Probiotic activity of PROBIOLACTIL®, SUBTILPROBIO® and its mixture in broilers

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ABSTRACT

Biopreparations with Lactobacillus salivarius and Bacillus subtilis are used as probiotics in poultry farming due to their beneficial effects on the intestinal ecosystem. Objective. To evaluate the probiotic activity of the biopreparations PROBIOLACTIL®, SUBTILPROBIO® and their mixture on microbiological, productive and health indicators in broilers. Materials and methods. An experiment with a completely randomized design was carried out for 42 days with four treatments: T1. Only based on diet (control group), T2. PROBIOLACTIL® (bioprepared with Lactobacillus salivarius C-65) + diet, T3. SUBTILPROBIO® (Bioprepared with Bacillus subtilis E-44) + diet and T4. Mixture of both biopreparations + diet. Results. The treatments where the probiotics were applied presented an increase in total lactobacilli and total anaerobes (p≤0.05) and a reduction in total coliforms. In the productive indicators, it was noticed that the live weight was higher in the birds treated with the mixture, as it showed an improvement in weight gain, average daily gain, feed conversion and carcass yield. The application of additives reduced mortality and increased viability in birds. Conclusions. The synergistic activity of Lactobacillus salivarius and Bacillus subtilis increases probiotic activity and favors the productive performance and viability of broilers.

Keywords: Poultry; Bacillus subtilis; Lactobacillus salivarius; chickens (Source: DeCS).

RESUMEN

Objetivo. Evaluar las propiedades probióticas del PROBIOLACTIL®, SUBTILPROBIO® y su mezcla como biopreparados en los indicadores microbiológicos, productivos y en la salud de pollos en ceba. Materiales y métodos. Un experimento fue realizado con diseño completamente aleatorizado durante 42 días con cuatro tratamientos: T1. Basado sólo en la dieta (control), T2. PROBIOLACTIL® (biopreparado con Lactobacillus salivarius C-65) + dieta, T3. SUBTILPROBIO® (Biopreparado con Bacillus subtilis E-44) + dieta y T4. Mezcla de ambos biopreparados + dieta. Resultados. Los
tratamientos donde se aplicaron los probióticos presentaron un incremento de lactobacilos y anaerobios totales (p≤0.05) y la reducción de los coliformes totales. En los indicadores productivos se observó que el peso vivo fue superior en las aves tratadas con la mezcla, y una mejora en el incremento de peso, la ganancia media diaria, la conversión alimenticia y el rendimiento de la canal. La aplicación de los aditivos redujo la mortalidad e incrementó la viabilidad en las aves. **Conclusiones.** La actividad sinérgica de *Lactobacillus salivarius* y *Bacillus subtilis* incrementa la actividad probiótica y favorece el comportamiento productivo y la viabilidad de los pollos de ceba.

**Palabras clave:** Avicultura; *Bacillus subtilis; Lactobacillus salivarius*; pollos (Fuente: DeCS).

**INTRODUCTION**

The use of antibiotics to maintain animal welfare, promote growth and improve efficiency has been practiced for more than 50 years. However, as early as the 1950s, researchers identified concerns about the development of bacteria resistant to the antibiotics streptomycin and tetracycline used in turkeys and broilers, respectively. This research laid the groundwork for agricultural officials to impose stricter regulatory parameters on the use of antimicrobials in poultry feeds (1).

Restricting the use of antibiotics in the diet to promote animal growth sparked interest in finding alternative approaches. These include probiotics, cultures of live microorganisms that can be formulated into different types of products, including feed, drugs, and dietary supplements (2).

Among the bacteria most commonly used as probiotics in poultry are those of the Bacillus spp. and *Lactobacillus* spp. genera (3,4,5). Different researchers report that these microorganisms improve the beneficial microbial composition of the gastrointestinal ecosystem, inhibit pathogenic bacteria, stimulate the immune system, produce enzymes that intervene in the decomposition of nutrients, improve the integrity of the mucosa, neutralize enterotoxins, decrease the production of ammonia, improve productive efficiency, as well as the viability of the animals (6,7).

**MATERIALS AND METHODS**

**Preparation of bacterial cultures.** In the preparation of 10 L of SUBTILPROBIO® and PROBIOLACTIL®, strains of *Bacillus subtilis* E-44 and *Lactobacillus salivarius* C-65 were used, as described by Milián (13) and Rondón (14) respectively. Counting colony forming units (CFU) per mL from seeding in petri dishes (containing MRS agar for L. salivarius and nutrient agar for B. subtilis), as well as pH measurement, were used to check the quality of the additives. These biopreparations were stored in sterile 1 L rubber-capped flasks at 5°C. For the application of the mixture the cultures were added in equal parts (50:50).

**Location and duration.** The experiment was carried out in March 2021 at the San Agustín Zapata farm of the Israel Cabrera Cooperative, belonging to the private producer Lucía Liset Marrero Tarifa, located in the municipality of Unión de Reyes, Matanzas, Cuba. The average temperature reported was 23.9±2°C and humidity was 50±2% (15). The trial lasted 42 days.

**Experimental design.** A completely randomized design from 1-42 days was used. Two thousand one-day-old male broilers of the Cornish breed, HEEP-55 line, weighing 42±2 g, supplied by the Incubator of the “Empresa Genética Avícola”, Matanzas province, were used. Eight 1.25 × 3.75 m pens were used in the experiment. Each pen contained 250 chickens with a density of 11...
birds per square meter. Four treatments with two pens of 500 chicks each were applied: T1. Control group, where only the diet was fed, T2. PROBIO LACTIL®+ diet, T3. SUBTILPROBIO®+ diet and T4. Mixture of the two biopreparations (50:50, v/v) + diet. The additives were applied in the drinking water (1 mL.L⁻¹). Each additive had a concentration of 10⁹ cfu.mL⁻¹.

Feeding. The feed was prepared from corn and soybean according to Technical Instructions No. 7 UCAN -IIA (16) for broilers. Water and feed were supplied ad libitum. The feeders used were cylinder feeders and manual plastic drinkers with a 6 L capacity. The temperature in each pen was controlled by 30x26 cm heaters (MARHD), fans and curtains.

Microbiological indicators

Sample collection. 1 g of cecal contents was taken from five broilers per treatment at 42 days. Samples were added to 9 mL of diluent medium (17), then preserved anaerobically (5% CO₂ atmosphere).

Selective media. Selective media were used to count different microbial groups in the chickens’ ceca, such as NRF agar for total anaerobes (18), red-violet bile agar (OXOID) for coliforms, and MRS agar (BioCen, Cuba) for Lactobacillus spp.

Microorganism counts. Serial dilutions (1:10, w/v) were made with the samples obtained in diluent medium until a dilution of 10⁻¹¹ was reached. Dilutions of 10⁻¹⁰, 10⁻¹¹ and 10⁻¹² were used to count lactobacilli and total anaerobes and 10⁻⁶, 10⁻⁷ and 10⁻⁸ for coliforms; these were replicated three times (0.5 mL) in roll tubes with 5 mL of selective culture medium (20). The Hungate technique was used for total anaerobes under strict anaerobic conditions (18). Microbial counting was performed after incubation at 37°C (Lactobacillus for 72 h, coliforms for 24 h and for total anaerobes for 7 days). The CFU count was done with a magnifying glass by observation of the colonies.

Evaluation of the effect of biopreparations on productive and health indicators. Live weight (LW) was recorded with a technical scale (Sartorius). Weight gain (WG), average daily gain (ADG), dry matter intake (DMI) and feed conversion (FCR) were determined at 14, 30 and 42 days, while mortality was observed throughout the experimental period. At the end of the experiment (42 days), carcass yield was calculated. The management of the animals and the calculation of the indicators were carried out according to the Technical Instructions: “Broiler, breeding technology and general sanitary regulations” (16).

Statistical processing. An INFOSTAT statistical program, version 1, was used for this purpose (19). The Tukey test (20) was used for comparison of means at 95% significance. The CompaPro test (21) was used to determine if statistical differences were present for mortality and viability with 95% confidence. In the analysis of the data, the effect of pens was assessed. CFU values were transformed to Log to achieve normality of the data.

Ethical aspects. Before the arrival of the chicks at the farm, all protocols for the disinfection of the barns were developed following the technical instructions of the Technical Instructions for the Rearing of Broilers (16). During the experiment, optimal conditions were taken into account to achieve the animal welfare of the birds, such as heaters (for the initiation stage), fans and curtains to avoid heat stress, water and feed with the nutritional requirements of each stage. The development of the experiment was endorsed at the University of Matanzas, by the Ethics Committee of the Department of Veterinary Medicine and it complied with the guidelines established in the Decree Law of Animal Welfare of Cuba (22).

RESULTS

An increase in live weight was noticed in all stages in the birds that received the biopreparation compared to the control group. Figure 1 shows the live weight of the evaluated animals. In animals where probiotics were administered, an increase in weight (p≤0.001) was seen at 14 days, in relation to the control group, but without showing differences between these groups. At 30 and 42 days, the differences became more evident (p≤0.001) in relation to group I or control, with greater weight being noticed in the birds that consumed the probiotic mixture.

Table 1 shows these calculation results for the diverse productive indicators. From 14 days on, it is appreciated that the birds that consumed the probiotic biopreparations, either in monoculture (T2 and T3) or their mixture (T4), showed higher ADG (Average Daily Gain) and PI (Productive Indexes) (p≤0.001), and after 30 days there
was an increase in these indicators in the group of birds that consumed the mixture, followed by those that were given PROBIOLACTIL® and SUBTILPROBIO® compared to the control group.

Table 2 shows the effects of the biopreparations on the health indicators of the animals. It was found that the birds that consumed the biopreparations showed a reduction in mortality and higher viability than the control group (p≤0.05).

Table 3 shows the population of coliforms, total anaerobes and lactobacilli in the cecum when using the diverse treatments. It can be seen that coliforms were higher in the control group and lower in the treatments with PROBIOLACTIL® and the mixture. However, total anaerobes and lactobacilli increased with the probiotic biopreparations (p≤0.001).

Table 1. Action of the biopreparations on the productive indexes of broilers throughout the experiment.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Days</th>
<th>Control</th>
<th>PROBIOLACTIL®</th>
<th>SUBTILPROBIO®</th>
<th>Mixture</th>
<th>P±EE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Daily Gain (ADG, g)</td>
<td>14</td>
<td>34.21b</td>
<td>43.34a</td>
<td>42.30a</td>
<td>44.93a</td>
<td>0.001±1.03</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>45.01c</td>
<td>49.93b</td>
<td>49.72b</td>
<td>55.27a</td>
<td>0.001±1.18</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>45.23c</td>
<td>51.01b</td>
<td>51.26b</td>
<td>56.06a</td>
<td>0.001±0.25</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>498.50</td>
<td>528.75</td>
<td>516.36</td>
<td>508.23</td>
<td>0.302±12.02</td>
</tr>
<tr>
<td>Dry matter Consumption (DMC, g)</td>
<td>30</td>
<td>2100.28</td>
<td>2233.18</td>
<td>2216.21</td>
<td>2218.45</td>
<td>0.241±10.02</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>3515.2</td>
<td>3543.0</td>
<td>3552.5</td>
<td>3578.5</td>
<td>0.201±14.08</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0.85a</td>
<td>0.77b</td>
<td>0.76b</td>
<td>0.71b</td>
<td>0.001±14.82</td>
</tr>
<tr>
<td>Feed Conversion (FC)</td>
<td>30</td>
<td>1.76a</td>
<td>1.60b</td>
<td>1.61b</td>
<td>1.48c</td>
<td>0.001±8.15</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>1.81a</td>
<td>1.62b</td>
<td>1.62b</td>
<td>1.49c</td>
<td>0.001±10.59</td>
</tr>
</tbody>
</table>

Table 2. Effects of the inclusion of probiotic additives on mortality and viability in broilers from 0 to 42 days of age.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Control</th>
<th>PROBIOLACTIL®</th>
<th>SUBTILPROBIO®</th>
<th>Probiotic Mixture</th>
<th>P±EE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality (%)</td>
<td>4.2a</td>
<td>2.4b</td>
<td>2.6b</td>
<td>1.8b</td>
<td>0.04±0.02</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>95.80b</td>
<td>97.60a</td>
<td>97.4a</td>
<td>98.20a</td>
<td>0.04±0.02</td>
</tr>
</tbody>
</table>

Table 3. Microbiological indicators in the cecum of birds at 42 days with the use of probiotic biopreparations.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Treatments (Log CFU.g⁻¹)</th>
<th>P±EE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coliforms</td>
<td>Control: 8.22a (1.71X10⁸)</td>
<td>6.60c (4.06X10⁸)</td>
</tr>
<tr>
<td></td>
<td>PROBIOLACTIL®: 11.69b (5.10X10¹¹)</td>
<td>12.80a (6.64X10¹²)</td>
</tr>
<tr>
<td></td>
<td>SUBTILPROBIO®: 10.70c (5.18X10¹⁰)</td>
<td>12.87a (7.38X10¹²)</td>
</tr>
<tr>
<td></td>
<td>Mixture</td>
<td>6.32c (2.18X10⁸)</td>
</tr>
</tbody>
</table>

DISCUSSION

The use of probiotics in cattle significantly improves health, immunity, performance, nutritional digestibility and intestinal microbial balance. These biopreparations are useful in regulating beneficial bacteria and microbial turnover by stimulating the host immune system through specific secretions and competitive exclusion of potentially pathogenic microorganisms from the gastrointestinal tract (23).

The activity of probiotic bacteria starts from the beginning of life of the chicks. Primarily lactobacilli colonize the intestinal tract of the birds to achieve a state of eubiosis against pathogens,
showed high tolerance to bile acids and salts, high hydrophobicity, self-aggregation and antibacterial activities. Similar results were obtained by Rondón (14) when she evaluated the probiotic potential of *Lactobacillus salivarius* C-65, the strain used in this work.

The effect of the mixture and the biopreparations independently confirm the results of Chen et al (31), who stated that supplementation with *Lactobacillus salivarius* improves productive indicators, liver function and meat quality of broilers, even when the animals are challenged with aflatoxin B1 (AFB1). Supplementation of this bacterium increased specific antibodies and IFN-g production and lymphocyte multiplication in broilers challenged with AFB1 after immunization against Gumboro disease, demonstrating its immune system stimulating activity and the protection it can exert against the attack of pathogenic microorganisms.

The inclusion of SUBTILPROBIO® (*Bacillus subtilis* C-31 strain) as a feed supplement in the birds also caused improvements in live weight in relation to the control group. Similar results were obtained by Mohamed et al. (32) when they applied *Bacillus subtilis* cultures to broilers and improved growth and immune function by modulating intestinal morphology and cecum microbiota.

According to Xu et al (33) *Bacillus* spp. strains have the ability to survive at various pH and bile concentrations, indicating that they will survive the environment of the gastrointestinal tract of chickens. These authors also state that probiotic microorganisms cause remarkable changes in the cytoskeleton of superficial mucosal cells. These changes in the cellular structure are due to the fact that *Bacillus* strains are able to inhibit the growth of pathogens that colonize this tissue and cause diseases in birds.

Wang et al (34) also evaluated the probiotic properties of *Bacillus subtilis* KC1 as a feed additive for poultry feed. These authors proved that supplementation with this strain increased body weight, relative weight of immune organs and decreased feed conversion. In addition, it was shown that this additive alleviated the adverse effects caused by heat stress and challenge with *Salmonella pullorum*.

All probiotic treatments improved bird viability. In general, *B. subtilis* is known to have the ability to protect the intestinal epithelium thus improving their biological indicators (26). Probiotics can also have a beneficial influence on productivity and animal welfare. These biopreparations stimulate the native microbiome and the elaboration of short-chain fatty acids (SCFA), with proven antimicrobial, hypocholesterolemic and immunomodulatory effects, which contribute to a better nutrient absorption capacity and higher responses in cattle performance (24).

Similar results to this research were obtained by Vera et al (25) when they supplied broilers with monocultures of probiotic strains or mixed biopreparations: *Lactobacillus salivarius* and *Bacillus subtilis*. These researchers reported that the best treatment was T4 or mixture and that all probiotic biopreparations improved broiler production indicators. It is important to highlight that the effect of the application of the mixture was evidenced after 30 days. Authors such as Aliakbarpour et al (26) report that broilers fed with probiotics based on *Bacillus subtilis* and lactic acid bacteria (LAB) show higher live weight in relation to the control group. It is known that *Lactobacillus salivarius* is one of the predominant bacteria in the digestive tract of birds (27) and *Bacillus subtilis* is part of the microbiota commonly found in these animals (28). According to these results, it is proved that from the joint action of these microorganisms or the established synergy, the probiotic activity is enhanced.

In the literature there are other papers where *Lactobacillus salivarius* is used as a probiotic. In this sense, Blajman (29) administered a fresh culture of *L. salivarius* DSPV 001P to estimate its effect on the intestinal microbiota and on the productive parameters of broilers. The degree of colonization and in vivo persistence of this bacterium, which was fed in feed to broilers for 16 days, was evaluated. It was found that *L. salivarius* could be isolated 28 days after the cessation of supplementation and that its inclusion increased feed intake and weight gain in the birds; on the other hand, its application notably decreased broiler mortality.

The probiotic potential of *Lactobacillus salivarius* was also supported by Xu et al. (30), who isolated 57 strains of *Lactobacillus* from the intestinal microbiota of 17 different chicken breeds in China. These authors chose *Lactobacillus salivarius* CML352 as the strain that most influenced productive and intestinal health indicators in laying hens. *L. salivarius* CML352
against infection by Salmonella, E. coli and other pathogens by reducing the adhesion and invasion of these microorganisms by enhancing the intestinal barrier and attenuating inflammatory responses of enterocytes. In this way, it activates the immune system with the production of antibacterial substances (35).

Medina-Saavedra et al (36) analyzed the immune behavior in broilers with the use of B. subtilis. These authors report that this bacterium acts on the innate immunity of birds and that this feature is related to the production of high levels of nitric oxide (NO). This molecule regulates vascular tone, activates platelets and the immune response acting as an intercellular messenger or also as a neurotransmitter of the central nervous system; it is also considered a cytotoxic molecule involved in the elimination of bacteria, viruses and protozoa, as well as tumor cells.

The mortality of the birds was mainly associated with the presence of diarrhea; however, it was found that this indicator was lower in the groups where probiotics were applied. It is known that many bacteriocins originate from lactic acid bacteria, which are highly effective against food-borne pathogens such as Staphylococcus aureus, Escherichia coli O157:H7, Salmonella typhi, Clostridium botulinum, Pseudomonas fluorescens, P. aeruginosa, Shigella flexneri and Listeria monocytogenes (37). Juricova et al (38) studied the inhibitory action of four species of Lactobacillus against S. Enteritidis and E. coli on agar plates and it was found that L. salivarius was the only isolate that inhibited the growth of both pathogenic strains.

Arteaga et al (39) found that when they applied a mixture of Bacillus subtilis 20Bp and Lactobacillus brevis 40Lp to broilers, the performance of productive indicators and the health of these animals improved. It was showed that the birds that consumed the mixture obtained an average daily gain of 65.61 g, while in the control group 56.96 g were produced. The application of the mixture improved feed conversion to 1.70, compared to the results of the control group (1.93). On the other hand, in the control group there was higher mortality where the mixture was not applied, results analogous to those observed in the present investigation.

It was proved that the application of the additives improves the composition of the intestinal microbiota since it reduced the population of coliforms as potential pathogens and increased the population of Lactobacillus and total anaerobes, considered beneficial microorganisms. Iqbal et al (40) refer to the role of probiotic microorganisms when they control or inhibit harmful bacteria through the production of antimicrobial substances, competitive exclusion or activation of the immune response.

The synergistic activity of Lactobacillus salivarius and Bacillus subtilis increases probiotic activity and benefits productive performance and health of broilers.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

We would like to thank the producer Lucia Liset Marrero Tarifa for her cooperation in the execution of the experiment. We also thank CITMA for financing Territorial Project PT211MT001-001: “Evaluation of the effect of zootechnical additives in birds of the UEB Heavy Purebred Lines of the Empresa Genética Avícola y Pie de Cría”.

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