Evaluation of *Bacillus subtilis* biopreparations as growth promoters in chickens

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Bacillus subtilis bioprepations were evaluated as growth promoters in chickens. Two hundred chickens of the Cuban breeder hybrid EB34 were used, according to a completely randomized design. Four treatments were studied: diet (maize-soybean), diet + biopreparation C-31, diet + biopreparation C-34 and diet + biopreparation E-44, applied to a dosage of 109 endospores/g^{-1} of concentrate. The experiment was carried out for 42 d and microbiological and fermentative indicators were assessed. The study of these biopreparations provoked improvements in the microbial balance of the birds' caecum, when at 21 and 42 d was observed increase of the population of Lactobacillus, Bacillus endospores and total anaerobes, as well as reduction of coliforms at 42 d. In the treated groups with the *B. subtilis* biopreparations and their endospores the total and individual short chain fatty acids (SCFA) and the lactic acid content, were increased with a decrease of the intestinal pH at 42 d. It is concluded that *B. subtilis* biopreparations can be used as growth promoters in chickens.

Key words: *probiotic, Bacillus subtilis, chickens, growth promoters*

The addition of antibiotics to the feeds, as animal growth promoters or as prophylactic measure, is a common practice in modern poultry industry. However, its indiscriminate use can increase the number of strains responsible for human diseases, making them resistant to antibiotic therapies. Also, they can increase the possibility that these conditions are transferred from one microorganism to another (Morales 2007).

Different alternatives are proposed for the substitution of growth promoters, much more compatibles with feeding security and with the needs of the consumer (Farnell *et al*. 2006 and De Vuyst *et al*. 2008). Numerous natural products, among which are the probiotics based on sporulated *Bacillus spp*., are used by different enterprises of the sector to improve the intestinal microbial balance. This contributes to increase the concentrations of short chain fatty acids (SCFA) and the lactic acid (Steve 2006 and Opalinski *et al*. 2007).

The objective of this study was to evaluate *B. subtilis* biopreparations as growth promoters in chickens.

Materials and Methods

The experiment was carried out in the Poultry Unit of the Institute of Animal Science, Cuba. Two hundred chickens of the Cuban breeder hybrid EB24 were used from 1 to 42 d in a completely randomized design. Four treatments were studied: diet (maize-soybean) as negative control (NC), diet + biopreparation C-31, diet + biopreparation C-34 and diet + biopreparation E-44.

Feed supply was *ad libitum* and the drinking water was treated with calcium hypochlorite at 0.1 %. The diet varied in its composition for the different stages (starter, growing, finishing), according to NRC (1994) (table 1). The biopreparations were supplied at a dosage of 109 endospores g^{-1} of concentrates and adjusted according to the criteria of Frizza *et al*. (2003) and Mutus *et al*. (2006).

For determining *in vivo* the effect of the biopreparations on the microbiological and fermentative indicators, ten animals per treatment were selected and slaughtered puncturing the jugular vein. For the selection, birds with live weights in the range of ± 10 % of the actual average live weight of each group of animals were chosen.

Microorganism counts. The caecal contents of the birds were extracted at 21 and 42 d and one gram of it was taken. For carrying out lactobacilli, coliforms, yeasts and total anaerobe counts, serial dilutions of the samples (1:10, p/v) in thinner medium until 10^{-11} were made. From these dilutions, 10^{-9}, 10^{-10} and 10^{-11} were utilized for total anaerobe bacteria and lactobacilli; 10^{-6}, 10^{-7} and 10^{-8} for coliforms and 10^{-3}, $10^{-4}$and 10^{-5} for yeasts. Each one was replicated three times (0.5 mL) in rolling tubes containing 5 mL of selective culture medium (Hungate 1970). For total anaerobes the technique of Hungate (1966) was used under strict anaerobiosis conditions. The microbial count was made after incubating at 38º C (72 h for Lactobacilli, 24 for coliforms, 48 for yeasts and 7 d for total anaerobes). The number of CFU was determined with the aid of a magnifying glass, by visual colony count.

Sample preservation. Samples of the caecal contents of chickens were taken. They were diluted in water (1 g/10 mL) and preserved according to the determinations under evaluation. For the preservation of the samples destined for determining total and fractioned SCFA, 5

Table 1 Composition and contribution of the diets used

Raw materials (%)	Starter (0- 14 d)	Growing (15- 28 d)	Finishing (29-42 d)
Maize meal	42.43	54.32	60.27
Soybean meal	43.88	33.68	28.58
Sunflowerseed oil	8.80	7.28	6.52
Dicalcium phosphate	2.57	2.45	2.39
Calcium carbonate	0.74	0.72	0.71
Common salt	0.25	0.25	0.25
DL-methionine	0.33	0.30	0.29
Vitamin-mineral premix	1.00	1.00	1.00
Calculated analysis			
Metabolizable energy (MJ/kg)	13.38	13.38	13.38
Crude protein (%)	23.00	20.00	18.80
Calcium (%)	0.95	0.95	0.95
Assimilable phosphorus (%)	0.42	0.42	0.42
Methionine + Cystine (%)	0.92	0.87	0.82

mL were taken and 5 mL were added of a H_2SO_4 solution saturated with $MgSO_4$. For determining lactic acid, 0.3 mL of $HgCl_2$ to 5 mL of the sample was added. All samples were preserved at refrigeration temperature (4-8º C) until its processing.

pH determination. One gram of the caecum content was taken at 21, 35 and 42 d and diluted in 10 mL of distilled water (1:10). Samples were agitated and later the pH was read in the equipment pHmeter Sartorius Meter PP-25.

Lactic acid determination. The preserved samples of the caecal content were centrifuged at 42 d at 15000 rpm, for 15 min. From 1 mL of the supernatant of each sample, the acid lactic determination by colorimetry from the technique proposed by Taylor and Kimberley (1955) was realized. Firstly, a standard curve was prepared with increasing amounts of lactic acid (from 0 to 30 µg) for determining the cotangent. This allowed, from the absorbance reading at 570 nm in a spectrophotometer (Ultrospec 2100 pro), to estimate the lactic acid concentration in 1 mL of supernatant of the samples, once 3 mL of H_2SO_4 (96 %), 50 ŋL of $CuSO_4$ (4 %) and 100 µL of p-phenylphenol in ethanol at 95 % (1.5 %), were applied.

SCFA determination. For the SCFA study, samples of the caecal contents taken at 42 d were utilized and they were worked by gas chromatography. The preserved samples were centrifuged at 15000 rpm for 15 min. The supernatant (1 mL) was treated with 1 mL of a solution composed of 0.05 g of chrotonic acid (internal standard) and 2 g of metaphosphoric acid (deproteinizer), dissolved until 100 µL in HCL 0.5N. One µL was injected in a Perkin-Elmer Autosystem XL chromatograph, equipped with automatic injector, flame ionization detector (FID) and capillary TR-FFAP column, of 30 m x 0.53 mm x 1 µm (Supelco). The analysis conditions were: 140º, 250º and 250º C of temperature, in column, injector and detector, respectively, and He (carrier gas) flow of 13 mL min^{-1}. A 1/3 split relationship was applied. The analysis was made by gas chromatography (GC-FID).

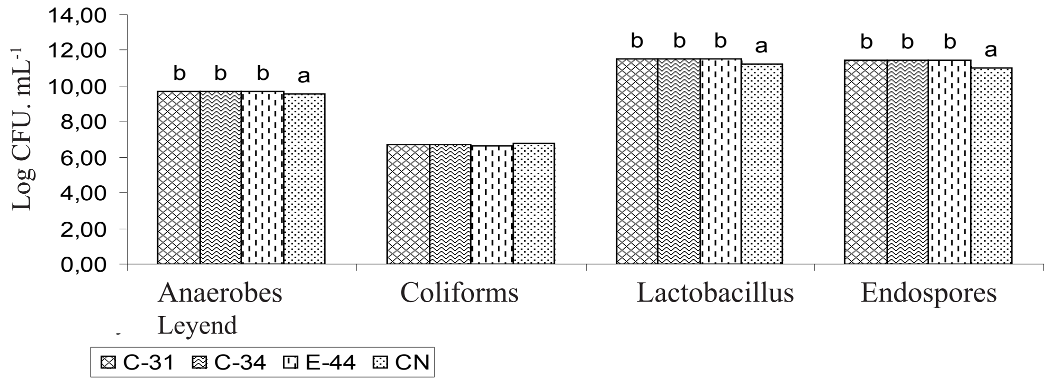
Statistical methods. For the analysis of the results the INFOSTAT, version 1 statistical program was used (Balzarini *et al*. 2001). For the treatment of the results an analysis of variance with a completely randomized design was realized. In the verification of the significant differences Duncan's (1955) multiple range test was used.

Results and Discussion

Figures 1 and 2 represent the performance of counts for total anaerobes, coliforms, Lacto *Bacillus spp*. and endospores, at 21 and 42 d in the caecum of birds, respectively. Total anaerobes showed higher values in the treated groups with biopreparations ($P < 0.001$) when compared to the control group in both ages.

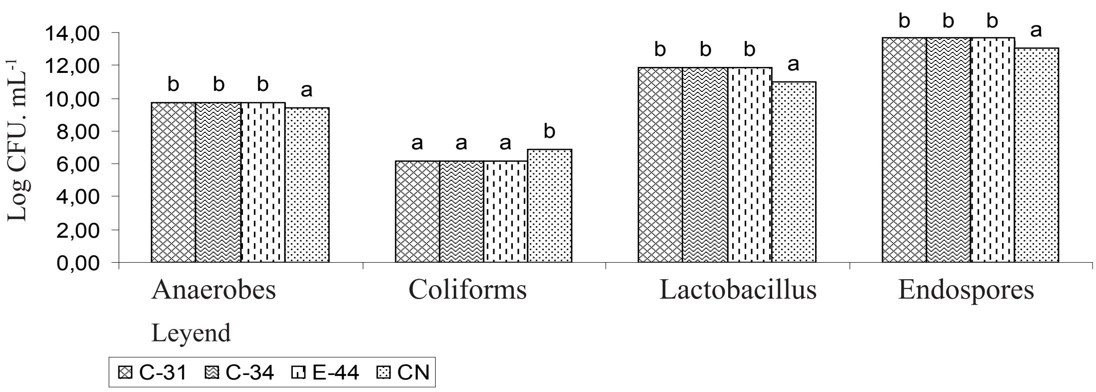
In coliform counts there were no differences between treatments at 21 d with *B. subtilis* cultures and the control group. This result could be related to the low acid levels, which was corroborated when it was assessed at 21 d. Rondón (2009) referred that one of the elements that makes possible the decrease of coliforms in the intestinal contents is the increase of SCFA and lactic acid in the caecum. Also the colonization and proliferation of lactobacilli added to the GIT of chickens are involved, provoking the competitive exclusion in face to other microorganisms of the normal microbiota or pathogen microorganisms. These criteria do not coincide with the results obtained. However, at 42 d it decreased ($P < 0.001$).

Lactobacillus and endospores counts showed higher



[ab]Different letters between treatment differ at P < 0.05 (Duncan 1955)
Total anaerobes SE ± 0.12 ***P < 0.001, coliforms SE ± 0.35**P <0.01,
lactobacillus SE ± 0.13 **P < 0.01 and endospores SE ± 0.001 ***P < 0.001

Figure 1. Count of microorganisms isolated in the caeca of chickens treated with three cultures of *B. subtilis* at 21 d.



[a,b] Different letters between treatments differ at P < 0.05 (Duncan 1955)
Total anaerobes SE ± 0.07 ***P < 0.001; coliforms SE ± 0.20 ***P <0.001;
lactobacillus SE ± 0.07 ***P < 0.001 and endospores SE ± 0.008 ***P < 0.001

Figure 2. Count of microorganisms isolated in the caeca of chickens treated with three cultures of *B. subtilis* at 42 d.

values in the treatments with *B. subtilis* cultures, at 21 (P < 0.01) and 42 d (P < 0.001). Caecum samples collected did not evidence the presence of yeasts in the evaluated dilutions (10^{-3}. 10^{-4} and 10^{-5}).

In the literature there is information on the application of probiotic products based on *Bacillus spp*. cultures in sporulated form, since they improve the microbial balance and the gastrointestinal tract health (Barbosa *et al*. 2005). Studies carried out by Gunther (1995), Samaniego *et al* (2004) and Rondón *et al*. (2008) define that one of the probiotic actions from the *Bacillus spp*. endospores is the decrease of enteropathogens, originated by the growth stimulation of the Lacto *Bacillus spp*. population.

The decrease of the number of coliforms present in the caecum content could be given by the inclusion of *B. subtilis* cultures. These cultures propitiate better microbial balance (eubiosis) and create favorable conditions for the development of beneficial microbial species, as Lacto *Bacillus spp*. Also, they promote a physiological effect in the organism beyond its nutritive value (Khaksefidi and Ghoorchi. 2006) and Laurencio *et al*. 2008).

Studies carried out by Spinosa *et al*. (2009), Hoat *et al*. (2001) and Casula and Cutting. (2002) demonstrated that the endospores of *Bacillus spp*. can germinate at GIT level (specially in the jejunum and ileum) and produce specific enzymes which favor the absorption of nutrients and of antimicrobial substances, capable of inhibiting the present pathogen microbiota.

Teo and Tan (2006) on evaluating the performance of the *B. subtilis* PB6 strain (CloSTAT) in chickens confronting an *E. coli* pathogen strain observed a decrease of this bacterium in animals consuming this probiotic. Also, they stated an increase in Lactobacillus count, from 1.4 to 4.5 x 10^{11} CFU g^{-1}. Maruta and Miyazaki (1996) evaluated the performance of *B. subtilis* cultures to decrease the presence of Salmonella and Campylobacter at GIT level. As result they obtained the decrease of these pathogens and increase in the number of Lactobacillus.

The important function that the intestinal microbiota plays in the resistance to the diseases was strengthened with the inclusion of these probiotic biopreparations of *B. subtilis* in sporulated form in the feed. This action was manifested in better preparation of the gastrointestinal tract to face the harmful bacteria challenging birds' health.

In the literature there are no studies on the activity of Bacillus endospores at GIT level in chickens. However, there are investigations regarding the performance of Bacillus endospores at GIT level in murine models with positive results in the intestinal microbiota balance (Spinosa *et al*. 2000 and Hoat *et al*. 2000).

In table 2 are shown the pH values at 21, 35 and 42 d. There were no differences at 21 and 35 d between animals treated with *B. subtilis* cultures or between these and the control animals. At 42 d there was a decrease (P < 0.001) of the pH of the caecal contents of chickens treated with *B. subtilis* cultures.

This result could be related to the production of acids occurring in the fermentative processes developed by the microorganisms in that ecosystem. These have an effect on a better physiological and microbiological state at GIT level, an increase in the levels of beneficial microorganisms, with possible improvement in nutrient assimilation, which could result in a betterment in the cell rechanging and maintenance processes. When the acid pH is maintained in the gastrointestinal tract, the metabolism and the lactic acid bacteria multiplication increase. These release enzymes that improve the host digestive capacity, inactivate more efficiently the toxic metabolites of the harmful biota and increase the absorption process, due to a better cell state of the villi. On increasing the secretion of antimicrobial substances (Segura and De Bloos 2000), there is greater vitamin synthesis and a more efficient control of enteropathogens is developed.

These results agree with those reported by Roe *et al*. (2002) and Pérez *et al*. (2005). These authors indicated that the decrease of the intestinal pH favors a greater digestive and absorptive activity by the host, besides making feasible the efficient control of intestinal pathogens.

In table 3 are set out the levels of some total and fractioned SCFA, as well as the lactic acid in the caecum at 42 d. There were values from P < 0.05 to P < 0.001 for chickens treated with *B. subtilis* cultures.

Results obtained coincide with Hungate (l966), who stated that among the final products of the microbial fermentation in the caecum are the SCFA, mainly acetate, propionate and butyrate. These constitute the main anions of the intestine. Also, they are included among the final products of the carbohydrate fermentation to lactate, ethanol, succinate, among others.

Caja *et al* (2003) and Linge (2005) demonstrated the intestinal pH decrease in studies carried out in birds with *Bacillus spp*. strains. This finding was related to the function of the acids as bacterial growth controllers.

Although not completely confirmed, one of the effects of the Bacillus cultures and their endospores is considered to be that of favoring the increase of the population of Lactobacillus species at GIT level, with the increase in the production of lactic acid and decrease of the intestinal pH (La Ragione *et al*. 2003). Results obtained in this work refute the hypothesis of the possibility of the *Bacillus spp*. cultures of influencing positively on the intestinal microbiota.

It can be inferred that the probiotic action of these biopreparations (C-31, C-34 and E-44) is associated

Table 2. The pH performance in the caecum contents of chickens treated with three cultures of *B. subtilis*

Indicator	Time (d)	Treatments				SE ± Sign
		C-31	C-34	E-44	CN	
	21	7.17	7.01	7.16	7.34	0.11
pH	35	6.81	6.88	6.85	7.38	0.17
	42	6.49^{a}	6.41^{a}	6.41^{a}	7.28^{b}	0.14***

^{ab}Different letters within the same row differ for P < 0.05 (Duncan 1955) ***P < 0.001

Table 3. SCFA levels in the contents of the caecum of chickens treated with three cultures of *B. subtilis* at 42 d

Indicator (mmol.L^{-1})	Treatments				SE ± Sign
	C-31	C-34	E-44	CN	
Acetic	11.56^{b}	11.32^{b}	11.36^{b}	9.09^{a}	0.27***
Propionic	2.02^{b}	1.88^{b}	2.03^{b}	1.43^{a}	0.13*
Total	14.85^{b}	14.65^{b}	14.87^{b}	11.85^{a}	0.32***
Lactic (µg,L^{-1})	156.92^{b}	157.38^{b}	160.85^{b}	133.74^{a}	7.06*

^{ab}Different letters differ between treatments for P < 0.05 (Duncan 1955) ***P < 0.001 and *P < 0.05

to the probiotic effect and to the action exerted in the cell rechanging and maintenance processes, metabolic and microbial of the GIT, mainly at caecum level. These responses allow concluding that the three biopreparations of *B. subtilis* could be used as growth promoters in chickens. This is an important aspect that must be considered, if positive results in some microbiological and fermentative indicators evaluated are expected.

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