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A Metagenomic Approach for Detection of the Bacterial Community Changes in Poultry as Affected by Various Feed Additives

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Abstract

Metagenomics is a culture-independent method that allows the identification and characterization of organisms from all types of samples. So, this study aimed to use a metagenomics approach to compare the benefits of two commercially available growth promoters namely synbiotic (Micro-BACLA)® and antibiotic (zinc bacitracin) ® on microbial biodiversity and immunity properties in poultry. In total, 120-day-old Ross chicks from an Egyptian commercial hatchery were divided into three groups (n=40) with four repetitions per treatment. The groups namely (G1) control group, (G2) antibiotic group, (G3) synbiotic group. At the phylum level, five different phyla of bacteria were detected, Firmicutes had the largest representation with 93.87, 90.94 and 91.38% in G3, G2, and G1, respectively. At the family level, Lactobacillaceae and Bacillaceae were dominant in G3, while Lachnospiraceae represented the largest percentage in G1. Furthermore, *Ruminococcaceae* was the highest family in the antibiotic group. At the genus level, *Lactobacillus* represented the highest percentage in G2 and G3 accounting for 23.91 and 35.28%, respectively. Contrarily, the *Lachnoclostridium* was more prevalent in the G1 at a rate of 29.95% compared to other groups. Regarding the immune response, the serum IgG and total IgE levels were equal at 50 ng/ml and 0.20, respectively, and did not change in response to synbiotic or antibiotic supplements in fodders. Whereas, IgA and IgM in chicken' serum were affected by the supplement of fodder with synbiotic compared to antibiotic and control groups, both of them were decreased. The current study concluded that adding synbiotics to the chicken broilers diet could increase nutrient digestion and growth performance by minimizing the negative effects of antibiotics and maximizing the synbiotic's advantages in the gut.

Keywords: Synbiotic, broiler chickens, bacterial diversity, immune response.

Introduction

The gastrointestinal tract (GIT) microbiota of humans and animals is made up of diverse communities that comprise a wide variety of bacterial species and smaller numbers of fungi, protozoa, and archaea **(Stanley et al., 2016).** Our understanding of the various functional roles and interactions of the gut microbiota has improved over the past years. For instance, in a healthy microbiota-host relationship, the microbiota increases the host's metabolic capacity, aids in food digestion, creates micronutrients, modifies the immune system, and interacts with the central nervous system **(Gill et al., 2006, Mayer et al., 2015, Turnbaugh et al., 2007).** Due to the physiological, nutritional, and immunological activities of the gut microbiota in chickens, high-throughput sequencing technology has made it clear that diet significantly influences the makeup of the gut microbiota **(Stanley et al., 2013).** In this regard, the poultry industry is very interested in knowing how dietary elements alter gut bacteria. Antibiotic growth promoters (AGPs) have been extensively employed to boost food animal performance. Broiler chickens are given antibiotics to prevent and treat illnesses such as necrotic enteritis caused by Clostridium perfringens as well as to encourage faster growth and increase conversion rates **(Castanon, 2007).** Recently, this practice has sparked worries about the growth of bacterial strains resistant to antibiotics that could infect humans **(Maron et al., 2013).** The key advantage of a synbiotic is that it does not leave any residues in animal production or contribute to antibiotic resistance when consumed. Synbiotics are being researched as a way to fill this gap, and some farmers are already choosing to use them instead of antibiotics. As a result, several researchers now substitute synbiotics for various antibiotics to treat infections and promote growth **(Jha et al., 2020).** Due to the lack of research that studied the difference between using antibiotics compared to synbiotics on broiler productivity, this study aimed to shed light on the effect of bacterial synbiotics plus an enzyme mixture (Micro-BACLA) on immunity properties,

and microbial biodiversity in the cecum contents using Metagenomic approach.

Materials and Methods

1.1. Experimental location

This research was conducted during the period from November 20th, 2022 to December 25th, 2022 at a private chicken farm in Met Fadala, Aga, Dakahlia Governorate, Egypt (30°52'43.2"N; 31°20'58.3"E).

1.2. Experimental design

A total of 120 day-old Ross chicks were obtained from a commercial hatchery in Egypt, then allotted into three groups (n=40), with for repetition. G1: the control group without any additives, G2: the fodder amended with zinc bacitracin (15%) at rate of (55g/ton), G3: the fodder amended with Micro-BACLA at rate of (500g/ton). Both products were obtained from PROPYN International Inc., (https://www.probyn.com/microbacla.html).

1.3. Housing

In a spotless, well-ventilated space that had previously undergone burning, Virkon-S, and TH4 disinfection, broiler chicks were kept. The space was divided into nine sections, each measuring 1.0 m^2 for the first 10 days and 3.0 m^2 thereafter. Five centimeters of fresh, clean wheat straw bedding material were provided for each partition. With the help of a gas heater and a 200-watt electric bulb, chicks were brooded at a beginning temperature of 33°C during the first week, which subsequently decreased to 25°C by the end of the fifth week. Continuous natural and artificial light programs were used on the birds.

1.4. Feeding and medical care

Birds were fed on a starter mash ration for the first three weeks from Apex Feed Company in Egypt (https://www.apexcairo.net), followed by a grower diet containing 21.5% protein and 3150 kcal/kg of metabolized energy until the experiment's termination in the fifth week. Four fortifications were used to immunize the birds against New Castle disease namely Hitchner B1 live virus intra-ocular at day 7, ND plus AI killed virus S/C on day 9, Gumboro E228 live virus in drinking water on day 14, and ND Colon 79 live virus in drinking water at day 19. Both the grower feeder and fortifications were from Dakahlia for Poultry Company in Egypt (https://dakahliapoultry.com).

1.5. Metagenomics

1.5.1. Samples collection

Random samples from each group were used to butcher the birds after the trial (35 days of age), and then a mid-section of the cecum was cut at both ends before being immediately placed on ice. The gut content samples were stored at -20°C until the isolation of bacterial genomic DNA.

1.5.2. DNA Extraction and PCR Amplification

The Genomics and Epigenomics Lab of Hospital 57357's Children with Cancer in Cairo, Egypt, performed the DNA extraction, library preparation, and next-generation sequencing for chicken cecum Using the DNeasy® PowerSoil® Pro kit and adhering to the manufacturer's recommendations, the microbial DNA was extracted. Using 16S amplification (PCR), the V3–V4 hypervariable region of the 16S rRNA gene was amplified Forward primer =5'TCGT CGGCAGCG TCAGATGTGTATAAGAGACAGCCTACGGGNG GCWGCAG and 16S Amplicon PCR Reverse Primer = 5'GTC TCGTG GG CTCGGAGAT GTGT ATAAGAGACAGGACTACHVGGGTATCTAATC C.

AMPure XP beads were used in the PCR process to separate the 16S V3 and V4 amplicons from free primers and primer dimer species. Dual indices and Illumina sequencing adapters were connected using the Nextera XT Index Kit (Illumina, USA). To check the size, 1 µl of the finished library was placed on a Bioanalyzer DNA 1000 chip. The finished library should be about 630 bp in size if the protocol's V3 and V4 primer pairs are used. Illumina advises measuring libraries with a dsDNA-binding fluorometric quantification technique. The library is pooled and normalized following Illumina production guidelines (Illumina, USA).

1.5.3. Bacterial data analysis

In QIIME2 V2022.2, raw 16S rRNA gene sequences were imported and demultiplexed **(Bolyen et al., 2019).** Primers were deleted using cutadapt v2.6 **(Martin, 2011).** To get rid of low-quality bases (mean Q score 30), correct sequencing mistakes, and produce bacterial amplicon sequence variants (ASVs), DADA2 was utilized in the QIIME2 environment for filtering, denoising, and chimera testi **(Callahan et al., 2016)**. Using the silva-138-99-nb-weighted-classifier file V3-V4 region targeted for sequencing, taxonomy for each ASV was assigned using a SILVA database (version 138) **(Bokulich et al., 2018).**

1.6. Immunoglobulins content

For measuring blood immunoglobulin content, blood samples were processed and analyzed as described by **Pourhossein et al. (2014)**. Total immunoglobulin (IgE) and immunoglobulin G (IgG) titers to SRBC were also determined by hemagglutination assay; then the immunoglobulin M (IgM) titers to SRBC were calculated as total Ig minus IgG titers. Finally, immunoglobulin A (IgA) was also estimated.

1.7. Statistical analysis

Results were given as means and standard deviations (± SD). The data were analyzed using the General Linear Models (GLM) method of the SPSS software, version 25. All data were analyzed in a random manner using one-way ANOVA. Group

differences were evaluated using Duncan's multiple comparison tests **(Duncan, 1955)** when the P-value was less than 0.05.

Results and discussion

1.8. Changes in bacterial community in the chicken cecum

Using the Qiime2-2022.2 framework, this study examined the diversity of the microbial population in the chicken cecum **(Bolyen *et al.*, 2019)**. The total number of reads in the synbiotic group was 51787; 48946 readings were left after denoising and chimera filtering using DADA2. In the Antibiotic group, the total generated reads were 52173. After removing the low-quality sequence, the reminder reads become 15632 reads. And control was 48151, and after filtration, it became 17256. In all groups, there are five different phyla of bacteria. Firmicutes had the largest representation within the five phyla, with 93.87% in the synbiotic group, 90.94% in the antibiotic group, and 91.38% in the control group, respectively **Fig. (1)**.

Furthermore, Proteobacteria were also the highest in the synbiotic group with 4.73% compared to 3.80% and 3.36% in the control and antibiotic groups, respectively. On the other hand, Bacteroidota phyla were the greatest in the antibiotic group with 2.94% compared to 2.19% and 0.09% in the control group and synbiotic groups, respectively. Additionally, the antibiotic group had the highest levels of Actinobacteriota (2.05%), compared to the control and synbiotic groups' 1.03% and 0.55%, respectively. In contrast, the unknown phyla were the highest in the control with 1.16% compared to 0.76% and 0.72% in synbiotic and antibiotic groups, respectively. Additionally, cyanobacteria had a 0.44% representation in the control group and were absent from all other groups **Fig (1)**.

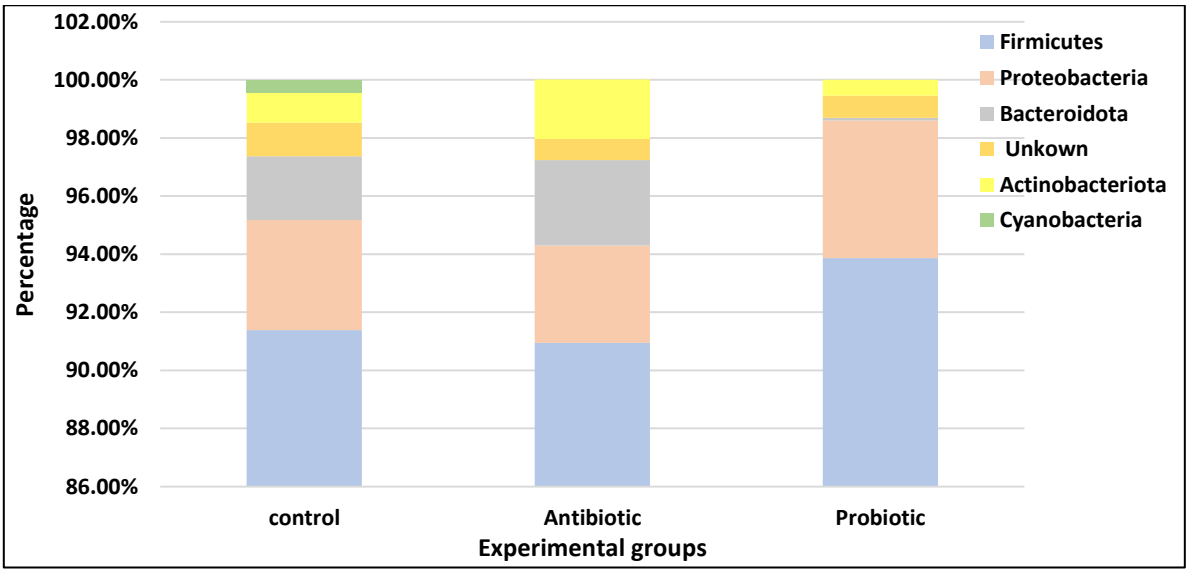

Fig. 1. Percentage of bacteria abundant on phylum level.

At the family level, Lachnospiraceae represented the largest percentage of all families, accounting for 36.65% in the control group and 18.34% in the antibiotic group, whereas synbiotics represented the lowest percentage at 12.58%. Next is Lactobacillaceae, with 35.28% in the synbiotics group, followed by 23.91% in the antibiotic group and 5.71% in the control group, respectively. Furthermore, the Bacillaceae family was the highest in the synbiotic group with 29.33% compared to 3.88 and 3.43% in the antibiotic and control groups respectively **Fig (2)**.

On the other hand, *Ruminococcaceae* was the highest family in the antibiotic group with 10.31% compared to 8.01% and 1.99% in the control and synbiotic groups, respectively. *Oscillospiraceae* was also the most prevalent in the antibiotic group, at 5.76%, as opposed to the control and synbiotic groups, which had 4.23 and 1.26%, respectively **(Fig. 2).** Contrarily, *Clostridiaceae* was most prevalent in the control group (6.87%) in comparison to the antibiotic and synbiotic groups (2.46 and 1.27%, respectively). Additionally, the *UCG-014* family made up 5.36% of the control group as opposed to 3.87 and 2.84%, respectively, in the synbiotic and antibiotic groups **(Fig. 2)**.

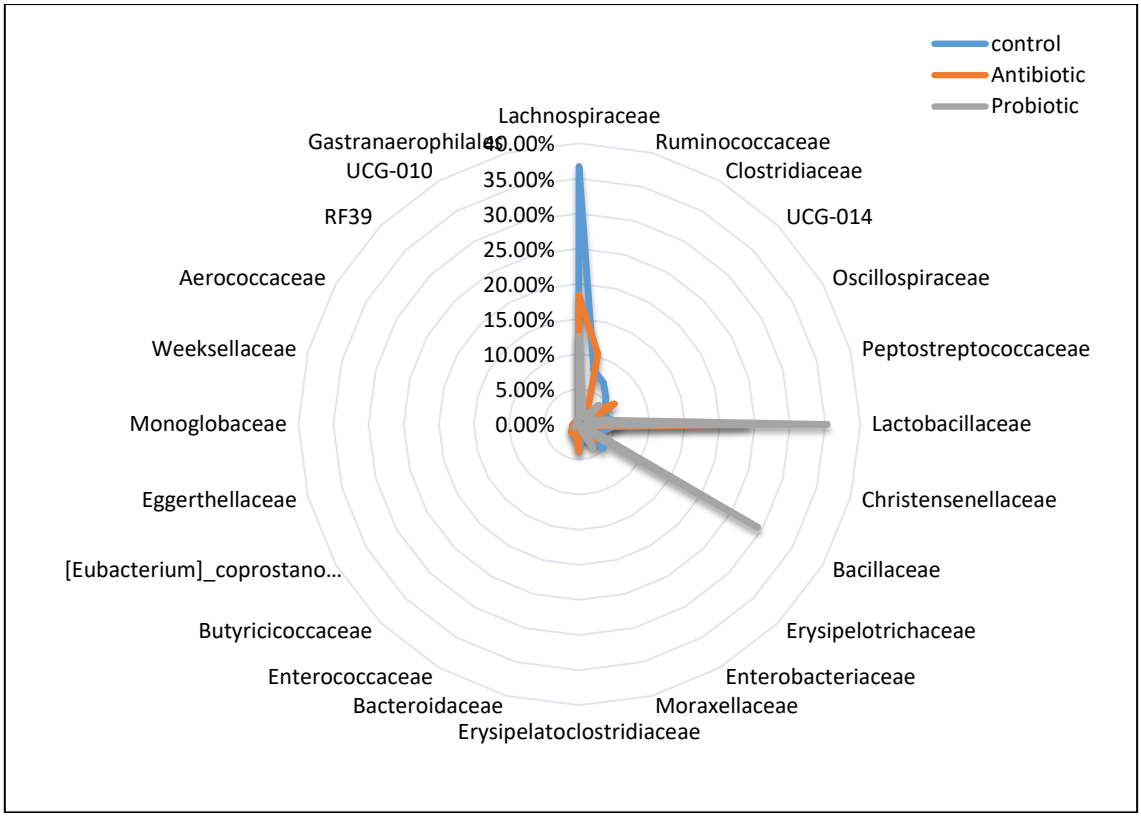

Fig. 2. Percentage of bacterial abundance on the Family level.

The two most prevalent families of Firmicutes identified in the chicken caecum are Lachnospiraceae and Ruminococcaceae, followed by Lactobacillaceae, Veillonellaceae, and Erysipelotrichaceae **(Rychlik, 2020).** Our study is also in raw with the previous study in the ratio of Lachnospiraceae and Ruminococcaceae in the control group On the other hand, Lactobacillaceae and Bacillaceae were the major in the synbiotic group (G3) due to the use of (Micro-BACLA). In the (G2) the major families were Lactobacillaceae, Lachnospiraceae, and Ruminococcaceae.

At the genus level, *Lactobacillus* represented the largest percentage of all genera, accounting for 35.28% in the synbiotics group and 23.91% in the antibiotic group, whereas the control group represented the lowest percentage at 5.71% **(Fig. 3).** *Bacillus* genus comes next with 29.33% in the synbiotics group in comparison with 3.88% and 3.43 in the antibiotic and control groups, respectively. On the other hand, the *Lachnoclostridium* genus was the greatest in the control group with 29.95% compared to 15.26 and 11.14% in the antibiotic and synbiotic groups, respectively **(Fig. 3).** Additionally, *Clostridium sensustricto* was most prevalent in the control groups (6.87%) compared to the antibiotic and synbiotic groups (2.46 and 1.07%, respectively). Also, *Blautia* had 6.70% in the control group compared to 3.08 and 1.43% in the antibiotic and synbiotic groups respectively. *Faecalibacterium*, on the other hand, was the most abundant in the antibiotic group, coming in at around 6.40%, as opposed to 5.30 and 0.56% in the control and synbiotic groups, respectively **(Fig. 3)**. *Clostridia UCG-014* is the next with 5.36% in the control and 3.87% in the synbiotic, and antibiotic was the lowest with 2.87%. Moreover, UCG-005 genera had 4.23% in the control compared to 0.96 and 0.43% in the antibiotic and synbiotic groups, respectively **(Fig. 3).** The two major phyla, Gram-positive *Firmicutes* and Gram-negative *Bacteroidetes* are the most abundant in the chicken gut, followed by two minor phyla Actinobacteria (Gram-positive) and Proteobacteria (Gram-negative), according to **(Oakley *et al*., 2014, Nordentoft *et al*., 2011).**

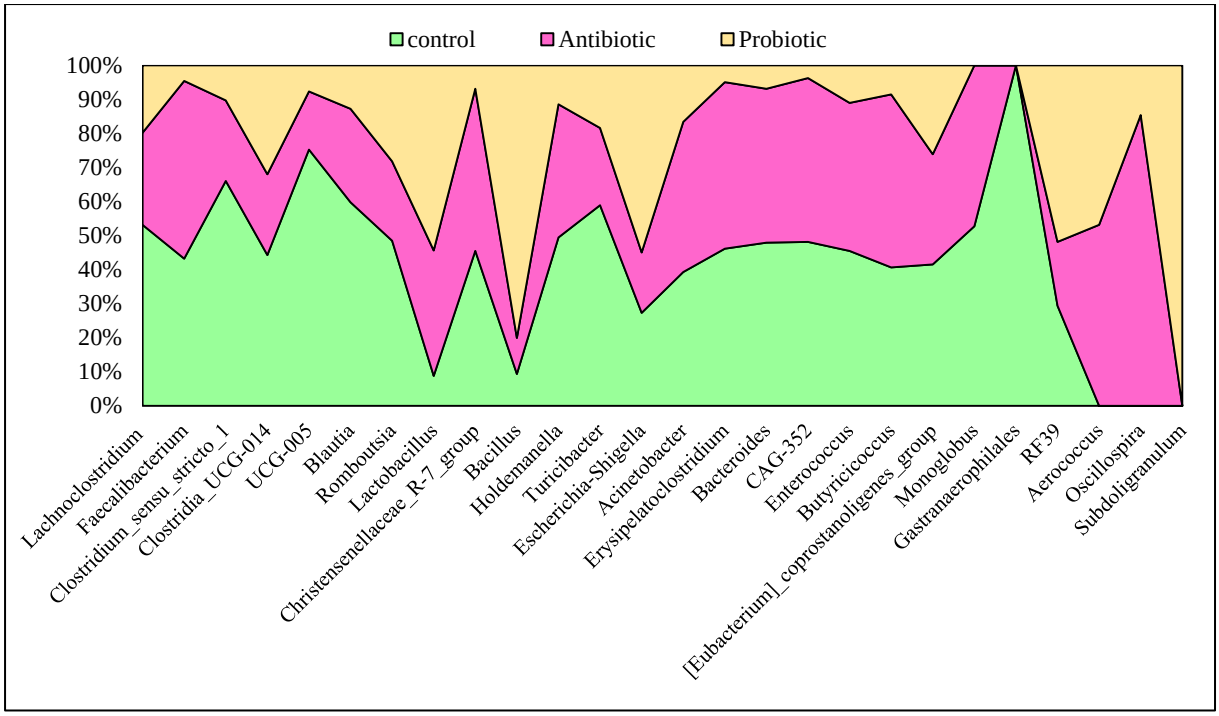

Fig. 3. Percentage of bacterial abundant at the genus level

The present study is in the same line with **Oakley *et al*. (2014) and Nordentoft *et al*. (2011)**, in the majority of *Firmicutes* but the ratio of *Proteobacteria* was higher than *Bacteroidetes* in the present study. Also, the present study is compatible with **Li et al. (2018)** in that *Lactobacillus* has effective roles in increasing body weight and tends to reduce mortality although the chicken was infected with *Clostridium perfringens*. Moreover, **Yan et al. (2017)** showed that the cecal microbiota played a significant role in the feed efficiency of chickens and suggested potential applications for *Lactobacillus* to increase host feed efficiency. In addition to producing acids like lactic acid, which lowers the pH of the gut, lactobacilli also create bacteriostatic substances that resemble bacteriocin. The processes by which lactobacilli species control the gut flora and inhibit the growth of harmful bacteria include competitive exclusion and antagonism **(Chateau et al., 1993, Fuller, 1989, Liu et al., 2014, Lin et al., 2008).** Also, *Lactobacillus* can inhibit pathogens and modulate immunity **(Lin et al., 2008, Lin et al., 2007).**

Furthermore**, Memon et al. (2022)** indicate that adding synbiotic *Bacillus subtilis* to a diet increased the abundance of some commensal genera, including *Clostridium sensu stricto 1, Corynebacterium, Enterococcus, Romboutsia, Subdoligranulum, Bacillus, Turicibacter, and Weissella*. These bacteria are involved in the production of butyrate, the reduction of inflammation, metabolic processes, and the alteration of defense mechanisms against pathogens. Additionally, Due to their ability to form spores, which produce resistance against high temperatures, pH, bile, and enzymes encountered in the gastrointestinal tract (GIT), withstand harsh conditions, and provide health benefits to the host, bacillus-based synbiotics are widely used as antibiotic substitutes in animal and chicken feed **(Haque et al., 2017, Xu et al., 2017).**

1.9. Immune response during the experimental period

Regarding the immune response to synbiotics compared to the antibiotic in chickens, four immunoglobulins namely IgG, IgM, IgA, and total IgE were estimated in the three experimental groups**.** As shown in **Table (1),** the level of serum IgG and total IgE didn't appear in any response to supplements in fodders (synbiotic or antibiotic) and were equal by 50 ng/ml and 0.20, respectively. Whereas, IgA and IgM in chicken' serum were affected by the supplement of fooder with synbiotics compared to antibiotics and chickens without any supplements, both of them were decreased.

Table 1. Immunoglobulins (Igs) profile during the experimental period.

Igs in serum	Experimental groups		
	G1 (Control)	G2 (Antibiotic)	G3 (Synbiotic)
IgG (mg/dL)	50.0	50 .0	50.0
IgA (mg/dL)	23.7	23.7	19.4
IgM (mg/dL)	25.0	25.0	2.0
IgE " total" (IU/mL)	0.20	0.20	0.20

a,b, and c values in the same row with different letters showed significant results (P ≤ 0.05).
G1: Control group (fodder without any additives).
G2: The antibiotics-feeding group.
G3: The synbiotics-feeding group.

The serum IgA was slightly decreased while the IgM was sharply decreased. Compared with that of the control birds, the synbiotic group had decreased serum IgA and IgM contents **(Table 1)**. On reverse, **Yu *et al.* (2022)** found that the level of serum IgM in both broilers supplemented with *Bacillus coaglulans* or *Lactobacillus plantarum* treatments was higher than that of the control treatment.

The beneficial effects of synbiotics in vivo have been proven, for example, increased peripheral immunoglobin production stimulated IgA secretion **(Villena *et al.*, 2008)**. As the biggest producer of immunity in vivo, the intestinal tract produces a large amount of IgA through its activated mucosal B cells, which play the role of the first-line immune defense **(Lycke and Bemark, 2017)**. The variations in immune response or intestinal physiology could be proposed as defense strategies against the microorganisms **(Agostini *et al.*, 2012)**.

Finally, it can be concluded that using synbiotics in conjunction with antibiotics could enhance nutrient digestibility and improve the growth performance of the broiler. By reducing the effects of the harm caused by antibiotics, and maximizing the benefits of the synbiotic directly in the gut (through the manipulation of intestinal microbes). This may lead to the elimination of harm to the consumer (the antibiotic residues that may still be in meat). Therefore, we recommend that broiler breeders supplement synbiotics to maximize economic benefits.

Conclusion

We can conclude that the application of a commercially available growth promoter namely synbiotic (Micro-BACLA)® has beneficial effects on microbial biodiversity and immunity properties in poultry compared to the commercial growth promotor antibiotic (zinc bacitracin)®. At the phylum level, five different phyla of bacteria were detected, Firmicutes had the largest representation in a symbiotic group than other two groups. While, at the family level, Lactobacillaceae and Bacillaceae were dominant in G3, while Lachnospiraceae represented the largest percentage in G1. On the other hand, *Ruminococcaceae* was the highest family in the antibiotic group. Additionally, at the genus level, *Lactobacillus* represented the highest percentage in both symbiotic and antibiotic groups compared to control group. Regarding the immune response, the serum IgG and total IgE levels were equal at 50 ng/ml and 0.20, respectively, and did not change in response to synbiotic or antibiotic supplements in fodders. Whereas, IgA and IgM in chicken' serum were affected by the supplement of fodder with synbiotic compared to antibiotic and control groups, both of them were decreased. The current study concluded that adding synbiotics to the chicken broilers diet could increase nutrient digestion and growth performance by minimizing the negative effects of antibiotics and maximizing the synbiotic's advantages in the gut.

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