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Effects of *Lactobacillus acidophilus* D2/CSL on laying hen performance

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**ABSTRACT**

In order to evaluate the effects of dietary addition of probiotic strain *Lactobacillus acidophilus* D2/CSL on hen performance and egg quality, 160 commercial Hy-Line Brown pullets, 17 weeks old, were divided in control group (C) (N=80) and treated group (T) (N=80), with 4 alternate replicates of 20 animals each per group. C was fed with a “standard diet”, whereas the T group received the same diet with an inclusion of 1x10⁹ CFU kg⁻¹ of *Lactobacillus acidophilus* D2/CSL (freeze dried cells). The experimental trial lasted 39 weeks after one week of acclimatization.

Hen performance and egg quality (egg production, FCR, egg specific gravity, shell thickness, Haugh Units) were recorded. The results show a higher overall egg production (P<0.01) and better FCR (Kg feed intake/Kg saleable eggs) (P<0.05) in the T birds, but no statistically significant differences were observed in egg weight. The eggs from the T birds were characterized by a higher specific gravity (ESG) (P<0.01) and albumen viscosity (Haugh Units) (P<0.05). No significant differences in egg shell thickness were recorded. In conclusion, *Lactobacillus acidophilus* D2/CSL improved some important parameters in laying hen performance and egg quality.

**Key words:** Hen, Probiotic, *Lactobacillus acidophilus*, Egg quality, Caecal microflora.

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**RIASSUNTO**

EFFETTO DEL *LACTOBACILLUS ACIDOPHILUS* D2/CSL SULLE PERFORMANCE DELLA GALLINA OVAIOLA

Al fine di verificare gli effetti del ceppo probiotico denominato *Lactobacillus acidophilus* D2/CSL, 160 pol-lastre commerciali Hy-Line Brown di 17 settimane di vita sono state divise in due gruppi omogenei di 80 animali ciascuno, controllo (C) e trattamento (T). Ogni gruppo era composto da 4 ripetizioni, con 20 animali/ripetizione. Il gruppo C era alimentato con la “dieta standard”, mentre il gruppo T riceveva la stessa dieta additivata con 1x10⁹ UFC kg⁻¹ di *Lactobacillus acidophilus* D2/CSL (cellule liofilizzate). La raccolta dei dati sperimentali è iniziata dopo una settimana di acclimatamento degli animali ed è proseguita per 39 settimane.
Introduction

Probiotics have been defined as live microorganisms producing health effects on the host upon ingestion provided that they are ingested in “adequate amounts” (Guarner and Schaafsma, 1998; Araya et al., 2001). The physiological status of the flock, the involved probiotic strain(s) (including its vitality and its liveability within the gastro-intestinal tract), the administered dose (CFU), and the treatment length are main factors influencing “on field” results. Today the European Food Safety Authority authorizes only: safe and effective microbial strains in target species. According to the EC Regulation No. 2003/1831, probiotics are feed additives of the functional group 4 (b) “stabilizers of the intestinal flora”. Usually, probiotic lactic acid bacteria (LAB) are strains selected from the intestinal mucosa of the host (symbiotic relationship). According to Stern (1987), these bacteria express antigens recognised as “self” by the system of the specific (adaptive) immunity. Other studies describe the immunological “tolerance” of the intestinal mucosa for commensal or symbiotic bacteria (e.g. probiotic strains) as the result of chemical signals that allow bacteria to modulate the immune response and to inhibit the inflammatory response (Blum et al., 1999; McCracken et al., 2002; Neish, 2002). Gastrointestinal microbiota has either metabolic, trophic or protective functions (Guarner and Malagelada, 2003). The main metabolic function is the intestinal mucin fermentation ingesta. In birds it is carried out specifically in crop (pre-gastric fermentation chamber) and caeca (post-gastric fermentation chambers) (Giardini et al., 1995a, 1995b). Trophic functions are due to the epithelium functionality stimulation through the production of SCFA (short-chain fatty acids) (Ziemer and Gibson, 1998; Montagne et al., 2003), and to GALT stimulation of the gut-associated lymphoid tissue, that is bursa of Fabricius, caecal tonsils, Meckel’s diverticulum, Peyer’s patches, intraepithelial lymphocytes and scattered immune cells of the intestinal lamina propria (McFall-Ngai, 2001; deVos et al., 2004). Furthermore protective functions are the result of the “barrier effect” (“bacterial antagonism” or “competitive exclusion”) towards the “allochthonous” microorganisms swallowed by the host (Berg, 1996). The barrier effect is part of the extra-epithelial innate defences, being the first obstacle to the microbial colonization of the mucosa. It is due to the occupation of potential binding sites (epitopes) or the secretion of inhibitory compounds (Stavric and Kornegay, 1995; Basset et al., 2003).

The “open ecosystem” composed by the gastro-intestinal tract (Savage, 1977) includes different habitats (crop, duodenum-jejunum, ileum, caeca) and microhabi-
tats (epithelium, crypts, mucus layer, lumen); within each habitat and between the different communicating habitats, this ecosystem must find a physiologic equilibrium as a result of interactions between bacteria, bacteria and epithelium, bacteria and mucus layer, bacteria and GALT, and bacteria and diet, to specify some principle factors. In poultry, such equilibrium can be altered by a high number of factors: endogenous causes (inflammations, perturbation of the gastro-intestinal motility, immunodeficiencies), low welfare (stress), lack of feed hygiene (ingestion of pathogenic microorganisms), changes in feeding regimen (feed composition, feeding restriction, forced moulting), pharmacological treatments (antibiotics), etc., that often lead to a reduction of the lactobacilli population (Fuller, 1989; Tournut, 1993; Berg, 1996).

According to Rowland (1992), the intestinal microflora is an “organ”. In this way the unbalance of the normal microbiota (dysbiosis), that is the uncontrolled growth of unwanted microorganisms, can be considered the “disease” of that organ. It consists of an increment of the intestinal putrefactions, with a loss of amino acids, and/or inflammation of the mucosa, with hypotrophy of the villi and reduced assimilation of the nutrients resulting in diarrhoea. This disease causes a reduction of the animal performance and a decrease in the quality of the products (Barrow, 1992).

In order to reconstitute a better ecological gastrointestinal balance (“eubiosis”), meaning a better ratio between probiotic (symbiotic) bacteria and “unwanted” (putrefactive and pathogen) ones, it is appropriate to intervene with the administration of properly selected probiotic LAB (Bianchi Salvadori et al., 1998; Patterson and Burkholder, 2003; Nousiainen et al., 2004). Autochthonous LAB, such as Lactobacillus acidophilus, are related in a positive way with the intestinal mucosa of the bird. Adhering to the intestinal mucosa, they strengthen the barrier effect (Fuller and Turvey, 1971; Fuller, 1977; Nuotio et al., 1992; Stavric and Kornegay, 1995) and perhaps they have an anti-inflammatory activity (Alterman et al., 2005). According to experimental results, the bacterial strain probiotic effects can vary from the control of intestinal infections, to the modulation of the immune response, and further to the improvement of the digestive functionalities and absorption of nutrients (Ca, P and N included). At present, the most interesting effects noticed on layers fed with probiotic microorganisms are the improvement of the bird’s performance (laying rate, conversion index) and egg quality (egg weight, egg specific gravity, shell thickness, Haugh Units, and cholesterol content) (Franchini et al., 1991; Giardini et al., 1993; Nahashon et al., 1994, 1996; Mohan et al., 1995; Tortuero and Fernàndez, 1995; Abdulrahim et al., 1996; Haddadin et al., 1996; Gusils et al., 1999a, 1999b; Panda et al., 2004).

Furthermore in vitro LAB antagonistic abilities towards several pathogenic bacteria, such as E.coli, salmonellae, shigellae, clostridia, etc. are well known (Juven et al., 1991; Helander et al., 1997; Patterson and Burkholder, 2003).

L. acidophilus D2/CSL is a probiotic strain isolated from the chicken intestine and capable of re-equilibrating the intestinal microbiota (Bianchi Salvadori et al., 1985). Up to now its probiotic efficacy has been investigated in broilers (Giardini et al., 1994; 1995c), turkeys (Bianchi Salvadori et al., 1993) and laying hens (Giardini et al., 1993) by drinking water administration.

The aim of this work was to investigate the effects of dietary addition of probiotic strain Lactobacillus acidophilus D2/CSL on hen performance and egg quality.
Material and methods

Groups and treatments

One hundred sixty commercial Hy-line Brown pullets, 17 weeks old, were housed in a four-deck single cages facility in an environmentally controlled room (16L:8D; 20÷22ºC). The cages were allotted in two parallel rows and equipped with nipples and anti-waste feeders. The trial lasted 40 weeks, including the first (acclimatization) week. Birds were fed ad libitum with a “standard” layer feed (Table 1).

Two experimental groups, with uniform weight distribution, control (C, no treatment) and treatment (T, probiotic treatment), were divided. Each group consisted of eighty hens, with 4 replicates of 20 birds per group. The T hens were fed with the “standard” diet with an inclusion of $10^9$ CFU kg$^{-1}$ of Lactobacillus acidophilus D2/CSL (freeze dried alive cells). A fresh feed stock and L. acidophilus D2/CSL was prepared every month (short-term survival in feed), and the feed was stocked in a cool room. A pharmaceutical mixer was used adding 25 g of a premixture composed of lactose and Lactobacillus acidophilus D2/CSL (1x10$^9$ CFU/g) into 25 kg of complete feed and mixing for 4 minutes. Five random feed samples (500 g each) were collected in sterile bags, and then were submitted to microbiological counting, aiming to ascertain both the actual number and the homogeneous distribution of LAB cells in feed. The same sampling and counting were carried out for the C feed.

Sanitary, quantitative, and qualitative data

The eggs of the 8 replicates were separately collected. The recorded parameters were the following:

- daily records: liveability and health

| Table 1. Feed composition$^1$. |
|-------------------------------|
| Crude protein                 | %   | 16.70 |
| Crude fat                     | "   | 3.20  |
| Crude fibre                   | "   | 3.00  |
| Ash                           | "   | 11.40 |
| Calcium                       | "   | 4.00  |
| Phosphorus                    | "   | 0.73  |
| Available phosphorus          | "   | 0.40  |
| Chlorine                      | "   | 0.23  |
| Sodium                        | "   | 0.18  |
| Choline                       | mg/kg | 1066.00 |
| Linoleic acid                 | %   | 1.45  |
| Lysine                        | "   | 0.93  |
| Methionine                    | "   | 0.39  |
| AME                           | MJ/Kg | 11.37 |

$^1$Ingredients: yellow corn, soybean meal, wheat, wheat middlings, sunflower seed meal, soybean oil, dicalcium phosphate, ground limestone, disodium carbonate, salt, magnesium, methionine, vitamins and minerals supplement.
LACTOBACILLUS ACIDOPHILUS IN LAYING HEN

conditions, egg number, egg mass (kg), number of unsealable eggs (eggs lacking the characteristics to be commercialised in category A, according to Regulations n. 1907/90/CE and 1274/91/CE), subdivided into: fragile eggs: uncollectable eggs, shell checks, broken eggs, dirty eggs: eggs soiled by faecal matter;

- Recorded every 10 days: Feed consumption

- Monthly records: egg weight: all the eggs were separately weighed, specific gravity of eggs (ESG): the eggs laid were weighed and divided in groups with the same weight range (±4 g). The ESG of a sample (25 eggs, 5 eggs at a time), having the same weight (range ±4 g), was evaluated with a special egg pychnometer (Giardini et al., 1996). Albumen quality (Haugh Units): each egg was shelled on a glass plate and the albumen height was evaluated by the Albumen Height Gauge AG-10, R. Brancker Research Ltd, Ottawa Canada. The HU value resulted from the mean of three measures of the albumen thickness applying the calculation of Haugh (1937).

Shell thickness (without the shell membranes): the measure was carried out on the same sample of eggs with a micrometer (Steinmeyer – mode “micro S”) at three points of the egg shell equator, after the shell membranes were removed. Counting of lactobacilli in feed: both treatment and control feeds were submitted to microbial counts by means of the IDF Standard method 117:2003/ISO 7889 (FIL/IDF, 2003). “Yogurt-enumeration of characteristic microorganisms - colony count technique at 37°C”:

- Weigh in aseptic mode 10 g of sample in a stomacher bag.

- Add 90 ml of sterile physiologic solution (0.9%) to the 10 g sample (concludes the 1st dilution) and submit into stomacher for 90 seconds.

- Continue with the following decimal dilutions until the 9th dilution.

- Inoculate aseptically in a rate of 1 ml of the diluted sample per each plate and pour 13-15 ml of MRS agar medium (45÷48°C).

- Shake and incubate in anaerobiosis for 3 days at 37°C.

- Count the colonies.

**Results and discussion**

In treated feed bacterial counting showed that the number of lactobacilli was equivalent to the dosage (1*10⁹ CFU/kg feed) just after probiotic inclusion and after 2-week storage of samples (4-6°C). In contrast control feed manifested in bacterial counting lower than 1*10⁶ CFU/kg.

Hen weight at 17 and 57 weeks are reported in Table 2. Homogeneous body weights have been recorded in both groups according to Hy-line brown ® production standard guide 2005-2007.

A small difference, not statistically significant, between C and T birds was observed at the 18th and 57th weeks of age.

Table 3 shows the weekly egg production and the average egg weight during the whole experimental period. The overall number of eggs produced was 18,725 for
group C and 19,051 for group T, a mean weekly egg production of 480.13±1.17 (mean ±SD) for group C and of 488.49±1.17 for group T was calculated (P<0.01). T flock average egg weight was higher, but calculated differences were not significant.

Figure 1 and Figure 2, respectively, represent the weekly egg production (%) and the weekly egg production difference between the 2 groups. Table 4 shows the number of unmarketable eggs (broken eggs, fragile eggs, and dirty shells). The broken eggs were 1.62% and 1.85% of the overall number of eggs for C and T, respectively. However many “broken eggs” were the result of birds pecking: thin-shelled eggs were nearly the same in both groups. Egg breakage of the shell was often an accidental fact. Unclean eggs were 126 in C vs 106 in T.

Table 5 reports feed intake of hens: higher in C birds than in T birds (P<0.01), 118.68 vs 117.43; an improvement of the FCR (Kg feed intake/Kg eggs): 1.97 for C vs 1.94 for T (P<0.05) was calculated.

The results of specific gravity of eggs, shell thickness, and Haugh units calculations are reported in Table 6. Mean ESG were different (P<0.01): 1.0799 vs 1.0811 for C and T groups, respectively. It is important to emphasise that egg specific gravity must be evaluated as soon as the egg is laid; only in that case it expresses the incidence of the shell weight on the overall egg weight (Hamilton, 1982). Thus, ESG is an index of shell thickness and porosity. Nonetheless, no significant differences were calculated between the shell thickness of C and T eggs (0.335±0.003 mm in C, 0.339±0.003 mm in T). Egg albumen (Haugh Units) mean viscosity reports higher values for T eggs (96.29±.33 HU) when compared to C eggs (95.29±.35 HU) (P<0.05).

All the positive results seem to be, first of all, the consequences of a better ratio
Figure 1. Weekly egg production (%).

Figure 2. Difference of the weekly egg production % between the groups (T% minus C%).
between probiotic (fermentative and symbiotic) bacteria and unwanted (putrefactive and pathogen) ones in the gastrointestinal tract of the birds. An improvement of the physiological conditions of digestion and intestinal absorption (or “gut health”) of treated hens could be supposed. In a previous study (Bianchi Salvadori et al., 1985), chickens fed with autochthonous lactobacilli (selected from the normal chicken gastrointestinal microflora) showed an increase in LAB and in anaerobic population, and a concomitant reduction of Enterobacteriaceae in their intestines. Other authors describe positive correlations between the probiotic administration and nitrogen, calcium and phosphorus retention in laying hens (Nahashon et al., 1994, 1996). ESG and shell thickness (including the shell membranes), besides the better gut health conditions, are probably due to the production of lactic acid and an increased calcium and phosphorus absorption (Haddadin et al., 1996; Nahashon et al. 1996) due to the acidification and solubilization (ionization) of salts. Probably the

Table 4. Unsaleable eggs (%).

| Group    | Broken eggs (%) | Thin-shelled eggs (%) | Dirty eggs (%) |
|----------|-----------------|-----------------------|---------------|
| Control (C) | 1.62            | 0.12                  | 0.67          |
| Treated (T)  | 1.85            | 0.11                  | 0.56          |

Table 5. Feed consumption and feed conversion (FCR).

| Group    | Hen-day feed consumption (mean±SE) (g) | Overall FCR (mean±SE) (kg feed/kg eggs) |
|----------|----------------------------------------|----------------------------------------|
| Control (C) | 118.68±0.34                          | 1.97±0.0093                            |
| Treated (T)  | 117.43±0.34                          | 1.94±0.0093                            |
| P value    | <0.01                                 | <0.05                                  |

Table 6. Egg specific gravity, shell thickness, and Haugh Units.

| Group    | Egg specific gravity (mean±SE) (g/cm³) | Shell thickness (mean±SE) (mm) | Haugh Units (mean±SE) |
|----------|----------------------------------------|-------------------------------|-----------------------|
| Control (C) | 1.0799±0.00005                      | 0.335±0.003                  | 95.29±0.3462          |
| Treated (T)  | 1.0811±0.00005                      | 0.339±0.003                  | 96.29±0.3539          |
| P value    | <0.01                                 | >0.05(ns)                    | <0.05                  |
LACTOBACILLUS ACIDOPHILUS IN LAYING HEN

Acidification is also responsible for the improvement of the albumen quality (HU). Similar results were previously reported by Tortuero and Fernandez (1995). *L. acidophilus* D2/CSL may improve the sanitary status of the hens and the hygienic quality of the eggs, considering results with respect to the reduction in the number of unmarketable eggs and the improvement of the eggs' specific gravity.

**Conclusions**

"*Lactobacillus acidophilus* D2/CSL" inclusion in laying hens diets at the recommended dosage of $10^9$ CFU/kg of feed improved some quantitative and qualitative parameters of eggs. In particular, an increase in the number of laid eggs ($P<0.01$), a decrease in the feed intake ($P<0.01$), a better FCR ($P<0.05$), an improvement of egg specific gravity ($P<0.01$), and an increase in the Haugh Units ($P<0.05$) have been recorded. Patterson and Burkholder (2003) underlined how probiotics seem to be more effective when animals are stressed, because stressors cause a decrease in LAB intestinal population. However, LAB cultures selection is the first step in ensuring objective results. Probiotic effects are strain-specific (FAO/WHO, 2002) and so selection criteria must consider specific efficacy of microbial species and strains. The dose (CFU/hen/day) beyond strain selection is important too and a continuous administration of the probiotic must be ensured. Nevertheless, the health status of the flock and/or the farm hygienic conditions may suggest modulating the dose. Since it is necessary to preserve the bacteria vitality until their ingestion, and assist their intestinal colonization, other very important variables that must be considered are: probiotic conservation and distribution technology, feed composition (presence/absence of prebiotic ingredients), the presence of ingredients, such as plant products, containing natural antimicrobial agents (Cowan, 1999), and current pharmacological treatments that could interfere with the probiotic liveability in the chickens' intestine.

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