and chicken macrophage-like cells (HD11) after infection with the most

representative serovars of *S. enterica*, including *S.* Infantis. Transcription levels of some genes (*IL6*, *IL8*, *IL10*, and *iNOS*) in *S.* Infantis-challenged chicken were reported to be the lowest among various serovars (*S.* Enteritidis, *S.* Typhimurium, *S.* Hadar, *S.* Gallinarum, and *S.* Pullorum); while expression of other genes were not altered (*IL4*, *LITAF*, and *IFNG*) or expressed at all (*IL13*) (50). When investigating the expression of the same genes in caecal tonsils of newly hatched and 3-week-old broilers, no differences were found between genes and other were not expressed (*IL13* and *IL10*) (70). No more information is available with regard to *S.* Infantis infection in birds.

Infection with *S*. Typhimurium in CKC cultures reported no differences in *IL1B* expression when compared to control cells (4). Comparable results were observed during infection with *S.* Enteritidis, where *IL6* seemed to play a role in eliciting inflammation, as it was found to be overexpressed (81). It has been observed that *IL15* expression in caecal samples of birds, infected with *S.* Enteritidis, were not altered when compared to unchallenged birds, while transcription of *IL17* was increased (66). Moreover, expression of *MYD88* was not affected in *S.* Typhimurium-challenged birds (82), while its expression during a *S.* Enteritidis infection was augmented (83).

A study correlating cytokine and chemokine expression with the invasiveness of intestinal tissues in the most prevalent NTS serovars (*S*. Enteritidis, *S*. Typhimurium, *S*. Hadar, and *S*. Infantis) concluded that *S*. Infantis is the weakest immune stimulator, and least invasive in comparison to the other *Salmonella* variations (84,85). However, *S*. Infantis was found to be able to survive in epithelial cells for as long as other serovars do (85). According to our results neither the infection with *S*. Infantis nor the administration of *L. fermentum* were able to influence any change in the expression of the reviewed genes. It is often suggested that this ability to suppress inflammatory responses is a bacterial strategy for the systemic invasion of the birds. This is not exhibited in other serovars, like *S*. Pullorum and *S*. Gallinarum that cause typhoidal diseases in chicken, characterized by a high mortality rate (50). The typhoidal *Salmonella* serovars are usually characterized by lower bacterial counts in the ceca and great changes in the expression of cytokines and chemokines, and therefore strong inflammation and disease (70). It could be argued that the severity of disease is not determined by the size of the bacterial population in the gut, but by the pathogenic features related to the infecting serovar instead.

It has been previously reported that administration of probiotic bacteria, like *L. fermentum*, is able to stimulate the production of antibodies and therefore modulate the immune response (86). For this reason, the present study also assessed the presence of antibodies (IgA and IgM) in the serum of chickens exposed to experimental conditions. While infection with *S.*

Infantis, as well as *L. fermentum* administration, did not induce changes in IgA and IgM production by themselves, co-administration triggered an increase in IgM concentration. Interestingly, *L. fermentum* has been observed to increase the level of IgM in caecum, which were observed to be correlated with *IL13* expression (45). Here, we have determined that the probiotic is able to increase IgM concentration in the serum. Immunoglobulins are known for being present in the sera for different periods according to the type. IgM can still be detected during the immune clearance stage, of a *Salmonella* infection, which takes place between 14- and 28-days post inoculation (81). No more information is available concerning antibody production during a *S.* Infantis infection in broiler chickens. In infection with *S.* Enteritidis, no significant differences were reported in IgM production (87).

Antibody-mediated responses in *Salmonella* infection in broiler chickens seems to work in a time-dependent manner. The classical pattern of antibody production after *Salmonella* primary infection describes an increase in IgM levels in serum shortly after inoculation, reaching its peak at 13 days post-inoculation followed by a decrease in concentration (88). Levels of IgA in serum go through a slighter increase after first contact with *Salmonella*, but its main role pertains to mucosa-associated responses during reinfections (89). Together with IgA, IgY concentration levels in serum significantly increase at day 21 after first inoculation with prolonged presence (89). The temporal dynamics of serum antibody following *Salmonella* infection previously described correlates to our results, regarding the presence in serum of IgM only, at 11 dpi. However, in our study this is not true for *S.* Infantis infection by itself, but for co-exposure of broiler chickens to *S.* Infantis and *L. fermentum*. We contemplate that this is due to the poor immune stimulator effects related to *S.* Infantis, which have been also previously described (84,85). Furthermore, this study demonstrate that the coexistence of both species is capable of eliciting innate immune responses in the gut of broiler chickens leading to antibody production, compared to unaltered humoral responses when administered independently.

Our study included IL-13 as a Th2 cytokine, in order to detect any influence in gene expression that could be later related to altered immunoglobulin production. While IgM concentration in serum was significantly increased in the co-exposure group (LfSe), no changes were reported in the relative expression of *IL13* for any of the treatments. We speculate that the reported increase in IgM levels might involve the participation of a different Th2 effector cytokine (e.g., IL-4, IL-10) or might have followed a different pathway leading to Ig production. A temporal aspect might be also related to this matter: while the influences in cytokine expression can quickly fluctuate in time, antibody responses can be detected for a prolonged period.

7. CONCLUSIONS

Gut microbiota and its composition play a key role in the development of immunological functions of the gastrointestinal tract of broiler chickens. Probiotic supplementation has been widely used for reducing intestine inflammation induced by unwanted bacteria and as a modulator of the immune system responses. Although, in this study no changes were reported in the transcription of immune related genes in *S.* Infantis infection and during the administration of *L. fermentum* in *S.* Infantis-challenged broilers; the increased concentration of IgM in serum, at 11 days post infection, reveals that the presence of the probiotic contributed to strengthen the immune response during a *S.* Infantis infection.

Undoubtedly, further research is required to elucidate the effects of probiotic treatment, in pathogen-infected chickens, on the dynamics of the immune response. A better understanding of the underlying strategies of the immune system in chicken and the host-pathogen interactions at cellular level is crucial developing treatments that help to mitigate the *S*. Infantis incidence, especially in Ecuador and the Andean region.

7.1. Recommendations

Recommendations for future research approaches with regard to the present work include:

- To assess immunoglobulin and cytokine levels on different stages of the infection, especially near critical stages of growth (e.g., weeks 5 and 6).
- To measure immunoglobulin levels in caecal content.
- To consider the initial (8 – 24 h post inoculation) responses in the *Salmonella* infection, as well as in the administration of *L. fermentum* in the assessment of cytokine expression and other immune related genes.
- To assess the Th1 lineage and related cytokines, considering that intracellular colonization is of great importance to a *Salmonella* infection.
- To determine if administration of the probiotic could reduce *S.* Infantis load in caecal samples.
- To measure effects of probiotic treatment on intestinal architecture, along with the assessment of goblet cells differentiation and expression of *MUC2.*

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9. APPENDICES

APPENDIX A – Antibody concentrations

A1. IgA concentration in serum

IgA conc. (ug/mL)				
Bird No.	**Control**	**Lf**	**Se**	**LfSe**
1	196.53	897.47	391.80	383.71
2	269.04	296.60	222.34	345.83
3	237.38	267.62	278.69	297.70
4	283.61	254.12	313.41	456.85
5	246.39	274.14	366.90	242.00
6	263.70	248.60	245.05	208.00
7	375.96	261.11	264.10	255.59
8	258.14	336.58	205.00	238.79

A2. IgM concentration in serum

IgM conc. (ug/mL)				
Bird No.	**Control**	**Lf**	**Se**	**LfSe**
1	77.11	79.71	76.60	139.77
2	77.46	63.86	175.03	173.87
3	95.27	87.19	46.49	130.48
4	61.61	70.52	79.45	216.55
5	156.10	84.94	58.16	122.11
6	51.85	67.71	111.63	115.76
7	74.67	56.72	41.14	115.76
8	79.71	102.15	56.99	62.76

APPENDIX B – Ct computed values and calculations for relative gene expression

B1. mRNA relative expression of *IL1B*

Gene of interest - *IL1B*								
	Control		Lf		Se		LfSe	
No,	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2
1	31.56	30.85	38.00	36.43	38.45	40.07	37.24	34.23
2	35.06	35.39	38.18	37.26	37.47	40.07	40.01	40.87
3	35.47	34.71	33.85	34.17	33.89	34.51	32.32	32.23
4	34.76	34.52	33.94	34.56	37.61	34.55	33.34	35.78
5	36.19	37.01	34.10	34.37	37.21	35.48	34.06	34.06
6	35.22	34.85	35.29	35.88	33.51	33.81	35.67	36.35
7	36.85	37.35	36.78	35.81	35.92	36.05	34.36	34.32
8	34.05	34.14	32.20	32.13	34.55	34.34	33.04	33.11
9	36.69	36.45	36.27	36.82	33.21	33.32	36.15	35.20
10	33.90	33.46	35.83	35.53	32.12	32.47	33.37	33.16

Gene of interest - *IL1B*			
Ct			
Control	Lf	Se	LfSe
31.21	37.22	39.26	35.74
35.23	37.72	38.77	40.44
35.09	34.01	34.20	32.28
34.64	34.25	36.08	34.56
36.60	34.24	36.34	34.06
35.03	35.59	33.66	36.01
37.10	36.30	35.99	34.34
34.09	32.16	34.44	33.07
36.57	36.55	33.27	35.67
33.68	35.68	32.29	33.26

REFERENCE GENE - GAPDH								
	Control		Lf		Se		LfSe	
No,	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2
1	25.12	23.60	25.47	25.84	29.47	32.85	33.30	32.59
2	26.36	26.28	31.84	31.91	29.46	29.24	28.79	28.52
3	23.34	23.59	24.08	23.62	26.95	26.69	26.78	26.46
4	28.30	26.85	27.50	27.33	27.15	26.30	25.66	25.45
5	28.24	29.60	28.98	28.84	29.34	29.25	28.19	34.40
6	23.58	23.74	28.36	27.74	26.84	30.12	25.26	25.03
7	24.82	24.68	24.98	24.72	25.43	25.02	25.11	25.20
8	27.21	27.25	23.01	22.99	23.03	22.71	23.86	23.71
9	26.13	26.26	26.21	25.95	25.47	25.51	26.87	25.88
10	24.64	24.63	24.91	24.83	24.77	24.60	25.72	25.26

REFERENCE GENE - GAPDH			
Ct			
Control	Lf	Se	LfSe
24.36	25.66	31.16	32.95
26.32	31.87	29.35	28.66
23.47	23.85	26.82	26.62
27.58	27.41	26.72	25.56
28.92	28.91	29.30	31.30
23.66	28.05	28.48	25.15
24.75	24.85	25.23	25.16
27.23	23.00	22.87	23.79
26.19	26.08	25.49	26.38
24.64	24.87	24.69	25.49

deltaCt			
Control	Lf	Se	LfSe
6.85	11.56	8.10	2.79
8.91	5.85	9.42	11.78
11.63	10.17	7.38	5.66
7.06	6.84	9.36	9.00
7.68	5.33	7.05	2.77
11.37	7.54	5.18	10.87
12.35	11.45	10.76	9.18
6.86	9.16	11.57	9.29
10.37	10.47	7.77	9.30
9.05	10.81	7.61	7.77

	delta Ct			
	Control	Lf	Se	LfSe
AVG	9.21	8.92	8.42	7.84
ST.DEV	2.11	2.35	1.88	3.13
ST.ERR	0.67	0.74	0.60	0.99
ddCT	0.00	-0.30	-0.79	-1.37
ddCt + SE		0.44	-0.20	-0.38
ddCt - SE		-1.04	-1.39	-2.36
2^-ddCt	1.00	1.23	1.73	2.59
2^-(ddCt+SE)		0.73	1.15	1.31
2^-(ddCt-SE)		2.05	2.62	5.14

B2. mRNA relative expression of *IL13*

Gene of interest - *IL13*								
	Control		Lf		Se		LfSe	
No,	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2
1	34.22	35.00	35.92	37.08	39.16	38.54	40.36	36.05
2	36.16	35.48	37.69	37.02	39.37	39.77	39.69	39.44
3	38.14	37.61	36.77	36.71	38.06	36.13	35.13	34.56
4	39.16	37.08	37.38	36.48	35.18	36.07	34.99	37.01
5	36.33	38.29	36.58	37.44	38.88	38.11	35.65	37.16
6	33.35	34.26	37.54	37.80	38.16	37.33	37.58	36.77
7	35.54	39.17	37.27	36.95	37.88	37.31	35.36	42.62
8	38.05	35.79	37.52	42.57	37.72	36.08	35.51	35.02
9	37.16	36.72	37.83	39.00	35.87	35.94	35.97	35.99
10	36.14	39.38	37.53	37.44	33.14	33.34	37.15	36.88

Gene of interest - *IL13*			
Ct			
Control	Lf	Se	LfSe
34.61	36.50	38.85	38.21
35.82	37.36	39.57	39.56
37.88	36.74	37.10	34.84
38.12	36.93	35.62	36.00
37.31	37.01	38.49	36.40
33.80	37.67	37.75	37.18
37.35	37.11	37.59	38.99
36.92	40.04	36.90	35.27
36.94	38.41	35.90	35.98
37.76	37.49	33.24	37.02

REFERENCE GENE - GAPDH								
	Control		Lf		Se		LfSe	
No,	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2
1	25.12	23.60	25.47	25.84	26.02	26.13	27.11	26.95
2	26.36	26.28	25.72	25.78	25.48	25.43	27.05	27.17
3	23.34	23.59	24.08	23.62	26.95	26.69	26.78	26.46
4	28.30	26.85	27.50	27.33	27.15	26.30	25.66	25.45
5	28.24	29.60	28.98	28.84	29.34	29.25	28.19	34.40
6	25.39	25.30	28.36	27.74	26.84	30.12	25.26	25.03
7	24.82	24.68	24.98	24.72	25.43	25.02	25.11	25.20
8	27.21	27.25	23.01	22.99	23.03	22.71	23.86	23.71
9	26.13	26.26	26.21	25.95	25.47	25.51	26.87	26.54
10	25.80	25.90	24.91	24.83	24.77	24.60	25.72	25.26

REFERENCE GENE - GAPDH			
Ct			
Control	Lf	Se	LfSe
24.36	25.66	26.08	27.03
26.32	25.75	25.45	27.11
23.47	23.85	26.82	26.62
27.58	27.41	26.72	25.56
28.92	28.91	29.30	31.30
25.34	28.05	28.48	25.15
24.75	24.85	25.23	25.16
27.23	23.00	22.87	23.79
26.19	26.08	25.49	26.71
25.85	24.87	24.69	25.49

deltaCt			
Control	Lf	Se	LfSe
10.25	10.84	12.77	11.18
9.50	11.61	14.11	12.46
14.41	12.89	10.28	8.22
10.54	9.51	8.90	10.45
8.39	8.11	9.19	5.11
8.46	9.63	9.26	12.03
12.61	12.26	12.37	13.83
9.69	17.04	14.03	11.48
10.74	12.33	10.41	9.27
11.91	12.61	8.55	11.53

	delta Ct			
	Control	Lf	Se	LfSe
AVG	10.65	11.68	10.99	10.55
ST.DEV	1.88	2.46	2.15	2.48
ST.ERR	0.60	0.78	0.68	0.79
ddCT	0.00	1.03	0.34	-0.10
ddCt + SE		1.81	1.02	0.69
ddCt - SE		0.26	-0.34	-0.88
2^-ddCt	1.00	0.49	0.79	1.07
2^-(ddCt+SE)		0.29	0.49	0.62
2^-(ddCt-SE)		0.84	1.27	1.84

B3. mRNA relative expression of *IL15*

Gene of interest - *IL15*								
	Control		Lf		Se		LfSe	
No,	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2
1	25.07	24.91	26.06	25.99	28.99	28.98	28.57	28.61
2	26.25	25.62	31.73	32.70	29.24	29.64	29.38	29.09
3	25.71	25.76	26.01	25.83	25.44	25.31	26.64	26.50
4	26.50	26.10	26.34	25.83	26.33	26.21	25.09	25.66
5	25.88	26.28	26.62	26.50	26.57	26.59	26.47	26.30
6	24.69	24.67	27.16	27.48	26.58	26.62	26.85	26.51
7	26.55	26.61	25.86	26.18	27.86	27.49	27.16	26.98
8	25.97	26.08	26.96	26.83	26.38	26.12	25.73	26.87
9	27.91	27.58	27.10	26.73	26.08	26.29	27.38	27.32
10	27.87	27.22	28.04	28.50	26.85	27.06	26.67	26.88

Gene of interest - *IL15*			
Ct			
Control	Lf	Se	LfSe
24.99	26.02	28.98	28.59
25.93	32.22	29.44	29.23
25.74	25.92	25.38	26.57
26.30	26.08	26.27	25.37
26.08	26.56	26.58	26.38
24.68	27.32	26.60	26.68
26.58	26.02	27.67	27.07
26.02	26.89	26.25	26.30
27.74	26.92	26.18	27.35
27.55	28.27	26.96	26.78

REFERENCE GENE - GAPDH								
	Control		Lf		Se		LfSe	
No,	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2
1	25.12	23.60	25.47	25.84	29.47	32.85	33.30	32.59
2	26.36	26.28	31.84	31.91	29.46	29.24	28.79	28.52
3	23.34	23.59	24.08	23.62	26.95	26.69	26.78	26.46
4	28.30	26.85	27.50	27.33	27.15	26.30	25.66	25.45
5	28.24	29.60	28.98	28.84	29.34	29.25	28.19	34.40
6	25.39	25.30	28.36	27.74	26.84	30.12	25.26	25.03
7	24.82	24.68	24.98	24.72	25.43	25.02	25.11	25.20
8	27.21	27.25	23.01	22.99	23.03	22.71	23.86	23.71
9	26.13	26.26	26.21	25.95	25.47	25.51	26.87	25.88
10	24.64	24.63	24.91	24.83	24.77	24.60	25.72	25.26

REFERENCE GENE - GAPDH			
Ct			
Control	Lf	Se	LfSe
24.36	25.66	31.16	32.95
26.32	31.87	29.35	28.66
23.47	23.85	26.82	26.62
27.58	27.41	26.72	25.56
28.92	28.91	29.30	31.30
25.34	28.05	28.48	25.15
24.75	24.85	25.23	25.16
27.23	23.00	22.87	23.79
26.19	26.08	25.49	26.38
24.64	24.87	24.69	25.49

deltaCt			
Control	Lf	Se	LfSe
0.64	0.37	-2.17	-4.36
-0.39	0.34	0.09	0.58
2.27	2.07	-1.44	-0.05
-1.28	-1.33	-0.45	-0.18
-2.84	-2.35	-2.72	-4.91
-0.66	-0.72	-1.88	1.54
1.83	1.17	2.45	1.91
-1.21	3.89	3.38	2.51
1.55	0.84	0.69	0.98
2.91	3.40	2.27	1.29

	delta Ct			
	Control	Lf	Se	LfSe
AVG	0.28	0.77	0.02	-0.07
ST.DEV	1.85	1.98	2.13	2.55
ST.ERR	0.58	0.63	0.67	0.81
ddCT	0.00	0.49	-0.26	-0.35
ddCt + SE		1.11	0.41	0.45
ddCt - SE		-0.14	-0.94	-1.16
2^-ddCt	1.00	0.71	1.20	1.28
2^-(ddCt+SE)		0.46	0.75	0.73
2^-(ddCt-SE)		1.10	1.91	2.23

B4. mRNA relative expression of *IL17*

Gene of interest - *IL17*								
	Control		Lf		Se		LfSe	
No,	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2
1	31.22	31.08	33.04	33.15	36.31	37.56	37.53	38.47
2	33.71	32.51	35.41	34.42	37.22	34.54	37.12	36.95
3	33.18	33.92	32.34	34.85	33.44	33.04	33.17	34.01
4	38.29	36.85	34.70	34.10	34.75	36.04	32.24	32.12
5	35.67	35.35	33.44	34.24	35.05	35.79	35.21	35.90
6	33.10	32.57	42.51	37.27	34.53	35.02	34.50	35.38
7	33.88	34.48	34.13	34.23	35.22	36.20	34.21	34.59
8	36.11	34.73	37.00	36.30	35.40	34.18	34.28	34.35
9	38.10	38.22	35.41	36.03	34.12	33.60	37.07	37.12
10	34.37	34.15	35.38	35.10	33.98	34.18	33.48	33.35

Gene of interest - *IL17*			
Ct			
Control	Lf	Se	LfSe
31.15	33.09	36.93	38.00
33.11	34.91	35.88	37.03
33.55	33.59	33.24	33.59
37.57	34.40	35.40	32.18
35.51	33.84	35.42	35.56
32.83	39.89	34.78	34.94
34.18	34.18	35.71	34.40
35.42	36.65	34.79	34.31
38.16	35.72	33.86	37.10
34.26	35.24	34.08	33.41

REFERENCE GENE - GAPDH								
	Control		Lf		Se		LfSe	
No,	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2
1	25.12	23.60	25.47	25.84	29.47	32.85	33.30	32.59
2	26.36	26.28	31.84	31.91	29.46	29.24	27.29	27.09
3	23.34	23.59	24.08	23.62	26.95	26.69	26.78	26.46
4	28.30	26.85	27.50	27.33	27.15	26.30	25.66	25.45
5	28.24	29.60	28.98	28.84	29.34	29.25	28.19	34.40
6	25.39	25.30	28.36	27.74	26.84	30.12	25.26	25.03
7	24.82	24.68	24.98	24.72	25.43	25.02	25.11	25.20
8	27.21	27.25	23.01	22.99	23.03	22.71	23.86	23.71
9	26.13	26.26	26.21	25.95	25.47	25.51	26.87	25.88
10	24.64	24.63	24.91	24.83	24.77	24.60	25.72	25.26

REFERENCE GENE - GAPDH			
Ct			
Control	Lf	Se	LfSe
24.36	25.66	31.16	32.95
26.32	31.87	29.35	27.19
23.47	23.85	26.82	26.62
27.58	27.41	26.72	25.56
28.92	28.91	29.30	31.30
25.34	28.05	28.48	25.15
24.75	24.85	25.23	25.16
27.23	23.00	22.87	23.79
26.19	26.08	25.49	26.38
24.64	24.87	24.69	25.49

deltaCt			
Control	Lf	Se	LfSe
6.80	7.44	5.78	5.05
6.79	3.04	6.53	9.84
10.08	9.75	6.42	6.97
9.99	6.99	8.67	6.62
6.59	4.93	6.13	4.26
7.49	11.84	6.29	9.79
9.43	9.34	10.49	9.24
8.19	13.65	11.92	10.53
11.97	9.64	8.36	10.72
9.63	10.37	9.39	7.92

	delta Ct			
	Control	Lf	Se	LfSe
AVG	8.70	8.70	8.00	8.09
ST.DEV	1.80	3.17	2.11	2.30
ST.ERR	0.57	1.00	0.67	0.73
ddCT	0.00	0.00	-0.70	-0.60
ddCt + SE		1.00	-0.03	0.12
ddCt - SE		-1.00	-1.36	-1.33
2^-ddCt	1.00	1.00	1.62	1.52
2^-(ddCt+SE)		0.50	1.02	0.92
2^-(ddCt-SE)		2.00	2.58	2.51

B5. mRNA relative expression of *MYD88*

Gene of interest - *MYD88*								
	Control		Lf		Se		LfSe	
No,	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2
1	33.65	34.03	34.58	34.41	38.28	38.44	36.48	37.01
2	35.86	35.47	43.96	41.13	37.97	41.87	35.57	35.43
3	34.93	34.47	35.21	35.87	34.66	34.92	35.42	34.89
4	36.33	35.30	34.81	34.50	34.83	34.60	33.34	33.90
5	35.09	35.06	36.16	35.41	33.66	33.75	33.70	34.52
6	32.01	31.88	34.91	35.04	33.46	33.79	33.56	34.03
7	34.28	34.76	33.55	34.29	34.65	34.32	34.92	35.06
8	33.77	33.97	34.11	33.82	35.22	34.82	34.43	34.18
9	34.82	35.18	35.73	35.19	34.29	34.83	34.67	33.89
10	36.48	35.68	36.66	37.07	35.52	35.10	35.26	36.23

Gene of interest - *MYD88*			
Ct			
Control	Lf	Se	LfSe
33.84	34.50	38.36	36.74
35.66	42.54	39.92	35.50
34.70	35.54	34.79	35.15
35.81	34.65	34.71	33.62
35.07	35.78	33.71	34.11
31.94	34.97	33.62	33.80
34.52	33.92	34.48	34.99
33.87	33.96	35.02	34.30
35.00	35.46	34.56	34.28
36.08	36.86	35.31	35.74

REFERENCE GENE - GAPDH								
	Control		Lf		Se		LfSe	
No,	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2	Ct1	Ct2
1	28.03	27.89	29.54	35.38	33.44	33.84	32.15	33.36
2	30.02	30.46	36.06	37.65	34.27	33.64	31.33	29.88
3	28.85	29.62	29.67	29.91	29.62	29.19	31.96	29.75
4	30.16	30.71	30.15	30.50	31.17	30.66	30.54	27.66
5	31.23	30.93	29.96	30.22	31.74	32.52	33.05	32.47
6	30.74	30.50	34.93	35.56	33.11	32.37	33.50	33.11
7	32.75	32.04	33.08	33.88	32.53	32.63	32.66	31.01
8	32.31	32.44	29.58	29.04	29.96	30.42	30.87	30.87
9	32.90	32.06	30.86	30.83	32.76	32.21	33.18	32.58
10	32.87	31.37	34.08	33.14	32.64	31.67	32.77	33.68

REFERENCE GENE - GAPDH			
Ct			
Control	Lf	Se	LfSe
27.96	32.46	33.64	32.76
30.24	36.85	33.96	30.60
29.23	29.79	29.41	30.86
30.44	30.33	30.92	29.10
31.08	30.09	32.13	32.76
30.62	35.25	32.74	33.30
32.40	33.48	32.58	31.83
32.37	29.31	30.19	30.87
32.48	30.84	32.48	32.88
32.12	33.61	32.15	33.22

deltaCt			
Control	Lf	Se	LfSe
5.88	2.03	4.72	3.99
5.42	5.69	5.97	4.90
5.46	5.75	5.38	4.30
5.38	4.33	3.80	4.52
3.99	5.69	1.58	1.35
1.33	-0.27	0.88	0.49
2.13	0.44	1.90	3.16
1.49	4.65	4.83	3.44
2.52	4.62	2.08	1.40
3.96	3.25	3.16	2.52

	delta Ct			
	Control	Lf	Se	LfSe
AVG	3.76	3.62	3.43	3.01
ST.DEV	1.77	2.20	1.77	1.51
ST.ERR	0.56	0.70	0.56	0.48

ddCT	0.00	-0.14	-0.33	-0.75
ddCt + SE		0.56	0.23	-0.27
ddCt - SE		-0.83	-0.89	-1.23

2^-ddCt	1.00	1.10	1.25	1.68
2^-(ddCt+SE)		0.68	0.85	1.21
2^-(ddCt-SE)		1.78	1.85	2.34