Effect of *In ovo* injection of probiotic, prebiotic and synbiotic on growth performance and gut health parameters of broiler chickens

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Abstract

Due to the claimed public health concerns, use of antibiotics as growth promoters (AGP) in the chicken feed is banned or regulated in several jurisdictions. Therefore, probiotics, prebiotics, and synbiotics are being evaluated as effective alternatives to AGP to improve growth performance and health of poultry. This study aimed to investigate the effects of *Bacillus coagulans*, Raffinose family oligosaccharides (RFO) and their combination on growth performance and gut health of broilers when injected *in ovo*. A total of 285 fertilized eggs were divided into 5 groups: i) No-injection group with intact shell, ii) 0.5 ml 0.85% normal saline, iii) Probiotic (*B. coagulans*) (2×10^6 CFU/egg) in 0.5 ml 0.85% normal saline, iv) Prebiotic (4.5 mg RFO) in 0.5 ml 0.85% normal saline, and v) Synbiotic (2×10^6 CFU/egg *B. coagulans* + 4.5mg RFO) in 0.5 ml 0.85% normal saline. The injection solution was deposited into the amniotic sac on d 17 of incubation. Hatchability of eggs were recorded. Altogether, 48 day-old chicks from each treatment were randomly allocated to 6 replicate floor pens (n=8/pen). All birds were raised on a standard commercial diet and management for 42 days. Body weight and feed intake of birds were measured weekly. Ileum samples were collected on d 0 and d 7 post hatch for total RNA isolation. Expression of immune/cytokines related genes in the ileum were determined using qPCR. The *in ovo* injection did not affect ($P > 0.05$) hatchability of eggs across the treatments. There was no significant effect of treatments on body weight, average daily gain and feed intake of broilers in different experimental groups. However, birds from normal saline treatment had significantly better ($P < 0.05$) feed efficiency and RFO group had the poorest feed efficiency in the first week of post-hatch period. No significant difference ($P > 0.05$) was found on relative organ weight of birds on d 21 and d 42. At d 7 of age, ileum villus height, crypt depth, and villus height: crypt depth ratio of RFO group were significantly better than other treatments ($P < 0.05$). On hatch day, expression of

IL4 (inducer of T-cells differentiation) was significantly higher ($P < 0.001$) in the ileum of probiotic group. On d 7, immune-related genes (*CD56*, *ChB6*, *TLR4*, *MCN2*) and cytokines related gene (*IL10*) were significantly higher ($P < 0.05$) in the ileum of saline-treated group, whereas glucose transporter (*SGLT1*) had lower expression ($P < 0.05$) in synbiotic group. In conclusion, *in ovo* injection of probiotic enhances gut immunity of chicken which would be beneficial for gut health. It is interesting to find that *in ovo* injection of saline also enhanced gut immunity.

List of Tables

List of Figures

TABLE OF CONTENTS

CHAPTER 1: LITERATURE REVIEW

1.1 Status of industry production

By 2050 the world's population will grow to more 9 billion, while patterns of food consumption are becoming more similar across the globe, shifting towards higher quality and more expensive foods such as meat and dairy products. Meat consumption in developing countries has risen from only 10 kg per person annually in 1964-66 to 26 kg in 1997-99. FAO expects that it is projected to rise to 37 kg per person per year in 2030 (FAO, 2010). Therefore, there is increasing demand for animal products, and growing consumer demands for more food safety, lower environmental impact, and better animal welfare conditions.

Poultry industry already become a leader in trends of industrialization in agriculture over the last 50 years for its highly specialized and efficient set of enterprises. In 2014, the U.S. poultry industry produced 8.54 billion broilers, 99.8 billion eggs, and 238 million turkeys. The combined value of production from broilers, eggs, turkeys, and the value of sales from chickens in 2014 was $48.3 billion, up 9 percent from $44.4 billion in 2013 (NASS, 2015). Compared to other meat animals like swine, cattle, lamb, poultry converts feedstuff to feed more efficiently. The feed conversion ratio of broilers can reach 1.8-2.2; As the short period requirements on growth and marketing, the poultry industry is dynamic, which can adjust rapidly to changing economic factors (feed, availability, cost, number of birds on feed). Other livestock industries require a longer length of time from birth to market like cattle. Poultry is more straightforward to feed, kind of by-product can feed to poultry, for example, blood meal, fish meal, meat and bone, distillers grains. And it not used for human consumption. Except for meat, poultry- layers also provided a continuous source

of food. Meat animals must be fed for a longer period. Layers produce several times its weight in eggs. Products form meat animals are restricted to final market weight. For the reason of religion, and in some countries, meat eaters are the minority, vegetarians consume eggs. From the marketing view, poultry products are relatively inexpensive, and poultry meats are one of the best meat buys in the supermarket. Poultry manure as fertilizer, organic farming, premium price, rich in Nitrogen and organic material, by-product feed for ruminants. All the above reason makes poultry industry becoming a more essential part in the whole agriculture industry. However, with the increasing human worries about meat consumption and food safety, many factors that will influence the evolution of the poultry industry in this coming decade. Technical considerations and the evolution of science and technology, the availability of natural resources and water (which are becoming increasingly limited), and the maintenance of trade barriers must be considered. Finally, consumer demands will have a strong influence as these demands are becoming increasingly concerned with animal welfare issues, food safety, and environmental impact relative to poultry production. New methods to assess the economic and environmental impact of poultry production have been developed. For the future of the poultry industry, the expectation of increasing biotechnology, (*in ovo* inject), mass production, increase attention to poultry behavior and welfare, enhance food safety and quality of products.

Antibiotics have been widely used in poultry production for decades, first primarily to control disease, and more recently as antimicrobial growth performance promoters (AGPs) to improve growth rate and feed conversion efficiency. However, it's use has been banned in many countries and has been severely limited, or will be eliminated as public health concern. This has been a challenge for animal nutrition increasing the need to find alternative methods to control and prevent pathogenic bacterial colonization. Ideally, alternatives to growth promoters should have

the same beneficial effect as AGPs. The most well-known mechanism to be proposed is that AGPs have antibacterial action that favours performance in different ways: 1) by reducing the incidence and severity of subclinical infections; 2) by reducing the microbial use of nutrients; 3) by improving absorption of nutrients because of thinning of the intestinal wall, and 4) by reducing the amount of growth-depressing metabolites produced by gram-positive bacteria (Huyghebaert et al., 2010). For that, several alternatives to AGP have been attempted with some success, such as organic acids, probiotics, prebiotics, essential oil compounds, and enzymes.

The modulation of the gut microbiota with new feed additives, such as probiotics and prebiotics, towards host-protecting functions to support animal health, is a topical issue in poultry production. Although the knowledge on the effects of such feed additives has increased, essential information concerning their impact on the host are, to date, incomplete. For the future, the most important target, within probiotic and prebiotic research, is a demonstrated health-promoting benefit supported by knowledge on the mechanistic actions. Potential combinations of suitable probiotics and prebiotics may prove to be the next step to reduce the risk of intestinal diseases and remove specific microbial disorders (Gaggia et al., 2010).

1.2 Probiotic

1.2.1 Classification

Probiotics used in animals are known as direct-fed microbial, many definitions have been proposed for the term. The more widely accepted and adopted one is “live micro-organisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2001). There are several different methods to classify the probiotics. Most commonly used was separate probiotics to bacterial and non-bacterial probiotics. In addition to certain yeast and fungal

probiotics, most of the micro-organisms used are bacteria. And according to the property of growth, they can be divided to spore-forming and non- spore forming probiotics. Although non-spore forming *Lactobacillus* and *Bifidobacterium* strains predominated initially, spore forming bacteria are now used, e.g., *Bacillus* species. Another method to separate probiotic can be classified by the number of strains, the multispecies probiotics or single- species probiotics. The microbial composition of probiotic products ranges from a single strain to multi-strain or species compositions. The microorganisms used as probiotics which are typically not present in the gastrointestinal tract (GIT) of animals are referred to as allochthonous (e.g., yeasts), while the microorganism normally present as indigenous inhabitants of the GIT are referred to as autochthonous probiotics (e.g., *Lactobacillus* and *Bifidobacterium*) (Bajagai et al., 2016).

1.2.2 Characteristics of probiotics

The mechanisms of action of probiotics as AGP appears to be different. Probiotics help to prevent and control GIT pathogens and improve the performance and productivity of production animals through varies mechanisms. One of the major determinants of maintaining a healthy GIT is the composition of the microbial population and their balance in the GIT. Probiotics can change the dynamics of bacteria in the GIT; it may increase the population of beneficial microorganisms including Lactobacilli and Bifidobacteria, which then inhibit growth of harmful microorganisms by producing inhibiting substances (bacteriocins or organic acids) and by competitive exclusion. The reduction in pathogenic microorganisms in the GIT may be attributable to the production of antimicrobial substances such as bacteriocins and adhesion of probiotic microbes to the intestinal epithelium, thereby excluding pathogens competitively or by including immune system response. Probiotics in the intestine may increase enzyme activity resulting in the promote of digestibility of

nutrients in diet (Zhang and Kim, 2014). And many experiments proved utilization of probiotics increased the villus height, crypt depth, and ratio of villus height to crypt depth thus increasing the surface area for nutrition absorption (Bajagai et al., 2016; Palamidi et al., 2016).

Another essential mechanism of probiotics is to produce the antimicrobial substances in intestinal. Many probiotic bacteria, especially lactic acid bacteria producing short chain fatty acids (SCFAs), particularly lactic and acetic acids, can inhibit pathogenic bacteria. SCFA reduce the pH in micro-environment within the intestinal, which can reach to a lethal level for some bacteria (Daskiran et al., 2012). Bacteriocin produced by lactic acid bacteria inhibits the growth of pathogenic microorganisms by inhibiting cell wall synthesis (Hyronimus et al., 1998). And some probiotics can produce other antimicrobial compounds that may inhibit harmful microbes in the GIT (Bajagai et al., 2016). According to the immune modulation, administration of probiotics before infectious or pathogens colonization play the most important role in gut immunity. Probiotics can improve the innate gut immunity through restitution of intestinal barrier function. And diets containing probiotics could modulate the host immune response, affect the expression of some anti-inflammatory cytokine or cell signaling proteins. Probiotics also increase serum immunoglobulin levels. A multi-strain probiotic containing *L. acidophilus*, *B. subtilis,* and *C. butyricum* increased serum levels of IgA and IgM in chickens (Zhang and Kim, 2014). One famous theory on probiotics mechanism is colonization resistance, which can be understood as following: the GIT of neonatal birds are colonized with microorganisms, generally originating from the mother, these microorganisms provide protection from enteric pathogens. Probiotics could mimic natural colonization in neonates, or colonize adult animals, preventing pathogenic organisms from colonizing the intestinal mucosa (Bajagai et al., 2016). Several beneficial claims have been

established for probiotics, but this evidence is not sufficient. In general, the growth promoting effects of probiotics are limited and variable.

1.2.3 Utilization of probiotics in poultry industry

Probiotics can improve growth performance, gut health, and immune function of broiler chickens (Bajagai et al., 2016; Palamidi et al., 2016; Zhang and Kim, 2014). Lactobacillus, Enterococcus, Bacillus, and Saccharomyces are the most used probiotics in poultry production (Gaggia et al., 2016). Probiotics have enhanced the growth rate in broilers better than AGP (avilamycin) (Bajagai et al., 2016; Palamidi et al., 2016; Zhang and Kim, 2014), which appeared in body weight (BW) gain. And these improvements in growth performance associated with increased feed intake (FI), and better feed conversion ratio (FCR), which is one of the modes of actions for improved growth. Another reason may be due to the different microbial population in the GIT resulting in increased production of SCFA and immune-modulation (Daskiran et al., 2012). Increased growth performance has also been related to increased villus height, crypt depth, and the ratio between villus height and crypt depth, which improve the absorption of nutrients from the intestine (Bajagai et al., 2016; Palamidi et al., 2016). Utilization of probiotics may results in the difference on the colonization of the GIT microfloral, which may prevent or control some enteric pathogens, like Salmonellosis, Campylobacteriosis, Necrotic enteritis, and coccidiosis (Gaggia et al., 2010; Palamidi et al., 2016). Some experiments evaluated the probiotics influence on egg production and quality. However, there is no consistent effect on the production and quality of eggs in laying hens. In general, probiotics could be a potential alternative to AGP to improve growth rate, have positive effects on FI, FCR, affect intestinal histomorphology, reduce intestinal colonization and spread of

pathogens, manage the enteric pathogen, but those outcomes from probiotic use are not consistent (Bajagai et al., 2016; Daskiran et al., 2012).

1.2.4 *Bacillus coagulans*

Spore-forming bacteria, particularly various species from the genus Bacillus, are becoming increasingly popular as probiotics for use in animal feed, due to their robustness in withstanding high temperature making them easier to handle during manufacture, storage, and transportation of feed. Probiotic *Bacillus Coagulans* (*B. coagulans*) is a lactic acid-forming bacterial species. It exhibits characteristics typical of both genera *Lactobacillus* and *Bacillus*, its taxonomic position between the families Lactobacillaceae and Bacillaceae was often debated. It was finally transferred to the genus Bacillus. *B. coagulans* is a Gram-positive rod, catalase positive, spore-forming, motile, and a facultative anaerobe. It may appear Gram-negative when entering the stationary phase of growth. The optimum temperature for growth is 50 °C.

Many factors make *B. coagulans* a good candidate for probiotic use; *B. coagulans* is one of the most promising forming lactic acid-producing species. It produces organic acids, possesses the capacity to sporulate, and is easily cultured in bulk (Hyronimus et al.,1998). In addition, in the spore form, it is more resistant to high temperature, which facilitates the pelleting process used in the mass production of probiotic chicken feeds. Seldom study has been carried out on spore forming lactic acid-producing bacteria such as *Bacillus coagulans* as probiotics in broiler chickens. First experiments conducted in 1998 by Cavazzoni et al., the newly isolated *B. coagulans* as probiotic compared with no additive and add virginiamycin groups, showed that *B. coagulans* as a probiotic has a growth- promoting, prophylactic effect comparable to that of virginiamycin. Hyronimus et al., in 1998 proved *Bacillus coagulans* can produce a bacteriocin-like-inhibitory

substance coagulin. Zhou et al. (2010) evaluated, also, the effect of probiotic via the basal diet on growth performance of Guangxi Yellow chickens. It was clear that the dietary administration of probiotic (*Bacillus coagulans* ZJU0616) had beneficial effects on both final body weight and daily weight gain of chickens. Another experiment in 2011 proved supplementation of 0.05% and 0.04% *B. coagulans* significantly improved FCR over the 21- 42 days and the full 42 days in broiler chickens. However, the ADG during this period decreased. Intestinal microflora *Lactobacillus* was significantly increased on duodenum and cecum in 0.02% and 0.04% *B. coagulans* group. Additionally, the count of *E. coli* was significantly decreased in duodenum and cecum in 0.02% and 0.04% *B. coagulans* group (Lin et al., 2011). In contrast, Hung et al. (2012) found that dietary use of the probiotic *B. coagulans* reduced the average daily feed intake by 8% in the broiler grower-finisher phase (days 22–42) with a reduction in FCR by 10%. All in all, *B. coagulans* has the potential to be a probiotic that may control the population of pathogenic microorganisms, further may influence on gut histomorphology and hence improve the growth performance of broiler chickens.

1.3 Prebiotic

1.3.1 Classification

Prebiotics are defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Manning and Gibson, 2004). There are three criteria for the classify the dietary substrate as a prebiotic: 1) the substrate must not be hydrolysed or absorbed in the stomach or small intestine; 2) it must be selective for beneficial commensal bacteria in the large intestine such as the lactobacillus, bifidobacterial; 3) fermentation of the substrate should induce beneficial effects

within the host. Most identified prebiotics are carbohydrates and oligosaccharides, according to the criteria of prebiotics, most promising prebiotics are non-digestible oligosaccharides, like fructo-oligosaccharides, galacto-oligosaccharides. They are found in chicory, barley, wheat, beet leaves and belong to plants fructans which are fermented by beneficial bacteria. Grain legumes are the most common natural resources of oligosaccharides, which includes raffinose, stachyose, and verbascose (Huyghebaert et al., 2011).

1.3.2 Characteristics of prebiotics

The mechanism of action of prebiotics as alternative AGP is dependent on the nature of the compound. As the criteria of prebiotics, they are non-digestible feed ingredients that can have a beneficial action because of selective stimulation of the growth or metabolic activity of a limited number of intestinal microbiota species, such as lactobacillus, bifidobacterial. Thus they may have a similar mechanism of action as probiotics. Many actions make prebiotics be a beneficial supplements of broiler chickens. Prebiotics affect bifidobacterial proliferation and reduce harmful micro-organisms proliferation. It can also help to resistance to gastric acidity, to hydrolysis by digestive enzymes and to GIT absorption, fermentation by intestinal microflora. It can also remove harmful enzymes and toxic metabolites, effect on the synthesis of Vitamin, organic acids (Dankowiakowska et al., 2013; Manning and Gibson, 2004).

1.3.3 Utilization of prebiotics in poultry industry

The utilization of prebiotic on broiler chickens has not a long history, reports on the impact of prebiotics on the growth performance, gut health, activity of intestinal microflora are not numerous. Even though the effect is different and depends on the type of prebiotic, there already have many research proved the potential of prebiotics as AGP in poultry industry.

In 2003, Chen proved that feeding chicory fructans to broiler chickens showed improvement in body weight gain, feed conversion ratio, carcass weight and serum cholesterol decrease; Additionally, the supplementation of fructans resulted in increase of lactobacilli counts in the gastrointestinal tract and Campylobacter and Salmonella decrease. Similar results got from experiment with mannan-oligosaccharides that yeast cell wall containing the prebiotic reduced the Salmonella concentration in broiler chicks compared with chicks fed non-supplemented diet. For mannan-oligosaccharides, no weight gain was observed in turkeys fed two different concentrations of inulin and mannan-oligosaccharides, whereas another experiment proved, feeding turkeys a standard diet supplemented with MOS, reported an improvement on body weight (Gaggia et al.,2010; Dankowiakowska et al., 2013). Enrichment mannan-oligosaccharides in poultry diets have stimulating effect on lymphatic tissue of the gastrointestinal tract. Broiler chickens fructo-oligosaccharides supplemented diet significantly increases the number of bifidobacteria and lactic acid bacteria in the cecum and small intestine. Furthermore, population of *Clostridium perfringens*, *Escherichia coli* and *Salmonella* are significantly reduced. Fructo-oligosaccharides alleviates the effects of caecal epithelial necrosis, also stimulates growth of intestinal villi and crypts in the jejunum and iliac colon (Rehman et al., 2007).

The possible explanation of those variable influence of prebiotics may be related to different dose of prebiotic applied, concentration of prebiotics, methods of feeding (in water/ feed, or *in ovo* technology), time and duration of supplements. There still need a lot of work to reach a consistent influence of prebiotic on broiler chickens.

1.3.4 Raffinose family oligosaccharides

The raffinose family of oligosaccharides (RFO) is a trisaccharide composed of galactose, glucose, and fructose. It can be easily found in many plants and seeds. Raffinose can be hydrolyzed to

galactose and sucrose by a specific enzyme, which is not found in the digestive tract. As the soluble carbohydrates, they rank second only to sucrose. As the deficiency of enzyme, RFO cannot be broken down and digested in stomach and upper intestine. It can only be fermented in lower intestine by specific bacteria.

Experiment by Bednarczyk et al. (2011) reported a prebiotic effect of *in ovo* injection with RFO on growth performance and concluded that *in ovo* injection of raffinose could replace AGP as a non-antimicrobial enhancer additive. Similar experiment in 2017 proved that supplemented with RFO has beneficial effect on gut health, which appears in higher villus height, and increased villus height to crypt depth ratio. And this research also claimed including of RFO contributes to higher expression on related immune gene (Berrocoso et al., 2017).

1.4 Synbiotic

Synbiotics are defined as a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and persistence of living microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and /or by activating the metabolism of one or a limited number of health-promoting bacteria (Huyghebaert et al., 2011). This combination would thus combine substrate and bacteria. The acquisition of data on the efficacy of synbiotic products as feed additives in poultry needs further investigation. However, results on in vivo trials are promising, showing a synergistic effect coupling probiotics and prebiotics in the reduction of food-borne pathogenic bacterial populations (Gaggia et al., 2010). Awad et al. (2009) evaluated the effect of a symbiotic product (a combination of *E. faecium*, a prebiotic derived from chicory, and immune modulating substances derived from sea algae) on broiler chickens. The results showed significant increase in BW, average daily gain, carcass yields percentage, and FCR, whereas no increase in organ weight was found, with a reception for the small intestine; This

research also reported a significant increase in the villus height in both duodenum and ileum (Awad et al., 2009).

1.5 *In ovo* technology

1.5.1 History of *in ovo* technology

Recent research tends to eliminate the unwanted effects of many factors that may affect the action of supplements. A promising method that gives positive results is the method of administration supplements by injection *in ovo*. The *in ovo* technology is based on injection of substances with various activity to the eggs air chamber or directly into the growing embryo (Bednarczyk et al., 2011). *In ovo* technology was designed in the 1980s in the United States, in order to vaccinate chicks against Marek's disease, and later against the Gumbro disease, Newcastle Disease. Due to the ability of late-stage embryos and fetuses to support immune responses to viral and bacterial antigens, used the *in ovo* injection for the Marek's disease vaccine in embryonic chickens have greater protective indices when vaccinated at embryo stage (the 16th to 20th days of incubation; Vaccination at the 18th day revealed the greatest protection) compared to those vaccinated at hatch ($P < 0.05$), while having no effect on hatchability (Sharma and Burmester, 1982).

Because of the success with *in ovo* vaccination, *in ovo* technology has a wider application with the injection of various biologics, aims at including for example: stimulation of beneficial bacterial profile in the colon of chicken, stimulation of immunological response, stimulation of embryonic development, teratogenic effects testing, selection for sexual phenotype, injection of genetically modified cells, etc (Dankowiakowska et al., 2013). The use of feed additions such as prebiotics and probiotics or their combination- synbiotics influences health and chicken performance. Their

administration *in ovo* could have an advantageous impact on early development of immunological system in broiler chickens (Roto et al., 2016). With the development of this technology, there are more issues on this technique need concern, like the site of injection, solution injected, age of injection, and method of injection. In the late stage of embryonic development, there are five regions through which an *in ovo* injection may be delivered: the air cell, the allantoic membrane, the amniotic fluid, the yolk, and the embryo. Recently, the most acceptable injection time was late-term avian embryo with delivery to the amniotic fluid, which developed by Uni and Ferket (2003). And variable *in ovo* studies has focused on different recommended location and age as given by Uni and Ferket (2003), what supplement is injected, such as nutrients, hormones, immunostimulants or other biologics, attempting to promote growth and stimulate the immune system.

1.5.2 Utilization of *in ovo* technology

Numerous studies have been conducted investigating the efficacy of *in ovo* injection of various biologics in poultry, including nutrient supplements. Carbohydrate, proteins, and amino acids, vitamins, or other modulators are most common injection material. *In ovo* supplementation of carbohydrates might help in improving the growth of late-term embryos and chicks. When inoculated eggs with carbohydrates (maltose, sucrose, and dextrose), the results showed that the additional energy source enhanced the development of goblet cells and increased the villi surface area in the intestines. The same carbohydrate mixture was applied again in different studies; both indicated increased body weight and increased liver glucose at hatch (Smirnov et al., 2006). *In ovo* glucose could modulate humoral-related immunity, while fructose or ribose might help in improving the cellular immunity in broiler chickens (Bhanja et al., 2015). The results of the

influence oligosaccharides on the post embryonic development of organisms may provide a good basis to conduct further research to improve immune response, higher productivity, and reduce use of antibiotics in animal production. Experiments of Ohta et al. (1999) injected amino acids into the yolk sac at both 0th and 7th days; both injections resulted in increased body weight with no effect on hatchability. And the addition of amino acids stimulated the utilization and synthesis of amino acids with a simultaneous decrease in the degradation of amino acids (exact biochemical degradation not specified), when the amino acids injected were identical to those naturally occurring in the egg. Recently experiment conducted by Calik et al. (2016) proved combined intra-amniotic and dietary symbiotic treatment improved broiler intestinal integrity and increased cecal beneficial bacteria population. The villus height and goblet and proliferating cell nuclear antigen positive cell counts were positively influenced by the intra-amniotic and dietary symbiotic treatments. This treatment also increased lactobacillus colonization and decreased coliform population in broiler cecum (Calik et al., 2016). In summary, the experimental studies of injections with carbohydrate, amino acids, synbiotic indicate the potential benefits for the commercialization of *in ovo* injection of nutrients (Roto et al., 2016).

1.5.3 Advantages and limitations of the *in ovo* technology

In ovo technology, as one of the most essential measures of early nutrition programming, own its premium advantages and shortcomings. Earlier immunity against disease with minimal interference from maternally-derived antibodies. By *in ovo* inoculation, the chick has the best possible start when it hatches, and better disease resistance from day one. Minimal chick handling, which reduces chick stress, improves bird health, allowing chicks to be transported to the farm faster and into the ideal grow-out environment. However, there still exists some limitation of this

technology. First, it is economically suitable only for large-volume hatcheries. For this technology, it hard to make sure the exact site of injection, and due to variable size of each embryo in eggs, it enhanced the damage possibility when using *in ovo* injection machine. If operation by manual, it is also difficult to control the time, environment and other conditions. Most essential point is there are no consistent results on the ideal time, site, and volume of *in ovo* injection. Different injection material has specific optimal injection procedure. It is critical to evaluate best *in ovo* injection regular pattern. More research on *in ovo* injection technology will provide a convenient and economic method on the development of early nutrition programming.

1.6 Hypothesis of the study

The selected probiotic, prebiotic, and synbiotic have the desirable nutritional profile and immune function to be used as alternative growth promoters when utilized by *in ovo* technology on broiler chickens for early nutrition programming.

1.7 Justification of study

A healthy gastrointestinal tract is crucial for optimum performance, better feed efficiency and overall health of poultry. Due to the claimed public health concerns, use of antibiotics as growth promoters in the chicken feed is banned or regulated in several jurisdictions. Therefore, it is necessary to find alternatives to AGP. Probiotics, prebiotics, and synbiotics are being evaluated as effective alternatives to AGP to improve growth performance and nutrient digestibility, balance intestinal microflora, promote immune function, and improve the intestinal morphology of poultry. Probiotic *Bacillus Coagulans* is a lactic acid-forming bacterial species. It exhibits characteristics typical of both genera *Lactobacillus* and *Bacillus*, and it was finally transferred to the genus

Bacillus in the seventh edition of Bergey's. Many factors make *B. coagulans* a good candidate for probiotic use; It produces organic acids, possesses the capacity to sporulate, and is easily cultured in bulk. In addition, in the spore form, it is more resistant to heat, which facilitates the pelleting process used in the mass production of probiotic chicken feeds (Hyronimus et al., 2000). Raffinose family oligosaccharides are considered to be prebiotic compounds because they are not hydrolyzed in the upper gastrointestinal tract and are able to alter the colonic microflora favorably. The synbiotic is the mixtures of *B. coagulans* and raffinose, thus combine bacteria and substrate.

Early supplements application, especially *in ovo* technology, represent a means to take advantage of the crucial time and promote early colonization of beneficial microbiota in order to stimulate intestinal and immune system development. It is critical to determine the injection time and size. According to the patent of Uni and Ferket (2003), the ideal injection time is 17 d of incubation egg on the amniotic fluid. After hatch, raising 42 d to observe the growth parameter and health condition of different stage broiler chicken.

1.8 Objective of the study

The primary objective of this study was to evaluate the growth performance of broilers with *in ovo* injection of probiotic, prebiotic, and synbiotic. Secondly, to explore the effects of *in ovo* administration on gut health parameters, and to compare the extent of these influence.

CHAPTER 2: METHODS

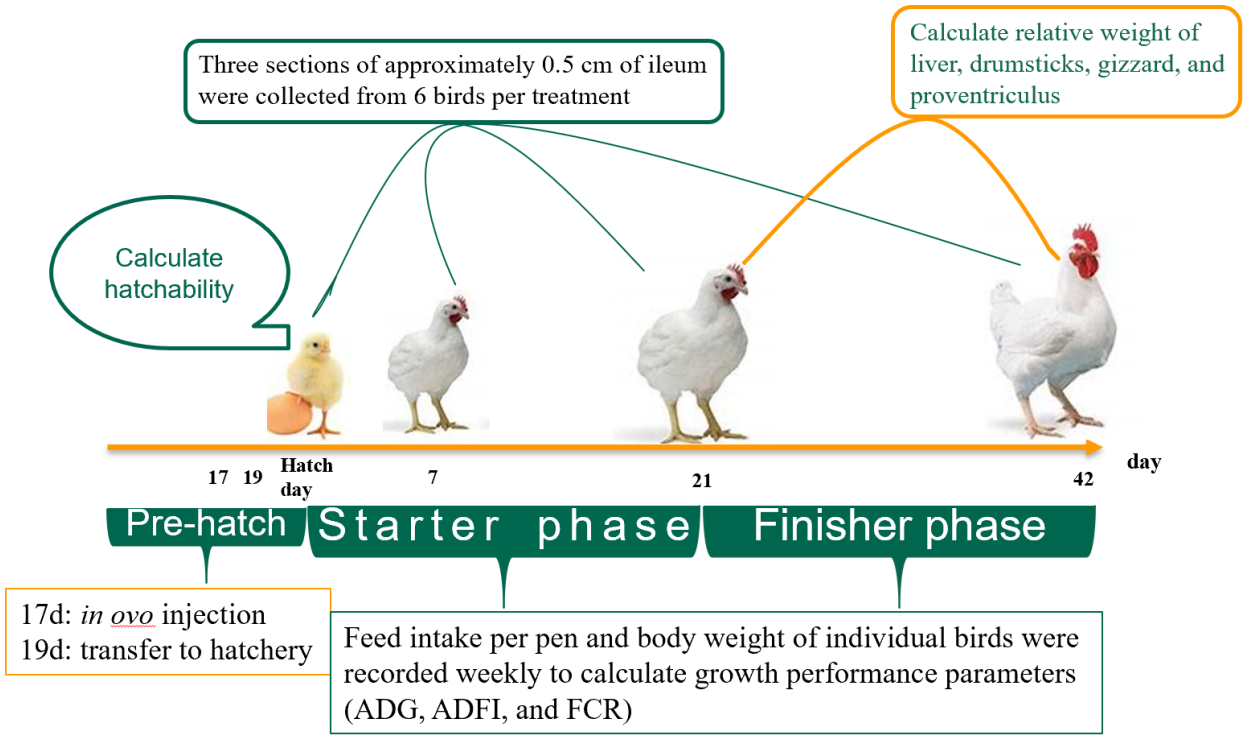
2.1 Materials and Methods

Egg incubation was conducted in the Animal Nutrition Laboratory of University of Hawaii at Manoa (Honolulu, HI). Growth performance was conducted at the Small Animal Facility (SAF) of UH Manoa. The study was conducted after approval by the Institutional Animal Care and Use Committee and following the Research Policy of UH Manoa.

2.2 Experimental design and egg incubation

275 fertile eggs (Cobb 500) from the 35-week breeding flock was obtained from a commercial hatchery (Asagi Hatchery Inc., Honolulu, HI) at 17th day of incubation. On arrival, the eggs were weighed and numbered and incubated at 37.5^{o}C and relative humidity of 58% in an incubator (GQF incubator, Savannah, GA). After the eggs are acclimatized in the incubator for >8 hours, 55 eggs were randomly assigned from different weight groups to each treatment. The eggs were numbered for each treatment groups and were randomly allocated to 5 replicates of each of 5 treatments by location on the setter trays. The eggs were transferred on d 19 to hatcher set at 37^{o}C and relative humidity 75% following the instructions for pre-set hatcher (GQF incubator, Savannah, GA). Each replicate group of eggs from setter was again randomly assigned to different hatcher trays separated by divider. After hatching, chicks from each treatment was weighed and tagged. Depending on the hatch, 48 chicks from each treatment was moved to the Small Animal Facility of the University of Hawaii at Manoa (Honolulu, HI) and randomly allocated to 6 replicate pens (8 chicks/pen). The whole process of the trial is showed in Figure 1.

Figure 1. Schematic diagram of the whole experimental trial



2.3 *In ovo* injection

For *in ovo* injection, each replicate group of eggs was taken out for injection in a biosafety cabinet and placed out of incubator for less than 15 minutes. At d 17.5 broad end site of all eggs was disinfected with 10% povidone-iodine solution and then a tiny punch hole (shell perforation) was made with a stabbing awl with a fixed depth of 1mm made with a pipette sheath/sleeve. After every punch, the tip of the awl was disinfected with 70% ethanol and wiped with sterile gauze. Each egg was injected in their amniotic sac using blunt tip 21 gauze sterile needle inserted to 1.1-inch length from the longest axis through the broad end and passing beyond the air sac to amnion fluid. All the eggs were sealed using non-toxic parafilm/glue. Probiotic *Bacillus coagulans* ATCC 7050 (KWIK-STIK unit contains lyophilized pellet) were bought from Microbiologics Inc, MN, USA. All together there were five treatment groups: 1) No-injection group with intact shell, 2) 0.5

ml 0.85% Normal Saline, 3) Probiotic (*B. coagulans*) (4×10^6 cfu/g) in 0.5 ml 0.85% Normal Saline, 4) Prebiotic (4.5 mg RFO) in 0.5 ml 0.85% Normal Saline, and 5) Synbiotic (4×10^6 cfu/g *B. coagulans* + 4.5 mg RFO) in 0.5 ml 0.85% Normal Saline.

2.4 Hatchability, growth performance, and organs relative weight

After hatch, the unhatched eggs were counted and opened to check for the cause of death of embryo. Hatchability was noted for all replicate and was being subjected to statistical analysis after arcsin square root transformation. The weighed and tagged chicks were placed randomly in 30 floor pens (7 birds per pen), making 6 replicates of each treatment. Birds in all the floor pens were raised under standard commercial broiler rearing environment (temperature, humidity, and light). The temperature in the first wk was maintained at 35ºC and gradually decreased to 28ºC by the end of the third week. All birds were fed with a commercial corn-soybean meal-based pellet diet during the starter 21-day post-hatch trial period (Table 1), and finisher 21-42 day post-hatch trial period (Table 2). The diet met or exceeded the nutritional requirements of broiler chickens (standard guidelines of breeder) and birds had unrestricted access to feed and water at all times. Body weight and feed consumption of the birds were measured by pen at 7, 14, and 21 d of age, and ADG, ADFI, and FCR were calculated from these data by period and cumulatively. Feed wastage was recorded daily, and the feed consumption was adjusted for wastage and bird mortality. On d 21, d 42, 6 birds per treatment (1 bird per pen) were randomly chosen for the determination of organ weights and were dissected after euthanizing with CO_2 gas. The weight of breast muscle, drumsticks, gizzard, and proventriculus were recorded, and the relative weight (% of live body weight) was calculated.

Table 1. Ingredient composition of starter diet fed to the broilers in the study

Item	Inclusion level
Active drug ingredients	
Amprolium	0.0125%
Guaranteed analysis	
Crude Protein (Min)	22.00%
Lysine (Min)	1.00%
Methionine	0.45%
Crude Fat (Min)	3.50%
Crude Fiber (Min)	4.00%
Calcium (Ca) (Min)	0.90%
Calcium (Ca) (Max)	1.40%
Phosphorus (P) (Min)	0.60%
Salt (NaCl) (Min)	0.30%
Salt (NaCl) (Max)	0.80%
Total Selenium (Se) (Min)	0.60 ppm
Total Selenium (Se) (Max)	0.72 ppm
Phytase (A. Oryzae) (Min)	227 FYT/LB

One phytase unit (FYT) liberates one micromole of inorganic phosphorus per minute from sodium phytate at pH 5.5 and 98.6 F. Contains a source of phytase, Ronozyme HiPhos GT, which can hydrolyze phytate increasing the digestibility of phosphorus in diets containing phytin-bound phosphorus.

Table 2. Ingredient composition of finisher diet fed to the broilers in the study

Item	Inclusion level
Active drug ingredients	
Amprolium	0.0125%
Guaranteed analysis	
Crude Protein (Min)	18.00%
Lysine (Min)	0.86%
Methionine	0.40%
Crude Fat (Min)	3.50%
Crude Fiber (Min)	5.00%
Calcium (Ca) (Min)	0.90%
Calcium (Ca) (Max)	1.40%

Phosphorus (P) (Min)	0.50%
Salt (NaCl) (Min)	0.20%
Salt (NaCl) (Max)	0.70%
Total Selenium (Se) (Min)	0.65 ppm
Total Selenium (Se) (Max)	0.78 ppm
Phytase (A. Oryzae) (Min)	227 FYT/LB

One phytase unit (FYT) liberates one micromole of inorganic phosphorus per minute from sodium phytate at pH 5.5 and 98.6 F. Contains a source of phytase, Ronozyme HiPhos GT, which can hydrolyze phytate increasing the digestibility of phosphorus in diets containing phytin-bound phosphorus.

2.5 Ileum Mucosa Histology

On d 0, ileal samples (n=3/treatment) and on d 7 & 21 (n=6/treatment) (1 bird per cage) after euthanasia was collected in fixatives solution for histomorphological study. Briefly, a section of approximately 1 cm of small intestine (between 1 cm posterior to the Meckel's diverticulum and 1 cm anterior to ileocecal junction) was collected in 2 replicates for each fixative. Ileal samples were stored in 1:10 volumes of each fixative. The fixatives were 10% neutral buffer formalin (NBF) kept in ice bath. The samples were fixed overnight. NBF samples were transferred to 70% ethanol. The samples fixed in NBF were sent to Histo-core at JABSOM for embedding and staining with Hematoxylin and Eosin (H&E). A total of 6 intact, well-oriented crypt-villus units were selected in triplicate (18 measurements for each sample). An upright light microscope (Olympus BX43, Olympus Co, Tokyo, Japan) was used for histological analysis. Villus height was measured from the tip of the villi to the villus crypt junction, and crypt depth was determined as the depth of the invagination between adjacent villi. Measurement of villus height and crypt depth was performed using image processing and analysis system of the software, INFINITY ANALYZE, specialized for the microscope. The objective magnification was used at 20X, 10X, and 5X at hatch, and 7 d post hatch for ileum histological slides for better measurement.

2.6 RNA Extraction, Reverse Transcription, and Real-Time Quantitative PCR

Three sections of approximately 0.5 cm of ileum were collected from 6 birds per treatment (1 bird per replicate). Ileum sections were collected and snap frozen in liquid nitrogen, later stored at −80◦C until total RNA isolation. For RNA isolation, the tissue was removed from RNA later solution to a micro-tube. Approximately 50-100 mg of the tissue were be homogenized directly in TRIzol RNA isolation reagent (Invitrogen, Carlsbad, CA). Total RNA was extracted using 1 mL of TRIzol per sample according to the manufacturer's instructions. After RNA extraction, RNA concentration was measured with NanoPhotometer® P330 (IMPLEN, Los Angeles, CA). RNA quality was determined with the Agilent 2100 Bioanalyzer (Agilent Technologies, Massy, France). The synthesis of first- strand cDNA was performed by reverse transcription of 1 µg total RNA (20 µl reaction of RT mixture) using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). cDNAs were further diluted with nuclease free-water (1:25) and 3 µl per qPCR reaction was used for gene expression. PowerUp SYBR Master Mix (Applied Biosystems, Foster City, CA) on a StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA) was used for gene expression analyze. Each 10 µl of PCR reaction mixture of consisted of 5µl PowerUp SYBR Green Master Mix, 1 µl of each forward and reverse primers, and 3µl of cDNA. PCR reactions were carried out following standard cycling mode. Melting curve was also generated to confirm the sequence-specific PCR products. The cycle threshold (Ct) value was determined, and the abundance of gene transcripts was analyzed using the ΔΔCt method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the reference gene (Berrocoso et al., 2017), and additionally, we were using Beta-actin (β-actin), TATA-Box Binding Protein (TBP) as a reference and normalization gene. The target genes were analyzed in duplicates and expression

level was determined using Ct value following standard curve method after normalization with TBP.

Innate and adaptive immunity stimulation due to probiotics and prebiotics inclusion will be determined measuring gene expression of respective immune cell markers. The *CD56* gene marker for NK-cells, *TLR4* gene marker for macrophage, *IL-10* gene marker for anti-inflammatory cytokines and *IL-1β* gene marker for pro-inflammatory cytokines were amplified to see their relative abundance which can explain about the status of innate immunity and any ongoing inflammation. In order to access the influence of the treatment on adaptive immune response, the *CD3* (membrane protein expressed in T cell at all stage of development) and *ChB6* (also known as Bu-1 is expressed on early and mature B cells) gene markers of T-cell and B-cell were amplified using the respective primers (Table 3). Fold change for each gene was calculated using the $2^{-\Delta\Delta Ct}$ method. Data for fold change were presented as mean ± standard error. Values were subjected to one-way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple mean comparison to determine significance at $P < 0.05$ on a SAS platform.

2.7 Statistical analysis

Data were statistically analyzed using the MIXED procedure of SAS (SAS v9.2, SAS Institute Inc., Cary, NC) to compare the test variables. Means were separated by Tukey test using pdmix macro of SAS and were declared significant if $P < 0.05$.

Table 3. Nucleotide sequences of primers used in Real-time qPCR analyses

Gene	Primer	Sequence	Genbank Accession number	Amplicon Size (bp)
Immune cell:				
CD3	Forward	5'-GGACGCTCCCACCATATCAG-3'	NM_205512	180
	Reverse	5'-TGTCCATCATTCCGCTCACC-3'		
CD14	Forward	5'-TGGACGACTCCACCATTGAC-3'	NM 001139478	132
	Reverse	5'-CCATCTCCTGCACCTGAGTG-3'		
CD45	Forward	5'-TATTCTTGGTGTTCTTGATTGTTGTG-3'	NM_204417	120
	Reverse	5'-CTGCTACAAGGCTGATGACTTCA-3'		
CD56(NCAM1)	Forward	5'-GTTCATGAGCAGAGGGTGCT-3'	NM 001242604	196
	Reverse	5'-ACATGGCCTGGATGATGCAA-3'		
ChB6 (Bu-1)	Forward	5'-TACTTTGTCGGCCGAGTGTC-3'	NM 205182	197
	Reverse	5'-AGTCTGCAGTTCCATTGGGG-3'		
TLR4	Forward	5'-AGTCTGAAATTGCTGAGCTCAAAT-3'	NM_001030693	190
	Reverse	5'-GCGACGTTAAGCCATGGAAG-3'		
Cytokine:				
IFN-gamma	Forward	5′-CTGAAGAACTGGACAGAGAG-3'	NM 205149.1	264
	Reverse	5′-CACCAGCTTCTGTAAGATGC-3'		
IL-1β	Forward	5′-CGCTTCATCTTCTACCGCCT-3'	NM_204524	144
	Reverse	5′-GATGTTGACCTGGTCGGGTT-3′		
IL-4	Forward	5′-TGTGCCCACGCTGTGCTTACA-3'	NM 001030693	155
	Reverse	5′-CTTGTGGCAGTGCTGGCTCTCC-3'		
IL-10	Forward	5′-TGTCACCGCTTCTTCACCTG-3′	NM_001004414	105
	Reverse	5′-CTCCCCCATGGCTTTGTAGA-3′		
Reference:				
GAPDH	Forward	5'-AGCTTACTGGAATGGCTTTCCG-3'	NM_204305	122
	Reverse	5'-ATCAGCAGCAGCCTTCACTACC-3'		
TBP	Forward	5'- TAGCCCGATGATGCCGTAT-3'	NM_205103	147
	Reverse	5'- GTTCCCTGTGTCGCTTGC-3'		
β–actin	Forward	5'- GAGAAATTGTGCGTGACATCA-3'	X 00182	152
	Reverse	5'- CCTGAACCTCTCATTGCCA-3'		

Chapter 3 Results

3.1 Hatchability

The *in ovo* injection did not affect ($P > 0.05$) hatchability of chicks across the treatments (Table 4).

Table 4. Effects of *in ovo* injection on hatchability

	Non-injected	Normal Saline	*B. coagulans*	RFO	*B. coagulans*+RFO
Total eggs	51	49	49	49	49
Hatched eggs	49	45	45	42	46
Hatchability	0.96	0.91	0.91	0.85	0.93

3.2 Effect of *in ovo* injection on growth performance and relative organs weight

There was no significant effect of treatments on body weight, average daily gain and feed intake of broilers (Table 5). However, birds from normal saline treatment had significantly better ($P > 0.05$) feed efficiency and RFO group had the poorest in the first week of post-hatch period. The results of injection of *B. coagulans*, RFO, and *B. coagulans* + RFO on the relative weights of digestive organs are summarized in Table 6. On d 21 and d 42 of age, the relative weight of the proventriculus, drumstick, breast muscle, and gizzard were not affected ($P > 0.05$) by inoculation supplements.

Table 5. Effects of *in ovo* injection on growth performance of broiler chickens

	Non-injected	Normal Saline	*B. coagulans*	RFO	*B. coagulans*+ RFO	SEM (n = 6)	P-value
Initial BW	44.2	45.1	44.7	44.2	44.6	1.33	0.7
d 0 to 7							
ADG, g	14.8	15.7	15.5	15	15.7	0.78	0.225
ADFI, g	19.6	20.2	20.3	21.6	20.6	1.6	0.319
FCR	1.325	1.29	1.306	1.441	1.317	0.091	0.059
d 7 to 14							
ADG, g	44.7	45.2	45.5	44.8	45.8	1.71	0.76
ADFI, g	65.5	69.1	65.7	66.8	67.2	5.13	0.754
FCR	1.469	1.53	1.441	1.49	1.466	0.096	0.59
d 14 to 21							
ADG, g	75.7	76.7	77.2	76.2	77.2	4.468	0.971
ADFI, g	119.4	119.5	118.2	120.6	119.2	8.319	0.993
FCR	1.581	1.558	1.531	1.585	1.544	0.081	0.744
d 21 to 28							
ADG, g	78.3	82	81.2	82.7	81.5	6.359	0.799
ADFI, g	159.2	174.5	172.4	165.9	169.6	22.583	0.79
FCR	2.032	2.137	2.125	2.009	2.073	0.226	0.83
d 28 to 35							
ADG, g	80.2	77.3	83	86.2	85.7	9.145	0.311
ADFI, g	189.8	201.7	200.6	191.6	200.9	29.132	0.918
FCR	2.369	2.615	2.417	2.23	2.344	0.27	0.198
d 35 to 42							
ADG, g	89.7	95.5	89.5	96.3	94.2	11.591	0.757
ADFI, g	204	229.9	226.1	223.8	218.3	32.418	0.676
FCR	2.29	2.448	2.522	2.335	2.347	0.364	0.804

BW: Body weight; ADG: Average daily gain; ADFI: Average daily feed intake; FCR: Feed conversion ratio

Table 6. Effects of *in ovo* injection on relative weight (% of BW) of liver, gizzard, proventriculus, drumsticks

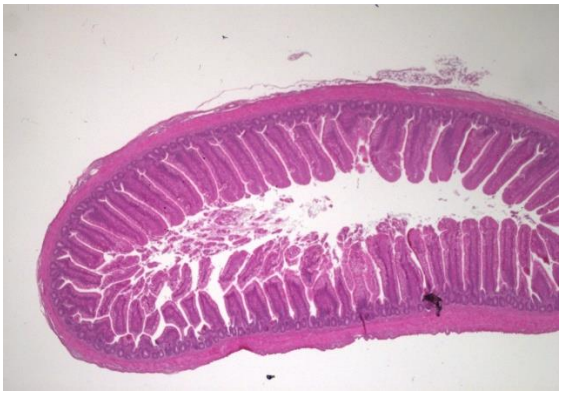
	Non-injected	Normal Saline	*B. coagulans*	RFO	*B. coagulans*+ RFO	SEM (n = 6)	P-value
d 21							
Liver, %	2.83	2.78	2.68	2.82	2.77	0.0034	0.942
Gizzard, %	1.95	2.16	2.07	2.01	1.92	0.00222	0.339
Proventriculus, %	0.5	0.57	0.61	0.53	0.51	0.00118	0.523
Drumsticks, %	8.34	8.17	8.2	8.06	8.32	0.00484	0.848
d 42							
Liver, %	2.53	2.04	2.2	2.22	2.45	0.0035	0.132
Gizzard, %	1.42	1.44	1.27	1.58	1.55	0.00269	0.324
Proventriculus, %	0.36	0.39	0.4	0.36	0.46	0.0009	0.352
Drumsticks, %	8.92	9.86	9.47	9.37	8.86	0.00826	0.23

3.3 Effects of *In ovo* injection on ileum mucosa morphology of chickens

At d 7 of age, ileum villus height, crypt depth, and villus height: crypt depth ratio of prebiotic groups were significantly better than other treatments ($P < 0.05$) (Figure 2; Table 7). On d 7 post-hatch, probiotic group had better villus height to crypt depth ratio, which suggests about better intestinal health, but its impact on growth may not be evident in healthy flock maintained in standard condition.

Figure 2. Effects of *in ovo* injection on ileum mucosa morphology of chickens at 7 d of age

Images were separately captured with light microscope (Olympus BX43, Olympus Co, Tokyo, Japan) at 10 X, 20 X magnification. a: villus ehight; b: crypt depth; a/b: villus height to crypt depth ratio.



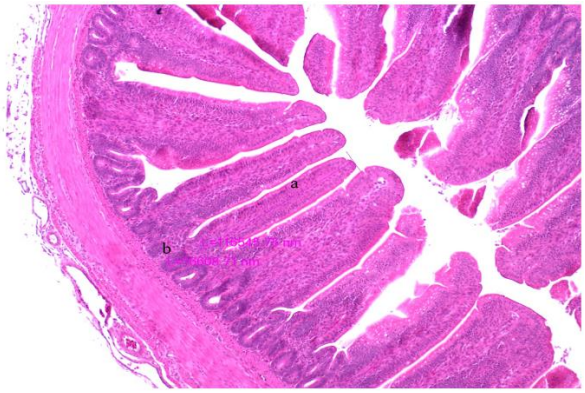


Table 7. Effects of *in ovo* injection on ileum mucosa morphology of broiler chickens.

	Non-injected	Normal Saline	Probiotic	Prebiotic	Synbiotic	SEM (n= 6)	P-value
VH	1230.1	1183.9	1237.8	1273.5*	1141.2	0.113	0.007
CD	196.1	179.1	198.7	156.1*	184.1	0.033	0.001
VH/CD	6.49	6.95	6.29	8.46*	6.28	0.001	<0.001

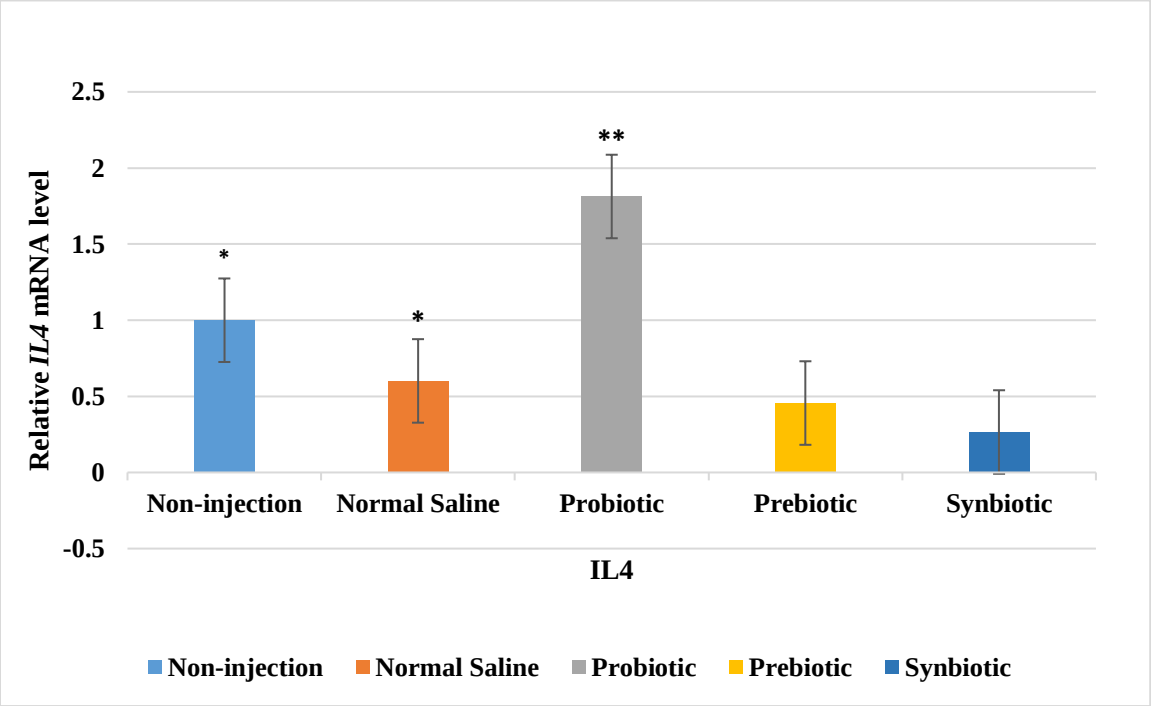
VH: villus height (μm); CD: crypt depth (μm); VH/CD: villus height to crypt depth ratio

3.4 Effect of *in ovo* injection on immune related gene expression in ileum tissue

IL4, a cytokine had significantly higher expression ($P < 0.001$) in the probiotic group's ileum sample on d 0 (Figure 3A). The expression of *IL10*, an anti-inflammatory cytokine was significantly higher ($P < 0.05$) in Normal saline and probiotic group's ileum sample on d 7 (Figure 3B). *SGLT1* had significantly higher ($P < 0.05$) expression on No-injection and probiotic treatments, while prebiotic and synbiotic group had lower expression (Figure 3B).

Figure 3A, 3B. Effects of *in ovo* injection on immunity of small intestine of broilers

Total RNA was extracted from the small intestine of broilers at hatch day and 7 d of age. The expression of each gene was examined using RT-qPCR and expressed as ratio to TBP, with the level being set to 1 in broilers treated without injection in each gene. A: Effect of *in ovo* injection on cytokine *IL4* on hatch day. B: Effect of *in ovo* injection on anti-inflammatory cytokine *IL10*, and *SGLT1* on 7d. ∗ indicates $P < 0.05$.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2
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Relative IL4 mRNA level
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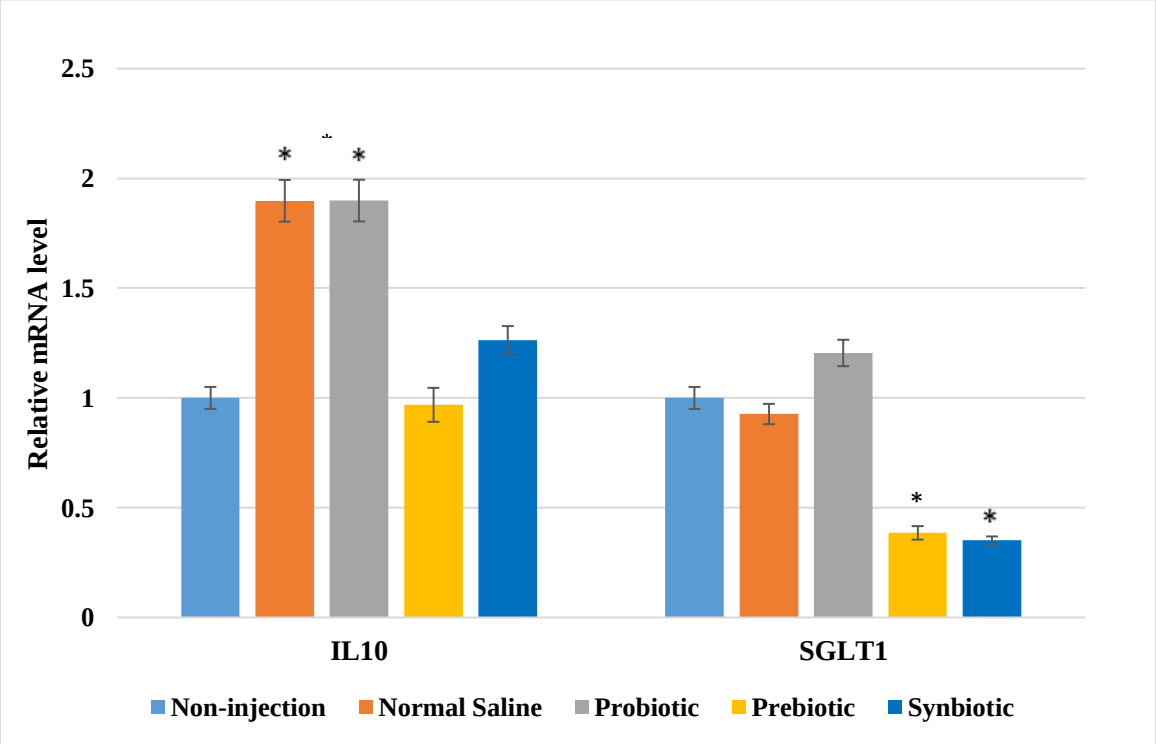
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Relative mRNA level
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IL10
SGLT1
Non-injection
Normal Saline
Probiotic
Prebiotic
Synbiotic

Chapter 4 Discussion

4.1 Growth performance

In ovo administration of *B. coagulans*, RFO, *B.coagulans* +RFO had no effect on the body weight, relative weight of the liver, proventriculus, gizzard, or drumsticks. Similarly, Berrocoso et al. (2017) reported that *in ovo* injection of RFO did not have a significant effect on d 21 broilers. Both experiments proved *in ovo* procedure did not influence hatchability. Feed efficiency was not much influenced by treatments, but it showed a strong tendency that birds from normal saline treatment had significantly better ($P > 0.05$) feed efficiency and RFO group had the poorest in the first week of post-hatch period. However, Hung et al. (2012) reported that dietary *B. coagulans* significantly improved FCR throughout the whole 42 days compared with negative control. It demonstrates that different supplementation methods and amount on *B. coagulans* may change their influence on broiler chickens. In general, RFO treatment in this experiment shows relatively negative trend in BW, FCR. There is evidence of negative effects on animal health and productivity from the use of RFO. The possible reason may be the raffinose series oligosaccharide remain undigested in the lower gut, can only be fermented by intestinal bacteria and release gases, due to the absence of a-galactosidase in the upper GIT of monogastric animal (Iji and Tivey, 1998). Including of RFO in diets may influence the absorption of diets energy value. Moreover, the digestion of raffinose may also alter the osmotic differences between the mucosa and plasma, and this may account for the diarrhea observed in animals on diets with high level of legume seeds (Saini et al., 1988).

4.2 Gut health

On d 7 post-hatch, RFO group had better villus height to crypt depth ratio, which suggests about better intestinal health. But when we go back to Berrocosa et al. (2017) experiment, they reported that *in ovo* injection RFO with an increasing dose increased ($P < 0.01$) the villus height on hatch day, and increased the villus height and villus height to crypt depth ratio ($P < 0.05$) at 21 d of post hatch. Therefore, the injection of RFO affected the ileum mucosa morphology of chickens differentially depending on stages of growth and concentration of RFO. Gut morphology and function determined the efficiency of digestion and absorption of nutrients, which impact growth of broiler chickens. Villus height, crypt depth, and villus length to crypt depth ratio are good indicators for functional capacity of the intestine (Fasina and Olowo, 2013). It has been proved that higher villus height is associated with a well-differentiated intestinal mucosa with high capacity of digestive and absorption. And deeper crypt depth represents on the faster tissue turnover and have the potential of higher demand for new tissue (Berrocoso et al., 2017). However, in the current study, the improvement in the development of the ileum mucosa, as was measured by villus length and villus height/crypt ratio, had no effect in improving growth performance from 0 to 7 d post hatch, indicating that the measure of intestinal morphology such as villus length and villus height/crypt ratio does not necessarily translate into improved growth performance, and its impact on health flock are not evident.

In this study, injection of *B. coagulans* upregulated *IL4 mRNA* expression on hatch day ileum tissue. *IL4* is a cytokine that helps in the differentiation of helper T cells to Th2 cells, stimulates proliferation of activated B- cell and T-cell, and induces the differentiation of B cells into plasma cells, which is a key regulator in humoral and adaptive immunity. And the presence of *IL4* in extravascular tissue promoted alternative activation of macrophages into M2 cells and inhibited classical activation of macrophages into M1 cells. The higher expression of *IL4* proved *in ovo*

injection of *B. coagulans* could boost early immunity. On d 7 post-hatch, expression of cytokine *IL10* in the ileum in normal saline and probiotic groups were significantly higher ($P < 0.05$); As an anti-inflammatory cytokine, *IL10* can impact on immunoregulation and inflammation by downregulation the expression of Th1 cytokines, and co-stimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. *In ovo* Supplementation with *B. coagulans* showed significantly high *IL10* expression on 7 d ileum sample proved that *in ovo* injection of *B. coagulans* did has the beneficial effect on immune function in poultry. However, this beneficial effect cannot reflect in the performance of healthy broiler chickens. Sodium/glucose cotransporter 1 (*SGLT1*) expressed significantly higher ($P < 0.05$) in Non-injection and probiotic groups, while prebiotic and synbiotic treatments had lower gene expression. Maybe early injection of RFO has some role in controlling blood glucose, and this function may also related with RFO cannot digested in the lower gut.

4.3 Conclusion

In conclusion, although *in ovo* injection of *B. coagulans*, RFO, *B.coagulans* +RFO did not significantly influence growth performance, RFO supplementation enhanced the ileum mucosa morphology, which are indicators of improved gut health. *In ovo* injection of *B. coagulans* improved growth performance, even not significant, and enhanced immune response indicators in the small intestinal, which would be beneficial for gut health. The combination of probiotics and prebiotics did not show some significant impact on broiler chickens. However, it is interesting to find that *in ovo* injection of normal saline also enhanced gut immunity. Thus, *in ovo* injection of *B. coagulans* can be a potential early nutrition programming strategy to improve gut development and immune function of broiler chickens.

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