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EFFECT OF THE PROBIOTIC ON BROILER GROWTH PERFORMANCE, MORTALITY RATE, INTESTINAL MICROBIAL POPULATIONS AND IMMUNE ORGANS

By

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ABSTRACT

The present research was carried out to evaluate the effect of *Saccharomyces cerevisiae* and *Bacillus subtilis* strain (QST 713) on broiler growth performance through the evaluation of live body weight gain, feed intake and feed conversion rate, mortality rate and weight of immune organs in chicks. The experiment consisted of a total number of 180 one day-old cobb chicks were brought from a commercial hatchery and distributed randomly into three groups of 60 chicken in each . Subsequently, the chicks in each group were distributed into 2 replicates with 30 chicks in each and were housed in separate pens in 6 battery cages. They were placed on one of the three dietary treatments. The afore-mentioned treatments were a basal diet (T1 control group). Two other diets composed of basal diet supplemented with probiotic as *Bacillus subtilis* spores strain (QST 713). To provide the concentration 10 ^{10}CFU /g feed and added by 100 gram per ton feed in the second group (T2 group).

Saccharomyces cerevisiae by concentration 10 ^{9}CFU/g feed was added by 1000 gram per ton in the last group (T3 group). All the chicks were allowed to have free access to starter diet during (1-21 day old) and then to a grower diet from 22 day old to the end of experiment at 42 day of age. Body weight gain, feed intake (FI), feed conversion rate (FCR) and mortality rates were recorded. At the end of the study, nine birds from each group were slaughtered for collection of intestinal and cecal contents for determination of intestinal microbial population. Then remove immediately the immune organs (bursa of fabrics, spleen) then record their weight. Our results showed increased body weight gain, feed intake in groups supplemented by *Saccharomyces cerevisiae* and *Bacillus subtilis* respectively as compared with control. Feed conversion rate was similar in probiotic supplemented groups at 42 day of age but both treated groups had FCR higher than that in broiler fed basal diet .bursa weight in probiotic added groups was affected and show heavier than it's similarity in control group .

Three experimented groups did not show any change in spleen weight during all experimental period .Therefore,our results support the addition of both types of probiotics firstly *Saccharomyces cerevisiae* then *Bacillus subtilis* , respectively which showed low mortality rate, increased body weight gain ,increase feed intake, feed conversion rate improvement , increase *Lactobacillus* population in broiler intestine and inhibit enteric pathogenic bacteria. Finally, the probiotics had a positive effect on broiler immune system by stimulate bursa weight, which might be attributed to improve bacterial populations.

Key words:

Saccharomyces cerevisiae,Bacillus subtilis, Lactobacillus,Salmonella, *E-coli*, feed conversion rate, feed intake, immune organ, intestinal microbiota, and broiler.

INTRODUCTION

Because of the emergence of multiple drug-resistant bacteria, the potential contamination of the environment with antibiotics, and increasing awareness of antibiotic use in poultry products by health mindful consumers **(Peric *et al*., 2010).** Increasing consumer awareness and preference for poultry products that are free from chemical residues has resulted in intense, global efforts to identify environmentally friendly and healthy replacements to improve animal health and performance **(Wolfenden *et al*., 2010)**. Therefore, currently in many parts of the world, feed additives alternatives, such as probiotics, prebiotics, are being experimented to alleviate the problems associated with the withdrawal of antibiotics from feed. Probiotics are defined as live microorganisms that have a beneficial effect on the health of their host when consumed in an adequate dose **(Manafi, 2015).** Among the probiotic microbial species available, *Lactobacillus, Saccharomyces, Bacillus, Streptococcus,* and *Aspergillus* species have been reported to have a beneficial role in poultry nutrition **(Lema *et al*., 2001; Tannock, 2001; Zhang *et al*., 2005; Chen *et al*., 2009, Manafi and Khosravinia,2013)**. Probiotics enhance the epithelial barrier through production of mucin, inhibit the pathogen adhesion, competitive exclusion of pathogenic microorganisms, production of anti-microorganism substances as bacteriocins produced by Lactic acid producing probiotics and defensine , modulation of the immune system modification of the gut **(Bermudez-Brito *et al*., 2012)** and Play an important role in correcting intestinal ecological imbalances and improving animal health **(Wolfenden *et al*.,2010; Chuka *et al*., 2014).** Probiotic *Bacillus* bacteria have been considered good candidates for feed additives

because their aerobic and endospore-forming nature gives them the capacity to survive environmental stresses, including storage, transport, and feed pelleting processes **(Setlow, 2006; Cartman *et al.,* 2008; Wu *et al.,* 2011).** *Bacillus subtilis* can be used to increase and maintain beneficial bacteria in the intestine **(Goldin *et al.,* 1998**). In addition to, improve intestinal microflora **(Hosoi *et al.,* 1999)**, enhanced BW gain, and improved feed efficiency **(Jiraphocakul *et al.,*1990).**Two vivo trials have shown that,the inclusion of *B. subtilis* PB6 as a feed additive improved the weight gain and feed efficiency of broilers **(Teo and Tan, 2006).** It also showed decreased pathogenic bacteria as *Clostridium perfringens* counts in intestine of *Bacillus subtilis* supplemented groups **(Teo and Tan, 2004)** .One other alternative is the addition of yeast and yeast products to poultry diets.

The inclusion of non-pathogenic yeast as *Saccharomyces cerevisiae* in the diet has been shown to improve bird performance and decrease mortality **(Miles and Bootwalla., 1991; Madrigal *et al.,* 1993; Bradley *et al.,* 1994; Santin *et al.,*2001)**. *Saccharomyces* could inhibit the pathogenic microorganism and this inhibition could potentially increase nutrient bioavailability, improved growth rate, and feed **efficiency (Manafi *et al*., 2016).**

Saccharomyces have also been shown to stimulate the immune system of chicks without decreasing growth performance **(Bai *et al*.,2013)**. The aim of the present study was to investigate the effects of supplementing the diet of broiler chickens with a probiotic containing *Bacillus* species and *Saccharomyces cerevisiae* on growth performance.

Also resulted in increased feed intake, feed conversion rate, intestinal bacterial population, immune responses .Second, they aimed to identify the potential of this probiotic supplement to replace antibiotic growth promoters in the broiler diet to reduce occurrence of antibiotic resistance in animal and human.

MATERIAL AND METHODS

Probiotics:

Yeast probiotic:

A probiotic commercially identified as organotech® was used as a test feed additive in this study. Organotech was purchased from organic chemical solutions, L.L.C, Georgia. The bacterial flora in the Organotech probiotic has mentioned to be *Saccharomyces cerevisiae* in a concentration of 10^9 CFU/g (colony forming unit).

Bacterial probiotic:

A probiotic commercially identified as Baymix ® was used as a test feed additive in this study. Baymix was purchased from Bayer Animal Health, USA. The bacterial flora in the Baymix probiotic has mentioned to be *Bacillus subtilis* in a concentration of 10^{10}CFU/g (Colony forming unit).

(Table 1): Composition of diet according to maintenance and production standard of Cobb.

Ingredient	Starter phase (1-21 d)	Grower phase (22-42 d)
Corn	441.05	562.05
Soyabean	340	263
Ddgs (dried distillers grains) as fat and protein source	60	60
Corn gluten	15	-
Soyabean (full fat)	-	26
Bakery	100	50
Limestone	13.1	12.4
Di - calcium phosphate	14.9	12.6
Vitamin and mineral premix	3	3
Soyabean oil	4	1
Atocs bio	0.3	0.3
Calpirine A	0.5	0.5
Lingobond (binder)	2	3
Salt	1.5	1.5
Frazime	0.5	0.5
Methionine	1.93	2
Lysine	1.37	1.3
Sodium bicarbonate (Naco3)	0.7	0.7
Garlysin	0.15	0.15
Total	1,000.00	1,000.00

Feed mixing procedures:

During the diet mixing, the probiotic was first mixed into the premix and then into the diet according to standard operating procedures for feed additive mixing at the factory.

All mineral and vitamin supplements (including limestone, inorganic phosphate source, trace mineral premix, and vitamin premix), known as the filler, were accurately weighed and then hand-mixed. The probiotic was then hand mixed with the filler at a predetermined ratio using the quartering technique. In this technique, the filler was divided into 4 quarters.

The probiotic product was first mixed with one portion of feed, and then mixed with the next filler portion. The resulting mix, probiotic filler, was blended in a small Hobart mixer for 10 min. eventually, the probiotic filler mix was added to the basal diet containing major ingredients and mixed in a horizontal mixer for another 5 min. All diets used in this study were cold-pelleted (65 to 70°C) and stored in airtight containers until use **(Teo and Tan, 2007).**

Birds and experimental design:

The study was conducted in three experimental trials. A total 180 one day-old cobb chicks brought from a commercial hatchery were weighed and distributed randomly into three groups of 60 chicken in each. Subsequently, the chicks in each group were distributed to two replicates with 30 chicks in each and were housed in separate pens in 6 battery cages.

Each replicate was assigned to a clean floor pen and birds were raised on new wood shavings as a litter. Heat was provided with a heating lamp per pen. The lighting program applied during the completely experimental period was 22 h light and 2 h dark, except during the first day till 2 weeks of age the lights were on for 24 h. The chicks were allowed continuous access to daily-cleaned water and warm room. They were placed on one of the three dietary treatments. These treatments were a basal diet (T1 group as a control) and two other diets which were same in composition as basal diet but supplemented with probiotic *Bacillus subtilis* spores strain (QST 713) to provide the concentration it was added by 100 gram per ton feed (T2 group) and *Saccharomyces cerevisiae* was added by 1000 gram per ton (T3 group). All the chicks were allowed to have free access to starter diet during (1-21 day old) and then to a grower diet from 22 day old to the end of experiment at 42 day of age.

Basal diet used in this study was a standard in house commercial formulation developed by Delta Masr Company Group according to the maintenance and production requirement of Cobb-Chicks.The ingredients of basal diet were presented in (Table 1) **(Palamidi *et al.*, 2016).**

Evaluation of growth performance:

Broiler growth performance responses such as body weight (BW) , feed intake (FI) and feed: gain ratio (FCR) were determined every 2 weeks during the six experimental weeks. Three birds from each replicate pen were selected according to the average BW within the pen after a 12-h fasting, and weighted individually at 14, 28 and 42 days of old by using traditional manual balance. Feed intake at 14, 28 and 42 days post feeding was calculated on a pen basis by dividing the amount of weekly feed consumption (corrected for the feed consumed by the birds that died during the week) by the number of birds alive at the end of the week. Performance data was presented on a growth period (i.e., starter, grower, and finisher) basis. Feed conversion ratio was weekly calculated as the amount (in grams) of feed consumed i.e. Feed Conversion = Feed intake / weight gain over the experimental period. Mortality number was recorded for each experimental group and presented as a percentage to total bird number **(Teo and Tan, 2017).**

Evaluation of Aerobic plate count:

Fresh intestinal content from broilers in each treatment group were obtained at the end of the experiment,and nine birds per treatment were slaughtered.To measure intestinal *Lactobacillus, E. coli, and Salmonella*, approximately 1 g of intestinal content samples was diluted 10-fold (1:9, w/v) in sterilized phosphate-buffered saline (PBS, 0.1 M, pH 7.0) and homogenized. Then, a 0.1-mL sample was serially diluted 10^3-10^6 and spread on MRS agar media, MacConkey's agar, and *Salmonella Shigella* agar media (SS agar) , for *Lactobacillus, E. coli* and *Salmonella*, respectively. Lactobacillus agar plates were incubated anaerobically at 37°C for 48 h, whereas *E. coli and Salmonella agar* plates were incubated under aerobic conditions at 37°C for 24 h. The colonies on each plate were counted using a colony counter, and the results are presented as log10 colony-forming units (CFU) per gram **(Manafi *et al*., 2018).**

Evaluation of lymphoid organs (bursa, spleen):

At day 14, 28 and 42 of age, the same 9 birds/ treatment from which intestinal content was taken were euthanized by severing the jugular vein **(Fujiwara *et al*., 2009).** The spleen and bursa of Fabricius were excised and weighed after adherent fat from these organs was removed for determination of The relative weights of the different organs were calculated as percentage of live body weight **(Ahmadi , 2011).**

RESULTS

Growth performance parameter:

Influence of feeds incorporated with *Bacillus subtilis* and *Saccharomyces cerevisiae* probiotics on The average weight gain , feed intake , feed conversion rate and percentage of mortality in all three treatment group at 14 ,28, 42 days post feeding are shown in (Table 2).

Treatment	Control group(T1)			*Bacillus subtilis* group (T2)			*S. cerevisiae* group (T3)		
Age	A.WT(g)	F.I(g)	FCR	A.WT(g)	F.I(g)	FCR	A.WT(g)	F.I(g)	FCR
14	439	489	1.11	450	500	1.11	456	542	1.16
28 days	1479	2100	1.41	1500	2200	1.46	1524	2500	1.64
42 days	2686	4157	1.54	2750	4605	1.67	2897	4785	1.67
Mortality %/ group	8.30%			3.33%			1.67%		

A .WT (average weight) was calculated by submission of body weight of three birds from each replicate divided by number of birds.

F.I (Feed intake) was calculated on a pen basis by dividing the amount of weekly feed consumption by the number of birds alive at the end of the week.

FCR (Feed conversion ratio) was weekly calculated as Feed Conversion=Feed intake / weight gain at 14, 28 and 42 days. There were obvious differences in BW among birds in different experimental treatment groups at 14, 28 and 42 days old (Table 2). Broilers fed with feed supplemented with *Bacillus subtilis* at 100 g/ton showed higher body weight than control birds. The BW of *Saccharomyces cerevisiae* supplemented group was higher during the completely experimental period compared with *B. subtilis* supplemented group and control group. The BW of the control group was lower than all other treatment groups at day 42. There were a differences in overall feed intake and FCR among experimental treatment groups. Broilers that consumed pyomex at 100 g/ton had the highest feed consumption and no difference in FCR as compared with control at day 14 but it was increased gradually at 28 and 42 day. All probiotic treatment groups showed a higher FCR than the control group by day 42. Broiler, which fed Organotech-supplemented diet, had higher weight gain, feed intake than other treated group but the same FCR as pyomex supplemented group by day 42.

Intestinal bacterial count:

A significantly large population of intestinal *Lactobacillus* was found in chickens fed with feed incorporated with *Bacillus subtilis* and *Saccharomyces cerevisiae* after 28 and 42 days (Table 3) as compared with control. However, there were slightly differences between the population of intestinal *Lactobacillus* between probiotic-supplemented groups during 28 and 42 days of age. Broiler that fed *Saccharomyces* from day one in the experiment had the higher *Lactobacillus* population as compared with control and *Bacillus* supplemented diet during the completely experimental period.

Table (3): Populations of intestinal *Lactobacillus* in broilers fed feeds incorporated with *Bacillus subtilis* and *Saccharomyces cerevisiae* at 14, 28, 42 days post feeding.

Population of intestinal *Lactobacillus* (log CFU /g)			
Treatment	**Day of age**		
	14 (d)	**28 (d)**	**42 (d)**
Control	**7.61**	**7.55**	**7.64**
Bacillus subtilis	**8.20**	**9.09**	**9.43**
Saccharomyces cerevisiae	**9.32**	**9.41**	**9.45**

Populations of intestinal *E.coli* in broiler fed feed incorporated with *Bacillus* and *Saccharomyces* were different from that of the control. As populations of intestinal *E.coli* in chickens fed *Saccharomyces* added to diet were highly decreased than *Bacillus* supplemented group and control group, respectively during all experimental trail as shown in (Table 4).

Table (4): Populations of intestinal *E.coli* in broilers fed feeds incorporated with *Bacillus subtilis* and *Saccharomyces cerevisiae* at 14, 28, 42 days post feeding.

Population of intestinal E.coli (log CFU /g)			
Treatment	**Day of age**		
	14 (d)	**28 (d)**	**42(d)**
Control	**7.28**	**7.46**	**7.58**
Bacillus subtilis	**6.96**	**6.90**	**6.89**
Saccharomyces	**6.25**	**6.08a**	**5.98**

Table (5) shows the occurrence of *Salmonella* in chickens fed three different added diets. Triplicate samples were taken from each groups at 14, 28 and 42 day of age. The result showed that, the *Salmonella* occurrence was not detected at 14 and 28 day of age in all three groups there were no *Salmonella* occurrence through all the experimental period in *Saccharomyces* treated groups. Moreover , there was a decrease in no of *Salmonella* positive samples in *Bacillus* probiotic added groups than in control respectively at 42 day of age .However, slightly differences in number of colonies were found among positive sample of probiotic supplemented groups and control group.

Table (5): Influence of *Bacillus subtilis* and *Saccharomyces cerevisiae* probiotics on occurrence of cecal *Salmonella* at 42 days post feeding.

Treatment	Occurrence of *Salmonella* (log CFU /g)		
	No of examined sample	No of positive sample	Log CFU / g
Control	**3**	**3**	**4.27**
Bacillus subtilis	**3**	**1**	**4.06**
Saccharomyces cerevisiae	**3**	**0**	**-**

Lymphoid organ:

Weight of lymphoid organ was recorded during the experimental period as shown in (Table 6). Spleen weight in all treated groups was not significantly different through all the experimental period. However, bursa weight was different in probiotic-supplemented groups from control. Broiler that fed diet supplemented with *Saccharomyces* probiotic had heavier bursa weight than Bacillus and control groups'. In addition, bursa weight in Bacillus added groups was higher as compared with control, which fed basal diet only.

Table (6): Influence of feed supplemented with f *Bacillus subtilis* and *Saccharomyces cerevisiae* probiotics on lymphoid organ weight at 14 to 42 days post feeding.

Treatment	Spleen weight (g)			Bursa weight (g)		
	Day of age			Day of age		
	14 (d)	28 (d)	42 (d)	14 (d)	28 (d)	42 (d)
Control	**0.43**	**0.78**	**2.13**	**0.56**	**0.72**	**1.06**
Bacillus subtilis	**0.46**	**0.79**	**2.16**	**0.58**	**0.74**	**1.1**
Saccharomyces cerevisiae	**0.47**	**0.79**	**2.23**	**0.65**	**0.78**	**1.3**

DISCUSSION

This study investigate effect of *Saccharomyces cerevisiae* and *Bacillus subtilis* probiotics on broiler growth performance as a growth promoting factor , mortality rate and their inhibitory effect on pathogenic bacteria as potential antibiotic alternatives. Probiotics are live microorganisms that when administered in adequate quantities have beneficial effects on the host by improving the intestinal bacterial balance **(Fuller 1989).** Dietary probiotics have been shown to improve weight gain, reduce mortality, and enhance feed conversion, resulting in increased broiler productivity **(Gerendai and Gippert 1988; Owings *et al.* 1990).** Our results showed that addition of *Saccharomyces cerevisiae* improved live weight gain, feed intake and feed conversion rate. Moreover, mortality was monitored throughout the course of the study and was found to be 8.3% in CON,3.33% in *Bacillus subtilis* group and 1.67 % in saccharomyces supplemented group.Such result agrees with those of Ezema ***et al,* (2007) and Abadeen *et al*, (2017).** Also it support their hypothesis that the use of *Saccharomyces cerevisiae* provided a source of proteins, vitamins, enzymes, and growth factors which improved nutrients absorbed in the digestive tracts of broilers so it increased body weight gain , FCR and decreased mortality rate even if chicks were fed aflatoxin contaminated feed as it bind with toxic compounds and prevent its absorption. The effects of *Bacillus subtilis* culture on growth of domestic avian has been studied extensively .*Bacillus* supplementation has also been shown to have beneficial effects on productivity, mortality, modulation of intestinal microflora, pathogen inhibition, and immune system stimulation in poultry **(LaRagioneand Woodward , 2003; Lee *et al.,* 2010).** Our results was similar to the findings by **Gadde *et al.* (2017), Park *et al.* (2018);Teo and Tan ,(2006) and Rajput *et al* ., (2013)** who reported that Bacillus *subtilis* improved broiler growth performance, weight gain and mortality rate was highly decreased as compared with control. In addition, **Khaksefidi and Gnocchi, (2006)** proved that administration of *Bacillus subtilis* in diet with 50mg/kg led to significant increase in live body weight, feed intake and feed conversion rate from 22 to 42 days in bird fed probiotic supplemented diet than bird fed control diet.

Our results support the above hypothesis and In contrast with **Lee *et al.* (2010)** who reported through their study that, the chickens that were fed a diet containing any of the *Bacillus subtilis* strain did not statistically exhibit altered body weight gains between 0 and 21 d post hatch compared with birds fed the non-supplemented control diet. **Jin *et al.* (1996)** reported that *Bacillus subtilis* culture when fed to poultry would associate with the gut wall and favor

an increase in the numbers of natural *Lactobacillus*. It will in turn , suppress undesirable enteric microorganisms such *E.coli* but had no effect on the occurrence of *Salmonella* in poultry .the present study support ,in part , the above hypothesis and showed that, the addition of *Bacillus subtilis* could increase intestinal *Lactobacillus* and decreased both *Salmonella* and *E.coli* population in chicken but it disagreed with findings of **saartchit and Sullivan , (1990)** which found that ingestion of *Lactobacillus* had no effect on intestinal population of *E.coli* and *Lactobacillus* in turkeys. Our results show that addition of *Saccharomyces cerevisiae* in broiler diet affected on intestinal microbial population by increasing intestinal Lactobacillus population and decreased pathogenic bacterial population as *E.coli* and *Salmonella*. These results agree with the results mentioned by **Koc *et al.* (2010**) that *Saccharomyces cerevisiae* supplementation substantially increased the population of Lactic acid bacteria and yeast in the cecum content and the population of *E. coli* was significantly decreased. *Saccharomyces cerevisiae* increased body weight, growth performance and immune host status. Fed supplement with *Saccharomyces cerevisiae* probiotic will increase globulin protein levels and stimulate immune response due to Chitin, mannan, and glucan, derivatives of the cell wall of *Saccharomyces cerevisiae* **(Abaza *et al*.2008).** In addition,MOS selectively prevent pathogen colonization of the gastrointestinal tract by offering competitive binding sites for undesirable microorganisms including *Salmonella* and *Escherichia coli* **(Newman, 1994).** Multiple strains of *E. coli* and *Salmonella* agglutinated to MOS in vitro **(Spring *et al*., 2000).** The MOS that found in cell wall of *Saccharomyces* is not enzymatically digested in the small intestine; therefore, bacteria bound to MOS likely exit the intestine without attaching to the epithelium **(Spring *et al.,* 2000).** The removal of potential pathogens from the intestinal tract of growing animals may provide a more favorable environment for the digestion, absorption, and metabolism of growth-enhancing nutrients **(Savage and Zakrzewska. 1996).** However, *Saccharomyces* show the best results during our in vivo experimental period in decreasing mortality rate and pathogenic bacteria also increased body weight and beneficial bacteria as *Lactobacillus.* Growth performance improvement may be attributed to the increase of populations of beneficial bacteria, such as *Lactobacillus* decrease the pH of the gastrointestinal tract due to increased production of lactic acid and volatile fatty acids. Therefore, the environment of the gastrointestinal tract becomes unsuitable for the proliferation of pathogens such as *Salmonella*. Furthermore, this inhibition is believed to be associated with the secretion of various antimicrobial compounds, such as organic acids,

hydrogen peroxide, and bacteriocins **(Reid *et al.* 2003).** Thus, these probiotic effects might influence the proliferation of the *Lactobacillus* and *Salmonella* populations.

In addition,Probiotics can affect the microbial stabilization in gastrointestinal system like antibiotics and this situation may be modify the microflora which those are the origin of some gastrointestinal sickness or favor the healthy intestine microflora **(Conway *et al* .1997; Choct. 2001).** Moreover, the balanced microbial population in the gastrointestinal tract which has an important role in the health and improve body weight gain and growth performance of the broilers **(Thongsong *et al.,* 2008).** The weight of bursa was significantly increased in probiotic-supplemented groups as compared with control. However, the relative weight of spleen was not affected by probiotic .our mentioned results was similar to **(Teo and Tan. 2007)** who recorded a heavy bursa of Fabricius in the birds supplemented with *Bacillus subtilis PB6* and *Saccharomyces cerevisiae* **(Ahmadi., 2011)** for 42 day compared with control groups. The reasons of this condition may be attributed to effect of *Saccharomyces cerevisiae* yeast on microbial population of gut. On the other hand, due to an increase in the number of follicles with high plasma cell reaction in the medulla which led to increase weight of bursa of fabricius in probiotic- treated group **(Shoeib *et al.* 1997).** In conclusion, the present study demonstrated that, the addition of *Bacillus subtilis* and *Saccharomyces cerevisiae* in the feeds of broiler improve the growth performance compared with control. A result that might be attributed to their probiotics effects, including the increase of beneficial microbial populations as *lactobacillus*, improved digestion, competitive exclusion of pathogenic bacteria, decrease the mortality rate and stimulate the broiler immunity.

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