IMMUNOLOGY, HEALTH, AND DISEASE

Effects of two different probiotics on microflora, morphology, and morphometry of gut in organic laying hens

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ABSTRACT The current study investigated the effects of *Lactobacillus acidophilus* and *Bacillus subtilis*, used as probiotics, on the microflora, morphology, and morphometry of the gut in organic laying hens. The birds (180 Hy-Line laying hens) were divided into 3 homogenous groups and received a pre-deposition diet from 16 to 20 wk of age and a deposition diet for the remaining 7 months of the experiment. The control group (**CTR**) was fed a corn-soybean cake-based diet, the second group (**L**) received the same diet supplemented with 0.1% of *L. acidophilus* while in the third group (**B**) the basal diet was supplemented with 0.05% of *B. subtilis*. At 18 wk of age (**T1**) and at 5 (**T2**) and 7 months (**T3**) from the beginning of deposition, 9 subjects per group were humanely killed for microbiological, morphological and morphometric analyses of the intestinal tract. The 2 probiotic-supplemented diets increased *Lactobacillus* spp. and *Bifidobacterium* spp. counts compared with the CTR diet. The lowest viable counts of *E. coli*, coliforms and staphylococci were observed in the L group ($P < 0.001$). *Clostridium* spp. decreased ($P < 0.001$) in both L and B subjects. The probiotic supplementation appeared to affect the intestinal microbial population, promoting the presence of beneficial bacteria such as *Lactobacillus* spp. and *Bifidobacterium* spp. and reducing potential harmful bacteria such as *E. coli*, clostridia and staphylococci. Morphological and morphometric analyses did not reveal substantial differences among groups. At T3, the plasma cell infiltrate in the villi of the CTR hens was more severe than that observed in the L and B groups ($P = 0.009$).

Key words: *Lactobacillus acidophilus*, *Bacillus subtilis*, intestinal microbiota, organic farming

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INTRODUCTION

In European organic farming, the use of any drug, even if for prevention or therapeutic purposes, is strictly regulated by EC Council Regulation No. 1804/99 (EC Council Regulation, 1999). Over the last 2 decades, probiotics, including *Lactobacillus* spp., *Bacillus subtilis* and enterococci, have been increasingly tested as alternative growth promoters (Patterson and Burkholder, 2003) in conventional rearing systems. Several authors have also reported the positive relationship between dietary *Lactobacillus acidophilus* and the intestinal mucosa of birds, since it strengthens the barrier effect (Fuller and Turvey, 1971; Fuller, 1977; Stavric and Kornegay, 1995). Chichlowski et al. (2007) demonstrated that a group of chicks fed probiotics, including lactobacilli, *Bifidobacterium thermophilum* and *Enterococcus faecium*, could increase the jejunal villus height and reduce the villus crypt depth compared with a salinomycin-treated group. *B. subtilis*-based probiotics were also investigated as growth promoters in poultry (Jiraphocakul et al., 1990; Santoso et al., 1995; Santoso et al., 2001). *B. subtilis* spores were shown to promote the intestinal function (Samanya and Yamauchi, 2002), favoring the balance of beneficial anaerobic species (Fiorini et al., 1985; Saatchi and Sullivan, 1990). Wu et al. (2011) showed that the dietary addiction of a new strain of *B. subtilis* (KD1) significantly improved intestinal flora by increasing lactobacilli and reducing the *Escherichia coli* count. Probiotics have also been seen to have a positive effect on the morphology and morphometric index of intestinal walls. Pelicano et al. (2005) reported higher villi in the ileum and jejunum in broiler chickens fed with *B. subtilis*-based probiotic. However, these results are not in accordance with other studies in which positive effects due to the use of probiotics, including *L. acidophilus*, *L. casei*, and *B. subtilis*, were not observed (Jin et al., 1998; Schwarz et al., 2002). The

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conflicting results related to the addition of probiotics in the diets have not definitely promoted their use in commercial feed. Variations in their efficacy may be due to differences in the microbial species and dose levels used, or in the methods of preparing supplements (Jin et al., 1998). The positive effect of bacterial strains might also depend on the adhesion and replication on the intestinal wall (Jin et al., 1996). To the best of our knowledge, the effect of probiotics in organic poultry farms has not been investigated. In an attempt to promote the use of probiotics as modulators of the microbial flora and to study a possible beneficial effect on the intestinal morphology, we examined the effects of 2 probiotics, *L. acidophilus and B. subtilis*, in an organic laying hens' farm, where the use of any drug is not allowed for the entire production cycle.

MATERIALS AND METHODS

Experimental Design

A total of 180 16-week-old Hy-Line layers were reared under organic conditions according to Council Regulation (EC) No. 1804/99. The hens were randomly assigned to 3 treatment groups of 60 birds, each divided into 3 replicates of 20 birds each. All the birds received a pre-deposition diet for the first 4 wk of the trial (from 16 to 20 wk) and a deposition diet for the rest of the trial. During both periods, the control group (CTR) was fed a corn-soybean cake-based diet (pre-deposition diet: C.P. 16.92%, M.E. 3.041 kcal/kg, Ca 2.94%, P 0.62%; deposition diet C.P. 18.01%, M.E. 2.810 kcal/kg, Ca 3.88%, P 0.62%), the second group (L) received the same diet supplemented with 0.1% of *L. acidophilus* (Lactomalt D2 Bio®, *L. acidophilus* D2/CSL CECT 4529, Zoo Assets Srl, Mantova, Italy) and the third group (B) was fed the basal diet supplemented with 0.05% of *B. subtilis* (Clostat® brand dry – 740210, *B. subtilis* PB6 ATCC-PTA 6737, Kemin®, Herentals, Belgium). All feeds were formulated to meet or exceed the National Research Council (1994) nutrient requirements. Before oviposition at 18 wk of age (T1), and at 5 (T2) and 7 months (T3) from the beginning of oviposition, 9 subjects per group (3 per replicate) were killed by cervical dislocation for morphological, morphometric and microbiological analyses of the intestinal tract. The animal care procedures were in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (NRC, 2010). The research project was approved by Council of the Department of Veterinary Medicine (University of Perugia, registration number 200/12).

Microbiological Analyses

The cecum and ileum were removed and a pool of content was obtained from 3 samples collected from each intestinal region of 9 subjects. One gram of intestinal content was placed into a sterile test tube with 2 mL 0.9% saline solution and brought to 10 mL volume with the saline solution. 0.1 mL of solution from each tube was tenfold serially diluted with 0.9% sterile saline solution. Each pooled sample (0.1 mL) was tenfold serially diluted (from 10^{-1} to 10^{-10}). Chromocult Coliform Agar (Merck, Milan, Italy) and Bile Esculin Azide Agar (ThermoFisher Scientific, Milan, Italy) were used for the enumeration of *E. coli* and coliforms, and enterococci, respectively. Mannitol Salt Agar (ThermoFisher Scientific) was used for the enumeration of staphylococci; all the plates were aerobically incubated at 37°C for 24 to 48 h. Reinforced Clostridial Agar enriched with 5% sheep blood and 1 mg/mL K1 vitamin (ThermoFisher Scientific), Brain Heart Infusion Agar (ThermoFisher Scientific), modified Man Rogosa Sharpe Agar (MRS; ThermoFisher Scientific), 0.3% (w/v) sodium propionate, 0.2% (w/v) lithium chloride, 0.05% (w/v) cysteine hydrochloride and 5% (v/v) defibrinated sheep blood included were used for the enumeration of *Clostridium* spp., total anaerobes and *Bifidobacterium* spp., respectively. Incubation was performed in anaerobic jars at 37°C for 48 to 72 h. MRS Agar (ThermoFisher Scientific) was used for the enumeration of lactobacilli. The plates were incubated at 35°C for 3 d, under microaerophilic (5% carbon dioxide, 5 to 10% oxygen) conditions. The number of colonies was counted and all the data were expressed as $\log_{10}$ cfu/g.

Morphological and Morphometric Analyses

Segments of approximately 2 cm in length were taken from the duodenum of 9 subjects (3 for each replicate) per group at T1, T2 and T3, fixed in 10% neutral buffered formalin and embedded in paraffin wax. From each sample, 3 serial sections of 5 μm thickness were cut, stained with hematoxylin and eosin and evaluated by light microscopy. Morphological and morphometric analyses of specimens were performed by 2 independent investigators (S. P. and E. M.) who were blind to the experimental data. A morphological examination was performed to evaluate the grade of inflammation. The crypts and the villi of the duodenum were independently investigated. To measure the intensity of the tissue inflammation, a scoring system was developed. A numeric value from 0 to 3 was given to the severity as follows: 0 = rare or no leukocytic infiltrate is present; 1 = mild (leukocytic infiltrate fills up to 5% of a microscopic field at 400× magnification); 2 = moderate (leukocytic infiltrate fills approximately 25% of a microscopic field at 400× magnifications); 3 = marked (leukocytic infiltrate fills approximately 50% or more of a microscopic field at 400× magnification). Morphometric evaluation was carried out on 5 selected random fields for each intestinal section for each sample at low magnification (5x). The measurements were villus height (VH – from the tip of the villus to the villus-crypt junction) and crypt depth (CD – from the base up to the crypt-villus transition region) using a Nikon DS-Fi1 digital camera (Nikon Corporation, Tokyo, Japan) connected

Table 1. Effect of *Bacillus subtilis* and *Lactobacillus acidophilus* supplemented diets[1] on intestinal *Enterococcus* spp., *Lactobacillus* spp. and *Bifidobacterium* spp. population (log_{10} cfu/g).

	Time[2]					
Item	T1	T2	T3	Overall	Effect[3]	P[4]
Enterococcus spp.						
Ileum						
CTR	6.046^{A}	7.240^{A}	7.886^{A}	7.145^{A}	Diet	<0.001
B	5.251^{B}	6.171^{B}	6.804^{B}	6.185^{B}	Time	<0.001
L	4.901^{B}	5.224^{C}	5.282^{C}	5.240^{C}	Diet × Time	<0.001
SEM	0.131	0.101	0.131	0.062		
Cecum						
CTR	7.773^{B}	10.741^{A}	10.982^{A}	10.096^{A}	Diet	<0.001
B	7.790^{A}	10.406^{B}	10.815^{B}	9.963^{B}	Time	<0.001
L	7.949^{A}	9.645^{C}	9.793^{C}	9.613^{C}	Diet × Time	<0.001
SEM	0.021	0.016	0.021	0.010		
Lactobacillus spp.						
Ileum						
CTR	6.161^{B}	6.410^{C}	6.584^{C}	6.397^{C}	Diet	<0.001
B	6.403^{A}	8.647^{A}	9.802^{A}	8.569^{A}	Time	<0.001
L	6.585^{A}	7.566^{B}	8.949^{B}	7.980^{B}	Diet × Time	<0.001
SEM	0.047	0.036	0.047	0.022		
Cecum						
CTR	9.882	8.325^{B}	8.380^{C}	8.770^{B}	Diet	<0.001
B	9.531	9.544^{A}	9.326^{B}	9.585^{A}	Time	<0.001
L	9.665	9.474^{A}	10.771^{A}	9.990^{A}	Diet × Time	<0.001
SEM	0.089	0.069	0.089	0.042		
Bifidobacterium spp.						
Ileum						
CTR	3.663	3.672^{B}	3.886^{C}	3.712^{C}	Diet	<0.001
B	3.784	5.333^{A}	5.956^{A}	5.167^{A}	Time	<0.001
L	3.629	4.145^{B}	4.805^{B}	4.329^{B}	Diet × Time	<0.001
SEM	0.041	0.032	0.041	0.019		
Cecum						
CTR	5.855	5.758^{C}	5.663^{C}	5.765^{C}	Diet	<0.001
B	5.685	6.575^{B}	6.771^{B}	6.442^{B}	Time	<0.001
L	5.714	7.766^{A}	7.942^{A}	7.341^{A}	Diet × Time	<0.001
SEM	0.044	0.034	0.044	0.021		

[1]CTR: control diet, B: CTR diet with 0,05% of *B. subtilis*; L: CTR diet with 0,1% of *L. acidophilus*.
[2]Sampling time: T1 = 18 wk of age; T2 = 5 months from the beginning of deposition; T3 = 7 months from the beginning of deposition.
[3]Only main effects are shown.
[4]*P*-levels from two-way ANOVAs.
[A–C]Within a column, means without a common superscript differ ($P < 0.001$).

to a Leica DMR microscope (Leica Microsystems, Milan, Italy) and using NIS-Elements Br-2 as software. The criterion for villus selection was based on the presence of intact lamina propria. The VH:CD ratio was subsequently calculated and recorded.

Statistical Analysis

Data obtained from microbiological analyses were subjected to an ANOVA procedure using the GLM procedure of SAS (2001). The model included the diet (C, L and B) and sampling time (T1, T2 and T3) as fixed factors, as well as their interaction. Data were reported as least squares means ± standard error. Differences were assessed by Tukey's test and a probability level of $P < 0.05$ was used to determine statistical significance. As for the intestinal morphometry, ANOVA was performed by using a mixed model, including the hens as the experimental unit and regarding the measurements per animal as subreplicates. Contrasts were used to compare the VH, CD and VH:CD among the groups of animals (L, B, C) within the same age, and among ages within the same group. A single-step correction for multiplicity was used (Bretz et al., 2011). Moreover, the Kruskal-Wallis rank sum test followed by the pairwise Wilcoxon rank sum test were used to compare the inflammatory infiltrate severity among the 3 groups of animals. The evaluations were carried out within the same age group. All statistical analyses regarding the intestinal morphology data were carried out by using R software (R Development Core Team, 2013).

RESULTS AND DISCUSSION

Although several investigations have also been conducted on the effects of probiotics on the productivity of commercial laying hens and egg quality (Kurtoglu et al., 2004; Mahdavi et al., 2005; Balevi

Table 2. Effect of *Bacillus subtilis* and *Lactobacillus acidophilus* supplemented diets[1] on intestinal *Escherichia coli* and coliforms population (log_{10} cfu/g).

	Time[2]					
Item	T1	T2	T3	Overall	Effect[3]	P[4]
Escherichia coli						
Ileum						
CTR	4.040^{A}	4.195^{A}	4.654^{A}	4.311^{A}	Diet	<0.001
B	3.522^{B}	3.771^{B}	3.721^{B}	3.710^{B}	Time	<0.001
L	3.179^{C}	3.638^{C}	3.633^{B}	3.546^{C}	Diet × Time	<0.001
SEM	0.036	0.029	0.036	0.018		
Cecum						
CTR	6.927^{B}	6.884^{A}	6.980^{A}	6.923^{A}	Diet	<0.001
B	7.392^{A}	6.352^{B}	6.826^{B}	6.792^{B}	Time	<0.001
L	4.697^{C}	5.148^{C}	5.643^{C}	5.212^{C}	Diet × Time	<0.001
SEM	0.031	0.024	0.031	0.015		
Coliforms						
Ileum						
CTR	7.078^{A}	7.204^{A}	7.756^{A}	7.359^{A}	Diet	<0.001
B	3.656^{B}	3.909^{B}	4.737^{B}	4.274^{B}	Time	<0.001
L	3.513^{C}	3.811^{B}	4.623^{B}	4.114^{B}	Diet ×Time	<0.001
SEM	0.032	0.024	0.032	0.015		
Cecum						
CTR	8.040^{A}	7.417^{A}	7.824^{A}	7.751^{A}	Diet	<0.001
B	6.578^{B}	6.108^{C}	6.646^{B}	6.464^{B}	Time	<0.001
L	5.873^{C}	6.614^{B}	6.417^{C}	6.337^{C}	Diet × Time	<0.001
SEM	0.032	0.025	0.032	0.015		

[1]CTR: control diet, B: CTR diet with 0,05% of *B. subtilis*; L: CTR diet with 0,1% of *L. acidophilus*.
[2]Sampling time: T1 = 18 wk of age; T2 = 5 months from the beginning of deposition; T3 = 7 months from the beginning of deposition.
[3]Only main effects are shown.
[4]*P*-levels from two-way ANOVAs.
[A–C]Within a column, means without a common superscript differ ($P < 0.001$).

et al., 2009; Mátéová et al., 2009), to the best of our knowledge, there are no published data concerning the use of probiotics in organic laying hens and their influence on the intestinal microbiota and histomorphology.

In our study, the use of probiotic supplementations may be able to affect the intestinal microbial population, in particular promoting the presence of beneficial bacteria such as *Lactobacillus* spp. and *Bifidobacterium* spp. (Table 1). The lactobacilli count was higher in the ileum of groups fed with probiotics than in that of the CTR ($P < 0.001$) in all sampling times (Table 1). In the cecum tract, the L group showed a higher ($P < 0.001$) count of *Lactobacillus* spp. than both the B and CTR groups at T3. With respect to *Bifidobacterium* spp., the highest counts ($P < 0.001$) were found in the cecum of the L group and in the ileum of the B group at T3 (Table 1). In relation to *Lactobacillus* spp. and *Bifidobacterium* spp., it should be noted that in both cases the trend is similar, but *Lactobacillus* spp. supplementation was more effective in the cecum while the *B. subtilis* supplementation in the ileum showed a time-dependent effect at T3. Recently, Abdelqader et al. (2013) demonstrated that commercial hens supplemented with rising levels of *B. subtilis* presented a linear increase of *Lactobacillus* spp. and *Bifidobacterium* spp. In contrast, Jin et al. (1998) reported no significant increase of the *Lactobacillus* spp. count in chickens fed *L. acidophilus* or a mixture of *Lactobacillus* spp. Knap et al. (2011) and Jeong and Kim (2014) confirmed that the intestinal microflora of broiler chickens can possibly be manipulated by dietary supplementation with *B. subtilis* C-3102.

Coliforms showed lower counts in the ileum of probiotic-treated groups than in the CTR group, with no differences between L and B. Considering the cecum tract at T1 and T3, *E. coli* and coliforms had the lowest count in L, followed by the B group, whereas the CTR showed the highest value ($P < 0.001$) (Table 2). Jin et al. (1998) observed that *L. acidophilus* and a mixture of *Lactobacillus* spp. increased the concentration of volatile fatty acids in the ileum and cecum in broiler chickens and reduced the pH value, which may be responsible for a decline of intestinal coliforms. Li et al. (2009) reported that *B. cereus* increased the number of beneficial intestinal microorganisms in chicks, thereby reducing the potential harmful bacteria such as *E. coli*. Similar results have been reported elsewhere (Teo and Tan, 2007; Mountzouris et al., 2010; Hassan and Ryu, 2012). Our work, showing a decrease in the *E. coli* intestinal population associated with a higher count of *Lactobacillus* spp. in the treated subjects, agrees with the results of Suzuki et al. (1989) and Jin et al. (1996). It supports the hypothesis that lactobacilli could compete with *E. coli* for intestinal colonization. Watkins et al. (1982) observed that a competitive exclusion against pathogenic *E. coli* strains occurred in gnotobiotic chicks fed *L. acidophilus*. The antagonistic abilities of

Table 3. Effect of *Bacillus subtilis* and *Lactobacillus acidophilus* supplemented diets[1] on intestinal anaerobes, *Clostridium* spp. and *Staphylococcus* spp. population (log_{10} cfu/g).

	Time[2]					
Item	T1	T2	T3	Overall	Effect[3]	P[4]
Anaerobes						
Ileum						
CTR	10.713^{A}	11.187^{A}	11.740^{A}	11.310^{A}	Diet	<0.001
B	7.928^{C}	10.721^{B}	10.645^{C}	9.992^{C}	Time	<0.001
L	8.580^{B}	10.804^{B}	10.851^{B}	10.268^{B}	Diet × Time	<0.001
SEM	0.044	0.034	0.044	0.021		
Cecum						
CTR	10.361^{A}	12.642^{A}	12.591^{A}	12.026^{A}	Diet	<0.001
B	9.818^{B}	12.322^{A}	12.660^{A}	11.871^{A}	Time	<0.001
L	10.078^{A,B}	11.285^{B}	11.113^{B}	11.019^{B}	Diet × Time	<0.001
SEM	0.099	0.077	0.099	0.047		
Clostridium spp.						
Ileum						
CTR	7.939^{A}	7.771^{A}	8.851^{A}	8.340^{A}	Diet	<0.001
B	7.884^{A}	6.354^{B}	6.457^{C}	6.835^{B}	Time	<0.001
L	7.636^{B}	6.106^{C}	6.707^{B}	6.725^{B}	Diet × Time	<0.001
SEM	0.028	0.022	0.028	0.013		
Cecum						
CTR	9.819^{A}	9.521^{A}	9.879^{A}	9.753^{A}	Diet	<0.001
B	9.659^{B}	8.655^{B}	8.634^{C}	8.990^{B}	Time	<0.001
L	9.796^{A}	8.612^{B}	8.732^{B}	8.987^{B}	Diet × Time	<0.001
SEM	0.029	0.022	0.029	0.014		
Staphylococcus spp.						
Ileum						
CTR	3.497^{A}	6.933^{A}	6.879^{A}	6.037^{A}	Diet	<0.001
B	2.065^{C}	3.499^{C}	4.014^{B}	3.305^{B}	Time	<0.001
L	2.668^{B}	3.680^{B}	3.826^{C}	3.427^{B}	Diet × Time	<0.001
SEM	0.039	0.030	0.039	0.018		
Cecum						
CTR	5.014^{A}	7.623^{A}	7.514^{A}	6.960^{A}	Diet	<0.001
B	4.636^{B}	6.150^{B}	6.194^{B}	5.755^{B}	Time	<0.001
L	4.482^{C}	4.753^{C}	4.380^{C}	4.533^{C}	Diet × Time	<0.001
SEM	0.029	0.022	0.029	0.014		

[1]CTR: control diet, B: CTR diet with 0,05% of *B. subtilis*; L: CTR diet with 0,1% of *L. acidophilus*.
[2]Sampling time: T1 = 18 wk of age; T2 = 5 months from the beginning of deposition; T3 = 7 months from the beginning of deposition.
[3]Only main effects are shown.
[4]*P*-levels from two-way ANOVAs.
[A–C]Within a column, means without a common superscript differ ($P < 0.001$).

probiotics towards several pathogenic bacteria, such as *E. coli*, *Salmonella* spp., and *Shigella* spp. have been well documented (Juven et al., 1991; Helander et al., 1997; Patterson and Burkholder, 2003; Knap et al., 2011). *Clostridium* spp., more represented in the cecum than in the ileum of all experimental groups, considering the overall effect declined ($P < 0.001$) in the treated subjects and showed the lowest ($P < 0.001$) value in the ileum of the L group at T2 (Table 3). This may confirm previous data from Kizerwetter-Świda and Binek (2009), which demonstrated that the addition of the *Lactobacillus salivarius 3d* strain, as well as a reduction in the number of *Salmonella enteritidis*, decreased the number of *C. perfringens*. Yaman et al. (2006), Mountzouris et al. (2007) and Higgins et al. (2007) also showed that probiotic microorganisms, such as *Lactobacillus* spp., *Streptococcus* spp., *Bacillus* spp., *Bifidobacterium* spp., *Enterococcus* spp., *Aspergillus* spp. and *Candida* spp., played a role in the modulation of intestinal microflora and pathogen inhibition. Anaerobes showed the highest counts in the ileum of the CTR group at any sampling times while the lowest value was noticed for the B group at T1 (Table 3). The CTR followed by the B group had significantly higher values than the L in the cecum tract. However, the increase of the anaerobic count should be highlighted for both the B and the L group from T1 to T2. The anaerobic population includes beneficial bacteria such as *Lactobacillus* spp. and *Bifidobacterium* spp. In a previous investigation (Casagrande-Proietti et al., 2009), lactobacilli and total anaerobia showed a similar trend in organic broiler chickens, indicating an additional interaction between rearing system and diet in microbiota. In our work, the treated groups showed lower staphylococci values than the CTR ($P < 0.001$), though these increased with time (Table 3). The lowest value was observed for L at the cecum level in all sampling times ($P < 0.001$). *Staphylococcus* spp. is not considered a

Table 4. Effect of *Bacillus subtilis* and *Lactobacillus acidophilus* supplemented diets[1] on intestinal inflammation.

	Time[2]		
Item[3]	T1	T2	T3
Intestinal crypt score			
CTR	1.0	1.0	1.6
B	1.0	1.0	1.8
L	2.0	1.0	1.6
p[4]	0.082	0.999	0.839
Intestinal villi score			
CTR	1.0	2.0	2.0
B	1.0	1.7	2.0
L	2.0	1.3	2.8
P[4]	0.082	0.564	0.009

[1]CTR: control diet, B: CTR diet with 0,05% of *B. subtilis*; L: CTR diet with 0,1% of *L. acidophilus*.
[2]Sampling time: T1 = 18 wk of age; T2 = 5 months from the beginning of deposition; T3 = 7 months from the beginning of deposition.
[3]Each datum represents the mean of 3 replicated severity scores, on a scale from 0 (Rare or no leukocytic infiltrate is present) to 3 (leukocytic infiltrate fills approximately 50% or more of a microscopic field at 400× magnifications).
[4]*P* levels refer to a nonparametric Kruskal-Wallis rank sum test.

beneficial species in the microbial gut population; indeed, numerous infections in chickens are caused by coagulase-positive staphylococci, in particular *S. aureus* (Jordan, 1996; McNamee et al., 1998). Staphylococci are also frequently found in poultry products intended for human consumption (Rosec et al., 1997; Manie et al., 1998).

Histologically, intestinal samples from the duodenum showed a variable grade of inflammation in all investigated birds (Table 4). The inflammatory infiltrate was constantly represented by lymphocytes and plasma cells and few polymorphonucleated cells. The lymphocytes were mainly localized in the crypts of mucosae with multifocal to coalescing distribution, whereas the plasma cells diffusely infiltrated the tip of the villi. The study of the inflammatory infiltrate severity both in the crypts and in the villi did not show any difference among groups at T1 and T2. However, at T3, the CTR group showed a significant ($P = 0.009$) increase in the severity of plasma cell infiltrate in the villi compared to the L and B groups (Table 4), suggesting that the use of a probiotic-supplemented diet may reduce intestinal inflammation in the long term. As for the morphometric investigations, no differences were observed in the duodenum morphology characteristics among periods within the same treatment group (Table 5). Similarly, no variations among sampling times were noticed in any of the groups as regards VH, CD and VH:CD ratio. It is known that the increase of VH is suggestive of a greater area addressed to the absorption of available nutrients (Caspary, 1992). In contrast, a shortening of the villi and deeper crypts could be responsible for a poor nutrient absorption, increased secretion in the gastrointestinal tract and reduced performance (Xu et al., 2003). According to observations by several authors in broilers, intestinal histomorphometry can be affected by dietary supplementation with either symbiotic or probiotic administration (Chichlowski et al., 2007; Awad et al., 2009; Gutierrez-Fuentes et al., 2013). A number of reports showed that probiotics containing more than one bacterial culture were required in order to improve duodenal morphometry with greater villi height (Pelicano et al., 2007). However, Dobrogosz et al. (1991) reported that the use of a single bacterial culture was sufficient to increase VH in broiler chickens.

In conclusion, the use of probiotics to obtain a balanced intestinal microbial flora and prevent enteric

Table 5. Effect of *Bacillus subtilis* and *Lactobacillus acidophilus* supplemented diets[1] on the intestinal morphology.

	Time[2]					
Item	T1	T2	T3	Overall	Effect[3]	*P*[4]
Villus height (VH), μm						
CTR	1765.1	1797.1	1453.4	1717.6	Diet	0.3010
B	1597.0	1633.8	1452.8	1561.4	Time	0.3643
L	1915.4	1719.2	1636.8	1770.4	Diet × Time	0.9662
SEM	209.832	171.334	132.715	157.324		
Crypt depth (CD), μm						
CTR	384.1	333.9	331.7	350.9	Diet	0.0558
B	476.5	428.0	414.7	439.7	Time	0.4210
L	352.4	306.9	393.4	351.2	Diet × Time	0.5429
SEM	48.613	39.692	30.755	37.687		
VH:CD ratio						
CTR	4.8	4.9	4.3	4.7	Diet	0.3016
B	4.3	4.4	3.9	4.3	Time	0.3646
L	5.2	4.7	4.4	4.8	Diet × Time	0.9662
SEM	0.572	0.473	0.367	0.455		

[1]CTR: control diet, B: CTR diet with 0,05% of *B. subtilis*; L: CTR diet with 0,1% of *L. acidophilus*.
[2]Sampling time: T1 = 18 wk of age; T2 = 5 months from the beginning of deposition; T3 = 7 months from the beginning of deposition.
[3]Only main effects are shown.
[4]*P*-levels from two-way ANOVAs.

diseases can be eligible (or considered) in organic laying hen farms, where antibiotics are not allowed in any period of production. However, further investigations are necessary to better confirm these preliminary results, because of the lack of literature related to the use of probiotics in organic farms. Moreover, it is difficult to directly compare studies on probiotics, as their efficacy depends on the bacterial strains used, administration level, basal diet, management system, and environmental stress factors (Ghadban, 2002).

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