

Effect of Chinese Propolis Supplementation on Ross Broiler Chicks: Microbial Population in Fecal matter and LitterManal A.M. Mahmoud^{1*}, Hosnia S. Abdel-Mohsein¹, Usama T. Mahmoud²¹Department of Animal Hygiene, Faculty of Veterinary Medicine, Assiut University 7156, Egypt²Animal Management and Behaviour, Department of Animal Hygiene, Faculty of Veterinary Medicine, Assiut University 7156, Egypt.

Accepted 23 March 2014

Abstract

Propolis has been considered a good candidate for health amelioration and disease prevention over a long period of time. Effect of feed supplementation with Chinese ether extract propolis was evaluated for the antibacterial action on (Ross 308) broiler chicks and their litter over 35 days. The addition of different levels of propolis (100, 250 and 750 mg/kg diet) on total aerobic and coliform bacteria as well as *Lactobacillus spp.* and bifidobacteria were determined. Supplementation of propolis significantly increased ($p < 0.05$) the desired stimulating effect of *Lactobacillus spp.* and Bifidobacteria in fecal samples of all treatment groups compared to control group. However, propolis did not have an impact to reduce the total aerobic and coliform bacteria compared to control group in both fecal and litter samples. In conclusion, Chinese propolis improved the beneficial normal gut microflora and had a limited effect on the total aerobic and coliform bacteria in broiler chicks and litter quality.

Keywords: Propolis; Bacterial count; Gut microflora; Litter; Broiler.

Introduction

For restriction of antibiotic used as a growth promoter in broiler chicken, the supplements of natural products as probiotics, prebiotics, organic acids, antioxidants, enzymes and plant extracts has been studied as a candidate replacement (Capcarova *et al.*, 2010). These alternative substances improved the feed conversion, performance, growth and health of broiler chickens through a mechanisms associated with gastrointestinal tract and bacteria colonization (Kročko *et al.*, 2012).

Propolis is a natural glue-like substance produced by bees from plant seedlings and buds. It is obtained as a result of the biochemical alteration of the resinous materials and plant excretion by the enzymes secreted from the glands of bees. Propolis proved to have numerous biological activities including antioxidant, anti-microbial, anti-carcino-

genic, anti-fungal, anti-viral, anti-ulcer, immunostimulatory and anti-inflammatory properties. (Atungulu *et al.*, 2007 and Barros *et al.*, 2007). The antibacterial activity of propolis is contributed to flavonoids, phenolic acids and their derivatives (Tosi *et al.*, 2007; Erkmen and Özcan, 2008). The antibacterial mechanism was achieved through suppressing bacteria cell development by blocking their cell mitosis and protein synthesis. Another mechanism is through direct destructing bacteria cells by disarranging their cell cytoplasm, cell membranes, and also their cell walls (Takaisi-Kikuni and Schilcher, 1994).

Poultry are very sensitive to pathogenic bacteria such as *E. coli*, *Salmonella spp.*, *Clostridium perfringens*. Some bioactive substances derived from higher plants have been reported to kill pathogenic bacteria species while increasing beneficial bacteria such as lactic acid and bifidobacteria (Gunal *et al.*, 2006). The stimulation of such beneficial bacteria results in an effective protection against pathogenic microorganisms and a balanced intestinal

*Corresponding author: Manal A. M. Mahmoud

E-mail address: egymanal@gmail.com

microflora (Vidanarachchi *et al.*, 2006). Intestinal microflora provide natural barrier against harmful bacteria that enter the intestine; they inhibit growth of exogenous and pathogenic bacteria, and produce bacteriocins or other substances thus enhancing the immune system (Tannock, 1988; Barrow, 1992; Gibson and Roberfroid, 1995; Gong *et al.*, 2002; Van der Wielen *et al.*, 2002; Lan *et al.*, 2005). The competition between intestinal microflora and exogenous or pathogenic organisms for limited carbon sources, the presence of antibacterial compounds, and production of volatile fatty acids can control the growth and translocation of the exogenous or pathogenic organisms (Barnes, 1977). A well-established normal intestinal flora compete with pathogens and hence decrease the risk of salmonellosis, *Clostridium perfringens*-associated lesions, campylobacteriosis, or colibacillosis (Weinack *et al.*, 1981; Soerjadi *et al.*, 1982; Fukata *et al.*, 1991; Barnes, 1997; Craven *et al.*, 1999; Kaldhusdal *et al.*, 2001) and/or protect against colonization of the intestine by pathogens (Mead, 2000).

In poultry production, litter is a potential reservoir and transmission vehicle for pathogens and potential pathogens (Montrose *et al.*, 1985, Weinack *et al.*, 1985, Willis *et al.*, 2002, Lu *et al.*, 2003). Intensive systems generate large quantities of litter that has been traditionally applied to agricultural soils for decades as an organic fertilizer (Moore *et al.*, 1995). In addition, animal and poultry manure is extensively used as a cheaper alternative to artificial fertilizer to increase phytoplankton production (natural fish food) in fish ponds from different countries (Knud-Hansen, 1993; Little and Edwards, 1999; Petersen *et al.*, 2002; EAHMI, 2008). The importance of enhancing a safe recycling of poultry litter and its nutrients increased opportunities to market their energy and nutrients to agricultural and non-agricultural uses. Based on the work of several authors propolis may have a beneficial effect on the gastrointestinal microflora of poultry (Nováková *et al.*, 2008; Kačániová *et al.*, 2011). However, its antimicrobial activity depends on the physical structure, concentration and the dose intensity (Dias *et al.*, 2012). In addition, there is no information about the antimicrobiological effects of propolis on broilers litter. Therefore, the objective of the present study was to determine the effect of ether extract propolis (EEP) supplementation on intestinal and litter mi-

crobiology in broiler chicks. It was expected that EEP could be efficient for replacing the wide therapeutic antibiotics and able to reduce cost of medication in broilers due to the antibacterial properties of these extracts.

Materials and methods

Birds and treatments

Sixty four, one day old broiler chicks (Ross 308) were divided into four groups of 16 birds each in a completely randomized design. The experiment was planned in accordance with animal welfare. Feed and water were offered to allow for ad libitum consumption. The groups were fed as follow: 1- Basal diet, no additives (Control), 2- Basal diet plus 100 mg propolis /kg diet, 3- Basal diet plus 250 mg propolis /kg diet, 4- Basal diet plus 750 mg propolis /kg diet. The addition of propolis to the diet was started at day 3. The ingredients of the diet and calculated energy uptake/ Kg diet were presented in Table 1. Birds were given a starter diet to 21 days of age followed by a grower diet till the end of the experiment. The experiment was conducted in an environmentally controlled floor house of clean softwood shavings as litter material. Bird density was 20 kg per square meter. The lighting cycle was maintained at 23 hours/day. The ambient temperature in the experimental house was maintained at 34°C during the first week and gradually decreased by 3 °C during the second and third weeks, and was fixed at 26 °C thereafter. Average relative humidity ranged between 40 to 60 %. Ventilation was provided by negative pressure with fans. Chicks were vaccinated via their drinking water against New Castle Disease at days 6, 14, 21, and 32 and against Infectious Bursal Disease at days 10, 18 and 25. Ether extracted propolis was purchased from Dalian Tianshan Industrial Co.TM, Ltd. Changjiang Road, Dalian, Liaoning, China.

Sampling and microbiological analyses

Eight Faecal samples and three pooled litter samples were collected from each group at day 8, 18 and 35 for microbiological analyses. Fresh fecal samples from birds were aseptically collected on a sterile sheet and immediately transferred to sterile plastic bags. Litter samples were collected from each group using sterile plastic bags. Samples were

Table 1. Composition of the experimental diets (g/kg) and energy uptake (Kg/diet)

Ingredients	Starter	Grower
Com	50.5	60.05
Fish meal	3.5	3
SBM	36.75	29
Sunflower oil	6	4.7
Dicalcium phosphate	1.5	1.5
Ground lime stone	1	1
Salt	0.3	0.3
Lysine	0.1	0.1
Methionine	0.1	0.1
Premix	0.25	0.25
Calculated energy uptake/ Kg diet		
E (Kcal/kg)	3202.465	3208.048
CP (%)	22.977	20.0363

placed on ice and transported to the laboratory in an ice box for bacteriological analysis that was carried out within 2 hours. Bacteriological analyses were performed with a total of 96 fecal and 36 litter samples. Fresh fecal microbial population were accessed for the total viable aerobic counts, coliform, *Lactobacillus spp.* and Bifidobacterium by using culture techniques described by Hu *et al.* (2012). Fresh fecal samples (1g) were placed into preweighed 15 mL sterile plastic tubes containing 9 ml sterile saline (0.85%) which was called the initial fecal dilution. Litter samples (1 g) were placed into sterile glass bottle containing 99 ml sterile saline. Tenfold dilutions were spread in duplicate onto standard plate count agar (Lab M Limited, Lancashire BL9 6As, UK) and MacConkey agar (Lab M Limited, Lancashire BL9 6As, UK). Standard plate count agar and MacConkey agar plates were incubated aerobically at 37°C for 24 hours for total aerobic bacteria and coliform counts in fecal and litter samples, respectively (Halkman *et al.*, 1994; Merk, 1998). For enumeration of *Lactobacillus spp.* bacteria and bifidobacteria, the initial fecal dilution was tenfold serially diluted in 0.85% sterile saline solution. From each dilution, 1 mL was inoculated on MRS agar (Biolife, Milan, Italy) and MRS agar supplemented with L-cystiene hydrochloride for *Lactobacillus spp.* and bifidobacteria, respectively. Lactobacilli and Bifidobacteria were anaerobically incubated (SHEL LAB CO₂ Incubator, SHELDON manufacturing, Inc, USA, Model no. 2406-2) at 37°C for 48 hours, counted and recorded as colony forming units per gram of fecal material. All microbiological analyses were performed in duplicate and the average values expressed as log₁₀ cfu g⁻¹ fecal and used for statisti-

cal analysis. *Lactobacillus spp.* and Bifidobacteria were counted in faecal samples collected at days 18 and 35 using MRS Agar (pH 5.4,) and MRS Agar supplemented with 0.25% L-cysteine hydrochloride, respectively. Incubation was performed under anaerobic condition at 37°C for 48-72 hours (Asperger and Saad, 1999; Zinedine and Faid, 2007).

Statistical analysis:

Statistical Analysis of data was carried out using SPSS version 11 statistical package programs. A one- way analysis of variance (ANOVA) was performed. Differences in mean values were accepted as being statistically significant if $P < 0.05$. When the effect was significant ($p < 0.05$), means were separated using Tukey's test. The statistical significance was determined at both $P \leq 0.01$ and $P \leq 0.05$ levels.

Results

The results of the application of EEP (100, 250 and 750 mg /kg diet) influenced *Lactobacillus spp.* and Bifidobacteria number in fecal samples at 18 and 35 days are shown in Fig. 1 (a & b).

Lactobacillus spp. and bifidobacterium, in all treatment groups at day 18, have significantly increased with respect to control group. At day 35, only counts of *Lactobacillus spp.* were significantly higher comparing with control group ($P = 0.013$). Moreover, non-significant differences were observed in *Lactobacillus spp.* and bifidobacteria counts between groups of different propolis concentrations. The highest *Lactobacillus spp.* count

(9.4 log₁₀ cfu/g and 13.38 log₁₀ cfu/g) was observed in broilers received 750 mg and 250 propolis/kg diet at days 18 and 35, respectively. However, the highest bifidobacteria count (8.75 log₁₀ cfu/g) was reported in the group with 100mg propolis/kg diet at day 18.

Effect of different levels of EEP (100, 250 and 750 mg /kg diet) on fecal samples for total aerobic and coliform counts at different ages (8, 18 and 35 days) of broiler chicks, were presented in Fig. 1 (c & d). The total aerobic count in the control group has a tendency to increase with bird age from day 18 to 35. The same pattern was observed in all treated propolis groups regardless its concentration in the diet. Similar observation in both control and treated propolis groups with the total coliform count in broilers fecal samples which was increased with increasing the age of birds from 8 to 35 days. Propolis administration has no significant impact on the total aerobic and coliform counts in broiler,

except at concentration 100mg/kg diet where a significant increase in total aerobic and coliform at day 35 was observed (Fig. 1 c & d).

The data of total aerobic and coliform count of litter samples collected at days 8, 18 and 35 of the experiment were presented in Fig. 2 (a & b). It can be observed that there was no significant effect of any propolis treatment on the bacterial counts in any litter sample, except, total aerobic count of the treatment group received 100mg propolis/kg feed showed a significantly higher count than the control group at days 8 and 18.

Discussion

The desired effect of propolis on *Lactobacillus spp.* and *Bifidobacterium* could be attributed to the regulating and protecting role of these bacteria on chicks. Gut microflora are nutritional “burden” in fast-growing broiler chickens (Lan et al., 2005;

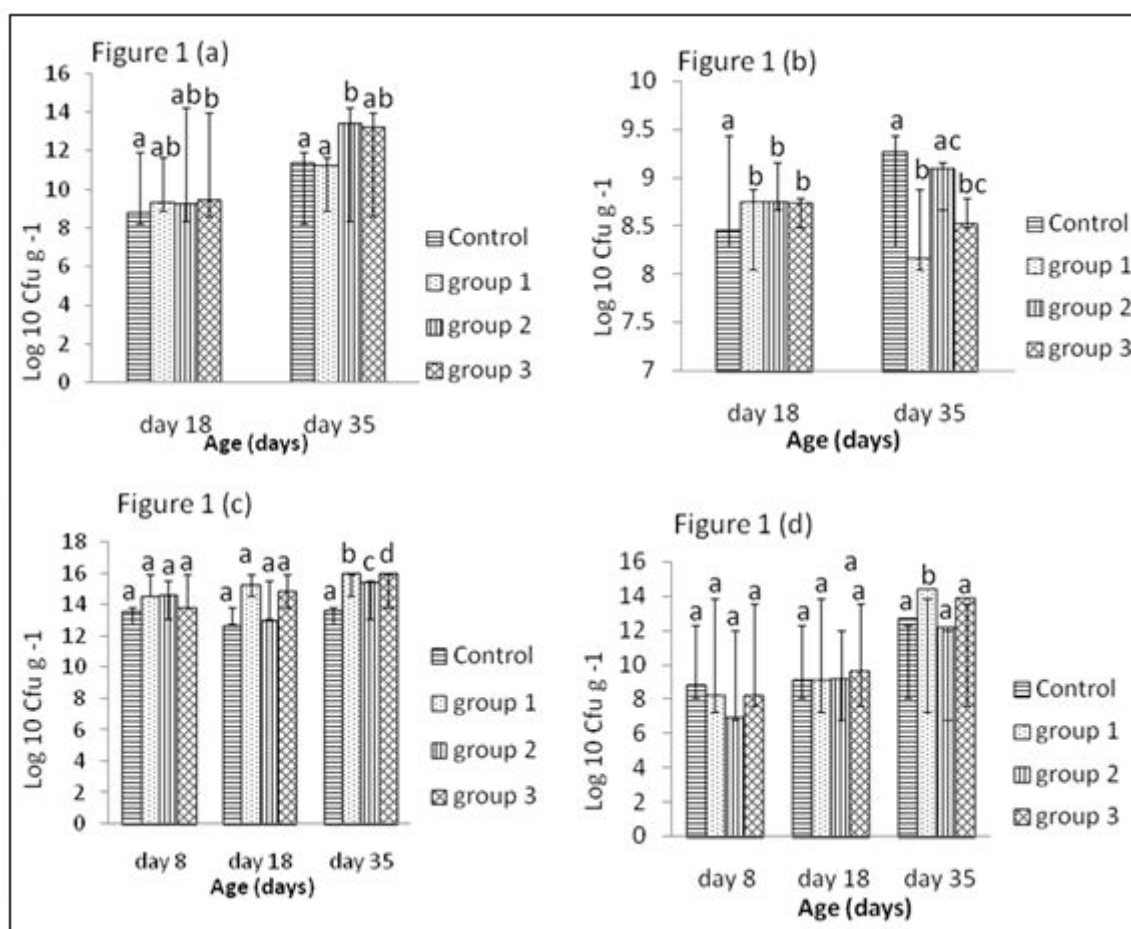


Fig. 1 (a, b, c and d). Log₁₀ cfu of the different types of bacteria in fecal samples of broiler chicken. a&b: Lactic acid and Bifidobacteria number in samples collected at day 18 and 35 of the experiment, respectively. c & d: Total mesophilic and coliform count in samples collected at day 8, 18 and 35 of the experiment, respectively. The groups were as follow: Control (Basal diet), Group 1, Basal diet plus 100 mg propolis /kg diet, Group 2, Basal diet plus 250 mg propolis /kg diet, Group 3, Basal diet plus 750 mg propolis /kg diet. abc: means with different letters are significantly different (p<0.05)

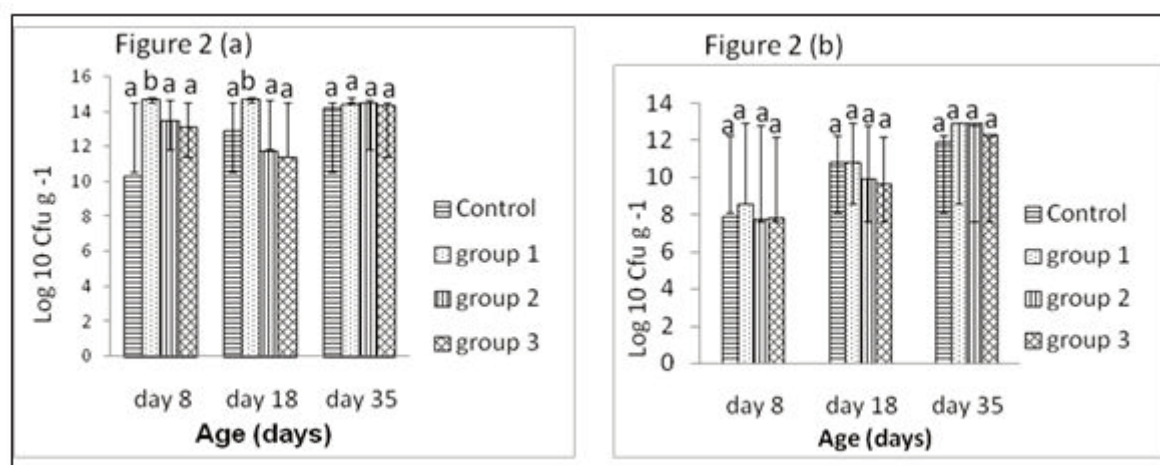


Fig. 2. Log₁₀ cfu of total mesophilic and coliform count of litter samples collected at days 8, 18 and 35 of the experiment as shown in a & b respectively.

Adil *et al.*, 2011), since active microflora component may have an increased energy requirement for maintenance and a reduced efficiency of nutrient utilization (Kročko *et al.*, 2012). The stimulation of such beneficial bacteria generates an effective protection against pathogenic microorganisms and a balanced intestinal microflora (Vidanarachchi *et al.*, 2006). The administration of *B. bifidum* to broiler chickens in drinking water significantly decreased the incidence of cellulitis and the increase of *Lactobacillus spp.* in newborn ducks and chicks preceded the development of weight gain (Estrada *et al.* 2001; Angelakis and Raoult, 2010). The results suggested that propolis level as low as 100 mg/kg diet, for 18 days, could be enough to enhance the growth and multiplication of beneficial gut microflora in broilers. This observation is in agreement with previous reports on the positive effect of propolis on gut microflora. Propolis extracts supplemented in the ration both separately and in combination with *Zingiber officinale* proved to stimulate lactic acid bacteria in broilers (Tekeli *et al.*, 2010). In another report, the number of beneficial lactic acid bacteria in chicken's crops with presence of propolis was increased while the number of this bacteria in chickens's ileum and caecum was decreased in both 400 and 800 mg propolis/kg diet (Kročko *et al.*, 2012). Many reports revealed the positive effect of some plant extracts on the gut microflora of birds. Plant extract additives increased the numbers of lactic acid bacteria (bifidobacteria ve lactobacilli) in the ileum and caecum of broilers (Guo *et al.*, 2004; Vidanarachchi *et al.*, 2006). On the contrary, *Lactobacillus spp.* number has no statistically significant differences in the

trial with chickens after application of propolis (Kačániová *et al.*, 2012).

The lower gastrointestinal tract of most animal species including poultry is normally populated by large number of microorganisms, and through various competitive niches and virulence capabilities, some are able to survive (Kačániová *et al.*, 2012). The effect of propolis was less pronounced, suggesting that the inhibition of total aerobic and coliform bacteria may be dose related. Lower concentration of propolis may contain reduced level of the active biological antibacterial substance/s. The presence of certain bacteriostatic/bactericidal chemicals in the growth medium at a concentration lower than a critical inhibitory level could enhance the growth of an organism that otherwise would have been inhibited/ killed by higher concentrations, a phenomenon known as hormesis (Calabrese and Baldwin, 2003). This situation alerts the importance of dose adjustment of propolis before using in broilers to be above the minimum inhibitory concentration (MIC) values, in order to minimize the risk of encouraging bacterial growth. In accordance with our results, Tekeli *et al.* (2010) recorded a significant increase in total aerobic bacteria after supplementation with of *Zingiber officinale* plant (240 ppm) and propolis (1000 ppm) comparing with the control ($P < 0.05$).

Coliform bacterial population in broilers displayed a reduced sensitivity especially at low propolis concentration. In accordance with other results, gram negative bacterium *E. coli* showed either very low sensitivity or total insensitivity against propolis (Digrak *et al.*, 1995; Marcucci, 1995; Bonvehí and Coll, 2000; Sforcin *et al.*,

2000). The most plausible explanation is their outer membrane that inhibits and/or retards the penetration of propolis at lower concentrations. After a certain threshold concentration, however, the impermeability of this membrane is disturbed resulting in the movement of bioactive components of propolis into the cell interior, resulting in cell inhibition or death. Another explanation may be the resistance of gram negative bacteria to propolis due to the possession of multidrug resistance pumps (MDRs), which extrude amphipathic toxins across the outer bacterial membrane (Tegos *et al.*, 2002). The MDRs could be very effective at lower concentration of propolis and extrude those molecules that crossed the outer membrane barrier. This pumping potential of the MDRs can be saturated at a certain threshold concentration and the rate of penetration and accumulation of antimicrobials in the cell interior increases, resulting in antimicrobial effect at higher concentrations (Lomovskaya and Lewis, 1992; Nikaido, 1999; Zgurskaya and Nikaido, 1999; Lewis and Lomovskaya, 2001). From these obtained results, it can be suggested that higher aerobic count in treated propolis groups may be attributed to the higher coliform counts in the examined fecal samples of poultry. In agreement with our results, Enterobacteriaceae count in ileum and caecum of chicks either remains unchanged or increases when propolis was added in a food mixture at concentrations 400 and 800 mg/kg diet (Kročko *et al.*, 2012). On the contrary, other researchers reported the antibacterial effect of propolis on coliforms. Results of Tekeli *et al.* (2010) revealed that supplementation of Zingiber officinale and propolis extracts alone or in combination significantly decrease ($P < 0.05$) the existence of coliform bacteria in all treatment groups. In addition, propolis significantly and dose dependently controls the microbial load, particularly the *E. coli* and clostridium rates comparing the control group (Rahmani *et al.*, 2006). Plant extract additives significantly reduced the numbers of total anaerobic, coliform and *C. perfringens* bacteria in the ileum and caecum of broilers (Vidanarachchi *et al.*, 2006). High aerobic and coliform bacterial count assumed to be responsible for the risk of developing clinical and subclinical infection as well as impairment of the absorption of nutrients in the intestine and depression of growth.

Our study found no influence of propolis on reducing litter microbiology. Large quantities of

poultry litter, have been produced by intensive poultry production systems, Average litter production rates were 228.2 g of dry litter material per kg of live broiler weight (g/kg) per flock (Coufal *et al.*, 2006). Poultry litter has been traditionally applied to agricultural soils for decades as an organic fertilizer, (Moore *et al.*, 1995). In addition, litter has the potential for being recycled as food nutrient in fish farms (Petersen *et al.*, 2002; EAHMI, 2008). However, because litter contains many essential nutrients, high bacterial loads could result if litter is not prepared at acceptable conditions. Therefore, improving litter microbial quality can help to reduce infection, improve production, performance and lower costs for poultry producers. Poultry litter containing high microbial population is considered of low value and unhygienic problems could result from reuse in plant or animal. A study on the pathogenesis of cellulitis demonstrated that scratching the skin of chicken broilers and then exposing them to litter seeded with *E. coli* cells induced cellulitis in many chickens (Macklin *et al.*, 1999). Litter and other management practices also can change microbial composition of the chicken gut directly by providing a continuous source of bacteria or indirectly by influencing the defense mechanisms of the birds (Apajalahti *et al.*, 2004). Studies with higher doses of propolis could be worthy of future work.

Conclusion

Under the conditions of this study, it can be concluded that *Lactobacillus spp.* and Bifidobacteria are positively influenced by supplementation of propolis. However, the highest propolis level in this experiment could be insufficient to exert an antimicrobial action on the total aerobic and coliform bacteria in broiler chicks. Further investigations are needed for justification the proper type, dose and regimen of propolis, as a natural additive, for efficient commercial application in organic chicken production.

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PLOS ONE 5(5), e.0010463.

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