Effects of dietary Lactobacillus acidophilus and Bacillus subtilis on laying performance, egg quality, blood biochemistry and immune response of organic laying hens

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Effects of dietary *Lactobacillus acidophilus* and *Bacillus subtilis* on laying performance, egg quality, blood biochemistry and immune response of organic reared laying hens.

Short title: Effects of probiotics on organic reared hens

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Keywords: laying hen, probiotic, physiological parameter, immune response, performance

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Comment [FB3]: enough

SUMMARY

The objective of this study was to evaluate the effects of two different probiotic microorganisms on the performance, egg quality and blood parameters of organically reared hens. A total of 900 16-week-old Hy-Line layer hybrids were randomly assigned to three groups of 300 birds each. The control group was fed a corn-soybean cake-based diet; the L group was fed the same diet supplemented with 0.1% *Lactobacillus acidophilus*, while the B group was fed the same diet supplemented with 0.05% *Bacillus subtilis*. Data were recorded at the beginning (weeks 5 and 6: T1) and at the end (weeks 19 and 20: T2) of the experiment, and no differences in hen performance were recorded between dietary groups or sampling time. All of the investigated clinical chemistry parameters, except GGT, were affected by diet (P<0.05), with the best results recorded for the probiotic-treated groups. The immune response values showed higher blood bactericidal activity in the B and L groups at T2 (P<0.05) and a lower lysozime concentration in the B group at T1. Higher antibody production against Newcastle disease virus was observed in the L group compared to the control (P=0.013). No differences in oxidative status were recorded, and no effects of diet on egg quality were observed. Among the physical egg characteristics, only the Roche scale colour was affected by diet (P<0.05); the egg yolk was paler in the L group. The age of the hen was the most relevant factor affecting physical egg characteristics. The chemical parameters of the egg were almost unaffected by supplementation with probiotics except for the lipid content, which decreased with the L diet (P<0.05). Both probiotic inclusions had beneficial effects on hen metabolism and welfare, and *L. acidophilus* induced the best immune response.

Key words:??

INTRODUCTION

In recent years, attention to nutrition-based health strategies has grown (Trovato, 2012), and because increased microbial resistance to antibiotics and residues in animal products can be harmful to consumers, the interest in alternative strategies to reduce antibiotic use in animal production has increased.

A probiotic is a non-pathogenic and non-toxic microorganism (e.g., bacteria, fungi and others) administered through the digestive tract. The FAO/WHO (2001) defines probiotics as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". The primary microorganisms used as probiotics in poultry nutrition are bacteria, such as *Lactobacillus* spp., *Enterococcus* spp., *Pediococcus* spp., *Streptococcus* spp., *Bifidobacterium* spp. and *Bacillus* spp., as well as yeasts, such as *Saccharomyces cerevisiae* and *Candida* spp (Ayasan, 2013).

Comment [FB4]: The below mentioned literature is related to this paper, but authors did not use them in the text. It must be added these references.

AYASAN T, 2013. Effects of dietary inclusion of protexin (probiotic) on hatchability of Japanese quails. Indian J Anim Sci, 83(1): 78-81.

The organic farming of laying hens has become an important economic activity in many countries due partly to increasing environmental awareness and partly to increasing consumer demand for "natural" products (Berg, 2001). In organic farming, the use of pharmacological products is strictly controlled, so it becomes extremely important to ensure a high level of well-being for the animal, an appropriate level of health and an effective defence against external *noxae* arising from ground breeding. Probiotics could help to ensure such results without breaking the rules of organic farming.

The introduction of probiotics to the diet of laying hens can result in many positive outcomes: increased feed consumption and improved feed conversion ratio (Nahashon et al., 1994; Haddadin et al., 1996), higher resistance to parasitic infections (Dalloul et al., 2003; Tierney et al., 2004), increased immune function (Dalloul et al., 2003), larger eggs (Davis and Anderson, 2002), higher egg production and quality (Kurtoglu et al., 2004; Gallazzi et al., 2009; Panda et al., 2008), decreased presence of cholesterol in the eggs (Mohan et al., 1995; Abdulrahim et al., 1996; Kurtoglu et al., 2004; Zhang and Kim, 2013), decreased ammonia emissions (Zhang and Kim, 2013), reduced serum cholesterol (Fathi, 2013; Aluwong et al., 2012) and reduced oxidative stress (Anwar et al., 2012).

In this study, two microorganisms, which differ in their ecology and metabolism, were selected: *Lactobacillus acidophilus* D2/CSL, a commensal bacterium normally present in the intestinal microbial population, and *Bacillus subtilis* (ATCC PTA-6737), a non-commensal, spore-forming bacterium. Both bacterial species are included in the European Union Register of Feed Additives (2013) pursuant to Regulation (EC) 1831/2003. The aim of this research was to investigate the effects of probiotic dietary supplementation on production parameters, immune function, oxidative stress and biochemical chemistry parameters of organically farmed laying hens.

Comment [FB5]: Is the problem significant and concisely stated?: Yes

MATERIALS AND METHODS

Birds, feeding and management

The study was conducted at a farm located in the Lazio region of Italy. A total of 900 16-week-old Hy-Line layer hybrids were reared under organic farming guidelines according to the Council Regulation (EC) No 1804/1999. The hens were randomly assigned to three groups of 300 birds each, which were each divided into three pens (experimental units) of 100 birds each. The hens where reared for a total of 20 weeks and received a pre-deposition diet for the first 4 weeks of the experiment and the same deposition diet for the rest of the experimental period.

The control (CTR) group was fed a corn-soybean cake-based diet (Table 1). The L group was fed the same diet supplemented with 0.1% *Lactobacillus acidophilus* (Lactomalt D2 Bio®, *Lactobacillus acidophilus* D2/CSL CECT 4529, Zoo Assets Srl, Mantova, Italy) while the B group was fed the same diet supplemented with 0.05% *Bacillus subtilis* (Clostat® brand dry – 740210, *Bacillus subtilis* PB6 ATCC-PTA 6737, Kemin®, Herentals, Belgium). The experimental diets and water were given to the birds *ad libitum*.

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The birds were managed under the same prophylactic procedures. Throughout the experiment, the natural photoperiod, temperature and humidity were maintained.

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Feed analysis

The chemical composition of the diets was determined according to AOAC methods (2000, 1990, 1996). Starch content was evaluated according to ISO 10520:1997, and metabolisable energy content was estimated using the equation of Carpenter and Clegg (1956).

Performance

Data for calculating the deposition rate, feed intake and feed conversion efficiency (FCE) were recorded per pen (3 pens/group) daily at the beginning (weeks 5 and 6: T1) and end (weeks 19 and

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20: T2) of the experiment after the hens reached the age of 30 and 58 weeks, respectively. Thirty eggs/pen were collected for analysis at both T1 and T2.

Chemical and physical analysis of eggs

The chemical composition of the yolk was determined according to the AOAC (1995). Total protein was calculated from Kjeldahl nitrogen using 6.25 as the conversion factor, and total lipids were twice extracted from 5 g of each sample homogenate and separated by gravity (Folch et al., 1957). Data recorded included the integrity, weight and thickness of the shell (Mueller and Scott, 1940), the weight and colour of the yolk (Roche scale), and the height of the albumen (Haugh unit) using an electronic gauge (Bukley *et al*., 1981). Colour coordinates (Commission International de l'Eclairage, 1976) were determined using a Minolta Chromameter CR400 (Minolta, Osaka, Japan — D65 light source calibrated against a standard white tile), and the results were expressed in terms of lightness (L*), redness (a*), and yellowness (b*).

Serum analysis

Fifteen hens/pen were sampled using vacuum tubes without an anticoagulant (Vacuette, Greiner Bio-one, Frickenhausen, Germany) from the brachial vein on the first day of each sampling period (T1 and T2). Blood samples were incubated at room temperature for 1 h and centrifuged at 1200 g for 10 minutes. The serum samples were stored at -80°C to determine the metabolic profile, innate immune parameters (lysozyme, serum bactericidal activity, and haemolytic complement assay), oxidative stress and immune response to NDV antigens.

Metabolic profile: Serum samples were tested for ALT, AST, γGT, cholesterol and triglycerides. These tests were assessed by a Konelab 2001 biochemical analyser using specific kits (Sentinel Diagnostics, Milan, Italy).

Comment [FB9]: Are the methods described comprehensively?: Yes

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The authors must include the Protocol number of the Ethics Committee of Animal Use or explain why this information wasn´t include in this paper, since the authors used layer for this experiment.

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Serum bactericidal activity (SBA). The SBA was performed according to a method previously validated for cattle (Amadori et al., 1997). The test is based on a serum challenge with non-pathogenic *E. coli*, and its concentration is expressed as a percentage.

Lysozyme. Serum lysozyme was measured with a lysoplate assay (Osserman and Lawlor, 1966) carried out in a moist incubator at 37°C for 18 minutes. The method is based on the *Micrococcus lysodeikticus* lyses in 1% agarose. The diameter of the lysed zones was measured with a ruler and compared with the lysed zones of a standard lysozyme preparation (Sigma, Milan, Italy, M 3770), and the value is expressed in µg/mL.

Haemolytic complement assay (HCA). The haemolytic complement assay (Barta and Barta, 1993) was carried out on microtitre plates. The complement titre is the reciprocal of the serum dilution that causes 50% of ram red blood cells to lyse, and its concentration is expressed as $CH_{50\%}$.

Haemagglutination inhibition test for NDV (HI). Hens were vaccinated at the hatchery against Newcastle disease. The test to determine the production of NDV antibodies is based on the principle that the haemagglutinin on the viral envelope can bring about the agglutination of chicken red blood cells and that this can be inhibited by specific antibodies. V-bottomed microtitration plates were used, and this test was performed according to the OIE Manual (2012). The HI titre was expressed as the $\log_2$, reciprocal of the highest serum dilution producing 10% inhibition of HI activity.

Oxidative status. The reactive oxygen substances (ROS) in the serum were evaluated with a commercial kit (Diacron, Grosseto, Italy) and are expressed as mmol H_2O_2.

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The serum antioxidant power (AP) was measured with a commercial kit (Diacron, Grosseto, Italy) that evaluates the ability of plasma to oppose the oxidative action of a hypochlorous acid (HClO) solution. The AP levels of the sample are expressed as µmol of neutralised HClO.

Statistical analysis

The data were analysed using the GLM procedure in SAS (2010). The ANOVA model included the diet (C, L, and B) and sampling time (T1 and T2) as fixed factors, as well as their interaction. Data are reported as least squares means ± standard error. Differences were assessed by Tukey's test and considered to be significant when $P<0.05$.

RESULTS

Comment [FB14]: does the RESULTS section provide adequate presentation of the authors' own results? YES

Hen productive performance

Productive performance of laying hens is shown in Table 2. No differences between dietary groups or sampling time were observed ($P>0.05$). At T2, an increase in egg size was recorded with the increased age of the hens.

Hen biochemical profiles

The results for the clinical chemistry parameters are reported in Table 3. All parameters except GGT were affected by the diet, and the cholesterol values of both of the supplemented groups were lower ($P<0.001$) at T2. The triglyceride content of serum was only lower ($P<0.05$) at T2 for the B group.

As for the enzymes, ALT followed a similar trend; the values of the treated groups were lower ($P<0.001$) than the control group at T2. The lowest AST levels were recorded in the B group at T1.

Immune response

The results of the innate immunity profiles are reported in Table 4. The bactericidal activity of the blood was increased at T2 by both of the probiotic supplementations. Lower HC values ($P=0.008$) were observed for the L group compared to the C group (overall effect), and the lysozime concentration was lower ($P=0.016$) for the B group at T1. An overall effect of the L diet on the production of antibodies ($P=0.013$) against NDV was observed, but these values were not affected by the age of the birds.

Oxidative status

Oxidative status data are reported in Table 5. No differences between the dietary groups were recorded.

Egg physicochemical characteristics

No effects of diet on egg quality were observed (Table 6). Among the physical characteristics of the eggs, only the Roche scale colour was affected (P<0.05) by diet; supplementation with 0.1% *L .acidophilus* produced paler egg yolks compared to both the C and B groups.

Sampling time had the greatest effect on the egg physical characteristics. Yolk weight, albumen weight, shell weight, yolk percentage, albumen percentage, edible percentage, Roche scale, Haugh unit and colour (a and b values) all increased (P<0.05) at T2.

The egg chemical characteristics (Table 7) were almost unaffected by supplementation with probiotics, except for the lipid content which was decreased (P<0.05) in the L group.

DISCUSSION

Hen productive performance

The overall results are similar to those found in other studies aimed at investigating the productivity of laying hens under organic conditions (Hammershoj and Steenfeldt, 2005; Mugnai et al., 2009; Minelli et al., 2007; ~~Hammershoj and Steenfeldt, 2005~~). According to Cunningham et al. (1960) and Funk and Kempster (1934), physiological ageing, with the consequent reduction in productive performance, increases egg size, and previous studies have shown that dietary probiotics, such as *Lactobacillus* spp. and *Bacillus* spp., could increase the performance of laying hens (Zhang and Kim, 2013; Xu et al., 2006; Abdulrahim et al., 1996; Nahashon et al., 1994). In contrast, other studies did not record any positive effects (Mikulski et al., 2012; Tortuero and Fernandez, 1995; Nahashon et al., 1996), but the values obtained in this study are difficult to compare with those data because they were not

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obtained under organic conditions. Other reasons for the discrepancies between studies could be the age of the study animals as well as the species and the dose of microorganisms used.

Hen biochemical profiles

Regarding lipid metabolism, the reduction in circulating cholesterol observed in the probiotic-treated groups in this study agrees with the results of other researchers (Onifade et al., 1999; Jouybari et al., 2009). Fathi (2013) found that supplementation with *Lactobacillus cultures* decreased serum cholesterol and triglyceride levels, and similar results were reported by Panda et al. (2006) who found that serum total cholesterol and triglycerides were reduced by dietary supplementation with *Lactobacillus sporogenes*. Hashemzadeh et al. (2013) found reduced serum cholesterol in groups treated with *Lactobacillus rhamnosus*. Probiotics could contribute to the regulation of serum cholesterol concentrations through the deconjugation of bile acids, and because the excretion of deconjugated bile acids is enhanced, more cholesterol molecules could be spent for the recovery of bile acids (De Smet et al., 1994). Other authors have ascribed this result to the reduced adsorption and/or synthesis of cholesterol in the gastrointestinal tract (Mohan et al., 1995; add another reference), but it has also been speculated that *L. acidophilus* could be able to decrease the cholesterol in the blood through the deconjugation of bile salts in the intestinal lumen thereby preventing them from acting as precursors in cholesterol synthesis (Abdularahim et al., 1996).

The trend observed for ALT and AST levels confirms the data presented by Fathi (2013), who reported that *Lactobacillus* supplementation can decrease ALT and AST plasma concentrations. On the contrary, other authors (Baidya et al., 1994; Capcarova et al., 2011;~~ Baidya et al., 1994~~) found that ALT and AST were not affected by the dietary addition of probiotic strains. In rats, however, dietary *Lactobacillus plantarum* and *Bifidobacterium infantis* decreased the level of ALT (Osman et al., 2007).

Immune response

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Probiotics are non-pathogenic bacteria that can promote animal health by reducing pathogen colonisation (Mead, 2005); these reductions are attributed to competitive exclusion, increased volatile fatty acid production, and the potentiation of the immune system (Nava et al., 2005, Donoghue et al., 2006). Fong et al. (2015) observed an improved immune response in the cells of healthy subjects treated with *Lactobacillus ramnosus* in vitro, and lactic acid bacteria can markedly increase the humoral immune response in chickens (Koenen et al., 2004).

The bactericidal property of blood may be due to the presence of complementary factors and a small quantity of natural antibodies (Michael et al., 1962) as well as other antibacterial substances, such as beta-lysine and lectin (Donaldson et al., 1964; Kawasaki et al., 1989). Our results showed an increase in serum bactericidal activity in T2 for both probiotic supplements. It is possible to assume that probiotics, which contribute to the maintenance of the integrity of the intestinal membrane, are able to enhance intestinal immunity (i.e., higher levels of IgA; Gorbach, 2000) and could be able to facilitate the intestinal absorption of antibacterial substances that are able to improve the AP parameters.

Unexpectedly, lower complementary values were registered for the L group compared to the C group in our study, and our data do not allow for a reasonable explanation of this trend.

Lysozyme is able to damage bacterial cell walls by attacking peptidoglycan. Because this enzyme is largely present in the granules of several types of cells, its serum concentration can provide useful information about granulocyte activities and the functionality of the monocyte-macrophage system; therefore, it can be reasonably considered to be a possible marker for the quantification of pathogens in the environment (Gordon et al., 1974). In this study, the concentration of lysozyme was influenced by the use of probiotics at T1, and it was lower in the B group compared to control, which is a sign of lower degranulation of granulocytes. In their study on rats, Vilahur et al. (2014) showed that by modulating the innate immune response, dietary *Lactobacillus plantarum* could be a natural option to protect against inflammatory disorders.

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As for the immune response to the NDV vaccine, the L group showed an antibody concentration higher than that observed in the control group in accordance with other studies (Khaksefidi and Ghoorchi, 2006). This difference in antibody production could be attributed to the probiotic microorganism, which might have influenced the intestinal ecosystem and aided the assimilation of nutrients essential for triggering the immune cells to produce antibodies (Panda et al., 2000).

Oxidative status

Probiotics have shown antioxidant properties in poultry reared under intensive conditions (Capcarova et al., 2010), but in the present study, no differences between groups were found in the oxidative parameters evaluated. We can hypothesise that the antioxidant properties of probiotics found by other authors might become evident in stressful environments, and the more "natural" organic farming conditions may have prevented this dietary supplementation from producing any favourable effects for animal metabolism.

Egg physiochemical characteristics

With respect to egg physical parameters (Table 3), probiotics have been reported to increase egg quality (decreased yolk cholesterol level, improved shell thickness and egg weight -Kurtoglu et al., 2004; Xu et al., 2006). The results obtained in the present study are in accordance with Yöruk et al. (2004) who found no effect of diet on egg quality.

In terms of albumen quality, Williams (1992) found that, excluding disease, Haugh unit scores decrease with advancing flock age, but they increased in our study.

The values registered for egg lipid content (Table 7) are in accord with the observed reduction in serum total cholesterol and triglycerides. Egg cholesterol content showed a time-dependent effect ($P<0.05$), which is considered to be physiological; with an increasing deposition period, hens reduce their productive performance while cholesterol concentration increases (dilution effect).

These results are in agreement with Minelli et al. (2007), who found lower percentages of lipids in organic eggs collected at the end of the deposition cycle.

CONCLUSIONS

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In this study, dietary supplementation with two probiotic microorganisms, *L. acidophilus* D2/CSL CECT 4529 and *B. subtilis* PB6 ATCC-PTA 6737, resulted in changes to the blood chemical parameters and immune response of organically reared hens. In particular, *L. acidophilus* and *B. subtilis* induced positive changes in metabolic parameters, such as serum cholesterol and triglyceride content and ALT values, without changing the productive performance, egg physiochemical characteristics and oxidative status of the birds. The administration of *L. acidophilus* resulted in an improvement in antibody production against the Newcastle disease virus. In conclusion, these probiotic strains could be used in organic farming to improve animal welfare and immune defence.

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Table 1. Ingredients and chemical composition of the experimental diets

	Predeposition	Deposition
Ingredients (%)		
Maize	55.00	43.50
Soybean cake	25.00	30.00
Wheat bran	7.00	7.00
Broadbean	5.00	
Wheat		5.00
Calcium carbonate	3.00	8.70
Soybean	2.50	3.00
Dicalcium phosphate	1.50	1.50
Sodium chloride	0.30	0.50
Sodium bicarbonate	0.20	0.30
Mineral and Vitamin Premix*	0.50	0.50
Calculated composition		
M.E. (kcal/kg) †	3.041	2.810
Analysed composition (%)		
Moisture	8.28	8.35
Protein	16.92	18.01
Lipids	4.61	5.48
Ash	8.51	11.48
NDF	13.85	13.51
ADF	3.92	3.84
ADL	0.79	0.72
Ca	2.94	3.88
P	0.62	0.62
Lys	0.89	1.01
Met	0.45	0.36
Na	0.17	0.16

*Vitamin and mineral premix supplied per kilogram of diet: Vitamin A, 14000 UI; Vitamin D3, 3000 UI; Vitamin E, 30.00 mg, Vitamin K, 2.00 mg; Vitamin B_1, 1.75 mg; Vitamin B_2, 12.00 mg; Vitamin B_6, 2.00 mg; Vitamin B_{12}, 0.015 mg; Niacin, 35.00 mg; Folic acid, 0.50mg; Pantothenic acid, 10.00 mg; Fe ($FeCO_3$), 44.51 mg; Cu ($CuSO_4.5H_2O$), 58.95 mg; Mn (MnO), 193.5 mg; Zn (ZnO), 69.75 mg; Se (Na_2SeO_3) 66.00 mg

†Metabolizable Energy content of diets was estimated using the equation of Carpenter and Clegg (1956).

Table 2. Effect of probiotic dietary supplementation and sampling time on performance of laying hens

	T1	T2	Overall	Main effect	P
Deposition rate (%)					
C	69.82	79.15	76.05	Diet	0.062
B	70.90	80.50	77.09	Time	0.069
L	71.10	80.81	77.15	Diet x Time	0.064
SEM	2.897	3.215	2.954		
Feed efficiency					
C	2.54	2.71	2.63	Diet	0.075
B	2.50	2.68	2.58	Time	0.062
L	2.47	2.66	2.56	Diet x Time	0.069
SEM	0.181	0.215	0.205		
Egg weight (g)					
C	57.81	62.56	60.18	Diet	0.948
B	56.12	63.[illegible]	[illegible]9.83	Time	<0.001
L	56.37	63.33	59.85	Diet x Time	0.500
SEM	1.063	1.344	[illegible]57		

N= 15 hens pen/group per time (T1 and T2).

[A,B...] Means within a column lacking a common superscript differ ($P<0.05$)

C: basal diet; L: basal diet + 0.1 % of *Lactobacillus acidophilus*; B: basal diet + 0.05% of *Bacillus subtilis*.

Table 3. Effect of probiotic dietary supplementation and sampling time on clinical chemistry parameters of laying hens

	T1	T2	Overall	Main effect	P
Cholesterol (mmol /l)					
C	2.86	2.85 A	2.86 A	Diet	<0.001
B	2.41	2.27 B	2.34 B	Time	0.329
L	2.42	2.21 B	2.32 B	Diet x Time	0.793
SEM	0.151	0.143	0.106		
Triglycerides (mmol /l)					
C	9.39	10.97 A	10.18 A	Diet	0.002
B	7.29	[illegible].75 B	7.88 B	Time	0.027
L	9.05	[illegible] AB	9.12 AB	Diet x Time	0.368
SEM	0.543	0.583	0.397		
ALT (U/l)					
C	13.85 A	18.24 A	16.05 A	Diet	<0.001
B	12.03 A	9.97 B	11.00 B	Time	0.277
L	7.40 B	9.26 B	8.33 B	Diet x Time	0.013
SEM	1.230	1.954	1.069		
AST (U/l)					
C	205.39 A	169.05	187.22	Diet	0.064
B	162.09 B	174.65	168.37	Time	0.023
L	188.33 A	167.82	178.07	Diet x Time	0.009
SEM	7.222	8.121	5.427		
GGT (U/l)					
C	50.34	46.27	48.31	Diet	0[illegible]
B	49.95	48.74	48.85	Time	0.7[illegible]
L	48.69	50.73	49.71	Diet x Time	0.495
SEM	2.472	2.721	1.844		

N= 15 hens pen/group per time (T1 and T2).

[A,B...] Means within a column lacking a common superscript differ (P<0.05)

C: basal diet; L: basal diet + 0.1 % of *Lactobacillus acidophilus*; B: basal diet + 0.05% of *Bacillus subtilis*.

Comment [FB26]: Ethical approval for the research was not informed in the manuscript.

The authors must include the Protocol number of the Ethics Committee of Animal Use or explain why this information wasn´t include in this paper, since the authors used layer for this experiment.

Table 4. Effect of probiotic dietary supplementation and sampling time on immune response of laying hens

	T1	T2	Overall	Main effect	P
SBA (%)					
C	42.77	43.97 B	43.37 B	Diet	0.010
B	43.36	58.16 A	50.76 AB	Time	<0.001
L	41.38	63.81 A	52.59 A	Diet x Time	0.004
SEM	3.161	2.963	2.257		
HCA ($CH_{50\%}$)					
C	27.56	31.53	29.54 A	Diet	0.008
B	22.46	31.89	27.17 AB	Time	0.003
L	21.83	24.16	22.99 B	Diet x Time	0.250
SEM	1.863	2.621	1.491		
Lysozyme (µg/mL)					
C	7.517 A	4.102	5.810	Diet	0.418
B	5.430 B	5.129	5.280	Time	<0.001
L	6.269 AB	4.067	5.168	Diet x Time	0.016
SEM	0.462	0.588	0.366		
NDV antibodies (HI titer in log_2)					
C	160.00	156.44	158.22 B	Diet	0.013
B	200.00	252.23	226.12 AB	Time	0.455
L	277.33	310.86	294.09 A	Diet x Time	0.834
SEM	41.605	50.956	30.896		

N= 15 hens pen/group per time (T1 and T2).

[A,B...] Means within a column lacking a common superscript differ (P<0.05)

C: basal diet; L: basal diet + 0.1 % of *Lactobacillus acidophilus*; B: basal diet + 0.05% of *Bacillus subtilis*.

SBA: serum bactericidal activity; HCA: haemolytic complement assay; NDV: Newcastle disease virus.

Comment [FB27]: Ethical approval for the research was not informed in the manuscript.

The authors must include the Protocol number of the Ethics Committee of Animal Use or explain why this information wasn´t include in this paper, since the authors used layer for this experiment.

Table 5. Effect of probiotic dietary supplementation and sampling time on ROM's and AP

	T1	T2	Overall	Main effect	P
ROM's ($mmol\ H_2O_2$)					
C	3.272	4.754	4.012	Diet	0.113
B	3.073	3.625	3.349	Time	0.078
L	3.115	3.087	3.101	Diet x Time	0.238
SEM	0.395	0.522	0.318		
AP (µmol/HClO)					
C	62.644	86.797	74.723	Diet	0.548
B	70.798	82.015	76.414	Time	0.891
L	85.765	77.111	76.442	Diet x Time	0.053
SEM	9.222	9.642	6.729		

N= 15 hens pen/group per time (T1 and T2).

[A,B…] Means within a column lacking a common superscript differ (P<0.05)

C: basal diet; L: basal diet + 0.1 % of *Lactobacillus acidophilus*; B: basal diet + 0.05% of *Bacillus subtilis*.

ROM's: reactive oxygen metabolites; AP: antioxidant power of serum

Table 6. Effect of probiotic dietary supplementation and sampling time on egg physical parameters

	T1	T2	Overall	Main effect	P
Yolk weight (g)					
C	13.58	16.38	14.98	Diet	0.192
B	13.42	16.14	14.78	Time	<0.001
L	13.33	15.70	14.51	Diet x Time	0.075
SEM	0.382	0.333	0.275		
Albumen weight(g)					
C	34.01	38.55	36.28	Diet	0.631
B	32.22	38.46	35.34	Time	<0.001
L	32.99	38.39	35.69	Diet x Time	0.697
SEM	0.794	1.310	0.701		
Shell weight(g)					
C	6.90	7.76	7.33	Diet	0.667
B	7.05	7.58	7.31	Time	<0.001
L	6.66	7.67	7.17	Diet x Time	0.469
SEM	0.199	0.163	0.141		
Shell ash (%)					
C	3.21	3.19	3.20	Diet	0.664
B	3.30	3.34	3.33	Time	0.082
L	2.94	3.53	3.23	Diet x Time	0.094
SEM	0.139	0.144	0.105		
Yolk (%)					
C	23.49	26.47	24.98	Diet	0.192
B	23.97	25.42	24.70	Time	<0.001
L	23.63	24.79	24.21	Diet x Time	0.075
SEM	0.412	0.379	0.301		
Albumen (%)					
C	58.77	62.13	60.45	Diet	0.411
B	57.46	60.47	58.96	Time	0.003
L	58.64	60.60	59.62	Diet x Time	0.810
SEM	0.631	1.730	0.789		
Shell (%)					
C	11.98	12.455	12.22	Diet	0.520
B	12.52	11.967	12.45	Time	0.723
L	11.81	12.129	11.97	Diet x Time	0.119
SEM	0.251	0.260	0.190		
Edible (%)					
C	82.26	88.60	85.43	Diet	0.204
B	81.43	85.90	83.66	Time	<0.001
L	82.27	85.39	83.83	Diet x Time	0.335

SEM	0.605	1.699	0.770		
Albumen/yolk					
C	2.53	2.38	2.45	Diet	0.582
B	2.43	2.39	2.41	Time	0.187
L	2.51	2.46	2.49	Diet x Time	0.690
SEM	0.059	0.092	0.0522		
Colour (Roche scale)					
C	5.72	8.32	7.02 A	Diet	0.007
B	5.82	7.92	6.85 A	Time	<0.001
L	4.83	7.30	6.06 B	Diet x Time	0.500
SEM	0.305	0.296	0.2260		
Hugh Unit					
C	120.08	123.95	122.02	Diet	0.125
B	120.74	124.41	122.58	Time	<0.001
L	118.25	123.90	121.08	Diet x Time	0.340
SEM	0.673	0.767	0.523		
Colour CIELAB					
L*					
C	56.11 B	57.81	56.96	Diet	0.095
B	57.24 AB	56.99	57.11	Time	0.175
L	57.86 A	58.01	57.93	Diet x Time	0.102
SEM	0.430	0.505	0.338		
a					
C	-4.41	-3.58	-3.96	Diet	0.078
B	-4.67	-3.52	-4.12	Time	<0.001
L	-5.01	-3.90	-4.46	Diet x Time	0.846
SEM	0.213	0.196	0.156		
b					
C	36.37	40.46	37.42	Diet	0.740
B	36.46	39.11	37.79	Time	<0.001
L	36.56	39.11	37.33	Diet x Time	0.018
SEM	0.612	0.513	0.438		

N=30 eggs/pen/group per time (T1 and T2).

[A,B...] Means within a column lacking a common superscript differ (P<0.05)

C: basal diet; L: basal diet + 0.1 % of *Lactobacillus acidophilus*; B: basal diet + 0.05% of *Bacillus subtilis*.

Table 7. Effect of probiotic dietary supplementation and sampling time in egg chemical parameters

	T1	T2	Overall	Main effect	P
Ash (%)					
C	1.76	1.78	1.77	Diet	0.8975
B	1.78	1.78	1.78	Time	0.0407
L	1.73	1.81	1.77	Diet x Time	0.1168
SEM	0.019	0.018	0.014		
Crude protein (%)					
C	16.00	16.20	16.10	Diet	0.1404
B	15.87	16.02	15.95	Time	0.1001
L	16.12	16.29	16.20	Diet x Time	0.9835
SEM	0.109	0.148	0.090		
Lipid (%)					
C	27.74 A	27.45	27.59 A	Diet	0.0009
B	27.70 A	27.56	27.63 A	Time	0.8460
L	26.80 B	27.31	27.05 B	Diet x Time	0.0407
SEM	0.138	0.193	0.115		
Cholesterol (mg/g yolk)					
C	12.80	12.52	12.66	Diet	0.6866
B	13.21	13.09	13.15	Time	0.7356
L	12.91	12.85	12.88	Diet x Time	0.9817
SEM	0.575	0.342	0.396		
Cholesterol (mg/egg)					
C	177.83	203.49	190.66	Diet	0.7495
B	180.15	211.49	195.82	Time	0.0016
L	174.01	201.94	187.97	Diet x Time	0.9645
SEM	10.859	6.577	7.446		

N= 30eggs pen/group per time (T1 and T2)

[A,B...] Means within a column lacking a common superscript differ ($P<0.05$)

C: basal diet; L: basal diet + 0.1 % of *Lactobacillus acidophilus*; B: basal diet + 0.05% of *Bacillus subtilis*.