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Impact of lyophilized *Lactobacillus salivarius* DSPV 001P administration on growth performance, microbial translocation, and gastrointestinal microbiota of broilers reared under low ambient temperature

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A R T I C L E I N F O

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A B S T R A C T

This study was undertaken with the aim of investigating the effects of dietary supplementation of probiotic strain *Lactobacillus salivarius* DSPV 001P on growth performance, microbial translocation, and gastrointestinal microbiota of broilers reared under low ambient temperature. Two hundred and forty, one-day-old male Cobb broilers were randomly distributed into two treatment groups, a probiotic group and a control group, with four replicates per treatment and 30 broilers per replicate. The temperature of the broiler house was maintained at 18–22 °C during the first three weeks, after which the temperature was at range of 8 °C to 12 °C. The results showed that probiotic treatment significantly improved body weight of broilers when compared with the control group. After 42 days, the weight means were 2905 ± 365.4 g and 2724 ± 427.0 g, respectively. Although there were no significant differences, dietary inclusion of *L. salivarius* tended to increase feed intake and to reduce feed conversion ratio during the six-week experimental period. Similarly, supplementation tended to reduce the rate of mortality, with 12 deaths occurring in the probiotic group, and 20 in the control group. However, no differences were observed in intestinal bacterial concentrations of *Enterobacteriaceae*, *E.coli*, and lactic acid bacteria in both crop and caecum among treatments. Through our study, it appears that *L. salivarius* DSPV 001P was non-pathogenic, safe and beneficial to broilers, which implies that it could be a promising feed additive, thus enhancing the growth performance of broilers and improving their health.

1. Introduction

In many areas of the world, regulatory pressures have limited antibiotic usage in livestock due to the risk of residues and the increasing rates of resistance in human population (Marshall and Levy, 2011). However, it is worth remarking that, over the last few decades, an alternative approach has emerged to sub-therapeutic antibiotics in animals: the use of probiotics, which are defined as live microbial food supplements that beneficially affect the host by improving intestinal microbial balance (Hill et al., 2014). Probiotics lead to considerable improvement in animal health, increasing production parameters (Salarmoini and Fooladi, 2011) and reinforcing host immunity (Wang et al., 2015). Each probiotic candidate should meet a number of requirements, including safety, functional and beneficial characteristics (FAO/WHO, 2002). In poultry production, emphasis has been placed on the selection, preparation, and application of probiotic strains, especially lactic acid bacteria (LAB) (Babot et al., 2014). Hence, many investigations have been conducted to determine the effects of *Lactobacillus* on the broiler performance (Olnood et al., 2015; Blajman et al., 2014). However, experimental results are contradictory; and variations in the effectiveness about the use of defined cultures may be attributed to differences in the bacterial strains used as dietary supplements.

To take maximum advantage of the benefits of microorganisms with probiotic capacity, it is desirable to use autochthonous strains, isolated from the same ecosystem of which they will be part once ingested (Rosmini et al., 2004). Gut inhabitants that share long evolutionary histories with their host species are likely to possess adaptive health attributes: these can be explored when those organisms are used as

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probiotics. It is therefore logical to consider that autochthonous strains constitute better probiotic strains for some applications (Walter, 2008). Furthermore, Hardin (1960) states that it is extremely difficult for a microorganism that is introduced into the gut to gain access. Some strains currently used as probiotics do belong to species which are likely to be allochthonous: their failure to persist might reflect a lack of competitiveness in the gut ecosystem (Walter, 2008).

Although poultry industry in Argentina has become one of the most dynamic and expanding sectors, all probiotics marketed in our country are imported. For this reason, our group aims at promoting the development of probiotic cultures from native strains. In a previous study, a *Lactobacillus* isolated from the jejunum of a local broiler was selected due to its *in vitro* probiotic properties, its capacity to colonize, its ability to integrate the indigenous microbiota and its aptitude to persist in the gastrointestinal tract (GIT) of broilers (Blajman et al., 2015). Consequently, the purpose of the present research was to determine the impact of administering lyophilized *Lactobacillus salivarius* DSPV 001P in promoting growth performance and intestinal microbiota balance in broilers reared under low ambient temperature.

2. Material and methods

2.1. Microorganism

L. salivarius DSPV 001P, a strain of avian origin with *in vitro* probiotic properties (Blajman et al., 2015), was isolated from the jejunum of a healthy broiler and identified based on the 16SrDNA gene sequencing. The 16SrDNA gene sequence was introduced to the GenBank database and was assigned the accession number KU295171. A spontaneous rifampicin resistant mutant *L. salivarius* DSPV 001P was selected in order to be able to trace down the bacteria during the *in vivo* study. The resistance of the strain to the antibiotic was selected from serial cultures in MRS agar (Oxoid, Basingstoke, United Kingdom), from low levels up to a concentration of 100 μg/ml rifampicin.

2.2. Bacterial growth

L. salivarius DSPV 001P was cultured in a fermenter of 4 l (Sartorius Stedim Biotech, Goettingen, Germany) with 2% of initial inoculum, and incubated 18 h at 37 °C. A feed-batch fermentation process was employed with 6% cheese whey permeate (Arla Food Ingredients, Porteña, Argentina), 0.8% yeast extract, 0.003 g/l $MnSO_4$ (Merck, Darmstadt, Germany), and 20 g/l of casein peptone (Sigma-Aldrich, San Luis, United States) as culture medium. Agitation was set at 120 rpm and temperature at 37 °C, while pH was adjusted to 6. After 6 h incubation, 15% cheese whey permeates (Arla Food Ingredients, Porteña, Argentina) and 10 g/l of casein peptone (Sigma-Aldrich, San Luis, United States) was added. The incubation was performed for 18 h. Afterwards, bacterial cells were harvested by centrifugation at 5000 × *g* for 10 min at 17 °C, supernatant was removed, and cell pellets were freeze-dried with skim milk as cryoprotective agent (Corlasa, Esperanza, Argentina). Bacterial cells were lyophilized at 0.044 mbar (Martin Christ, Osterode am Harz, Germany) for 27 h to obtain the probiotic product in a powder form. Lastly, the number of surviving cells after freeze drying was counted using the agar plate count method and expressed as cfu/g lyophilized powder.

2.3. Experimental design

Two hundred and forty, one-day-old male Cobb broilers were obtained from a commercial hatchery and randomly divided in two experimental groups of 120 broilers: the control group (C-G) and the probiotic group (P-G). There were eight replicates with 30 broilers per replicate. The experiment was conducted at an experimental broiler farm from the Faculty of Veterinary Science, Universidad Nacional del Litoral. Procedures used in this study were approved by the Ethics and Security Committee of the Faculty of Veterinary Science, mentioned above and consistent with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, Federation of Animal Sciences Societies (FASS, 1998). All broilers had similar initial weights (35 ± 0.73 g) and were reared in environmentally controlled conditions for 42 days (d) from May to June. The flock was placed in a broiler house with new litter. Surrounding environmental temperature was measured using a thermometer. The temperature of the broiler house was maintained at 18–22 °C for the first three weeks, after which the temperature was at range of 8 °C to 12 °C. A 23L:1D photoperiod was provided during the entire trial period. Broilers were fed on commercial basal diets (Avigan, Humboldt, Argentina) devoid of antibiotics or anticoccidials *ad-libitum*. The feed was formulated for starter (0–21 days) and grower (22–42 days) periods. The manufacturer supplied the composition of the basal diet, as shown in Table 1. Water was provided in continuous flow water troughs throughout the experiment. To ensure that P-G ingested all the inoculum, lyophilized probiotic bacteria were mixed thoroughly in half of feed which was estimated to be consumed daily by broilers. Then, broilers continued eating diet without inoculum *ad libitum*.

Table 1
Basal diet composition and calculated nutrient analysis of diets for broilers.

Starter feed (0–4 weeks)	Grower feed (5–6 weeks)
Ingredients	
Maize	Maize
Sorghum	Sorghum
Cultch	Cultch
Wheat bran	Wheat bran
Sunflower expeller	Sunflower expeller
Sunflower pellet	Sunflower pellet
Deactivated soybean beans	Deactivated soybean beans
Soybean expeller	Soybean expeller
Conchilla	Conchilla
Poultry oil	Poultry oil
Flour meal	Flour meal
Poultry feather meal	
	Barley
	Oat
	Gluten feed
	Wheat flour
	Calcium carbonate
	Phosphate
	Salt
Lysine	Lysine
Methionine	Methionine
Vitamins A, D3, E, K3, B1, B2, B6, B12	Vitamins A, D3, E, K3, B1, B2, B6, B12
Niacin	Niacin
Calcium pantothenate	Calcium pantothenate
Folic acid	Folic acid
Choline 70%	Choline 25%
Minerals: manganese, copper, zinc, iron, iodine, cobalt, selenium and antioxidant (BHT)	Minerals: manganese, copper, zinc, iron, iodine, cobalt, selenium and antioxidant (BHT)
Nutritional content	
Minimum crude protein 22%	Minimum crude protein 19%
Minimum ethereal extract 8.5%	Minimum ethereal extract 8.5%
Maximum crude fiber 3.2%	Maximum crude fiber 3%
Maximum moisture 12%	Maximum moisture 12%
Calcium 0.8 to 1.5%	Calcium 0.75 to 1%
Phosphorus 0.35 to 0.5%	Phosphorus 0.35 to 0.5%
Maximum total ash 6%	Maximum total ash 6%
Metabolizable energy 3.150 kcal/kg	Metabolizable energy 3.200 kcal/kg

2.4. Biosafety standards

A set of management practices were designed to prevent the entry and transmission of the probiotic strain to C-G: we agreed that different overalls and overshoes for P-G and C-G should be used, and made it compulsory to apply a disinfectant before entering and leaving each

group, place trays with lime at the entrance gates to each broiler house, ban visitors' entry, and dispose of dead broilers properly.

2.5. Necropsies

Programmed necropsies were performed on four randomly selected broilers from each experimental group (one per replicate) at d 0 and once a week after the administration of the probiotic bacteria. Necropsies were made 24 h after the last probiotic administration. Broilers were euthanized by cervical dislocation by a person with appropriate qualifications.

2.6. GIT microbial populations

The crop and caecum were collected using sterile instruments. With the aim of determining the GIT colonization by inoculated bacteria, the number of cfu/g of organ recovered from crop and caecum was calculated. The presence of bacterium in the digestive tract was interpreted as colonization by those bacteria. Samples of 1 g from crop and caecum were homogenized in diluted 1/2 Ringer solution (Biokar, Beauvais, France), each homogenate was serially diluted from initial 10^{-1} to 10^{-7}, and subsequently plated on selective agar media for enumeration of target bacterial groups. De Man et al. (1960) with rifampicin (MRSrif) agar plates were spread to recover only the administered probiotic strain. Petri dishes were incubated at 37 °C for 72 h in anaerobic conditions (10% CO_2 and 90% H_2; Indura, Buenos Aires, Argentina) and the characteristic colonies were counted.

Total lactic acid bacteria (LAB), yeasts, *E. coli*, and enterobacterias were also examined. Accordingly, homogenized samples were spread in the following media: MRS (Oxoid, Basingstoke, United Kingdom), modified HyL (Britania, Buenos Aires, Argentina), TBX (Oxoid, Basingstoke, United Kingdom), and VRBG (Oxoid, Basingstoke, United Kingdom), respectively. The number of bacterial colonies was counted at the end of each incubation period and results were expressed as the number cfu/g.

2.7. Microbial translocation

Samples of 1 g of liver were homogenized with a Stomacher Seward biomaster (Seward, Worthing, United Kingdom) in 1/2 Ringer solution (Biokar, Beauvais, France). In order to measure translocation in the internal medium, homogenized samples were spread in the following medium: MRSrif, MRS (Oxoid, Basingstoke, United Kingdom), modified HyL (Britania, Buenos Aires, Argentina), TBX (Oxoid, Basingstoke, United Kingdom), and VRBG (Oxoid, Basingstoke, United Kingdom).

2.8. Campylobacter isolation

During the first 14 d of the experiment, an aliquot of caecal content of broilers was transferred to a 4 ml tube of Bolton broth (Oxoid, Basingstoke, United Kingdom). The tubes were cultured under microaerophilic conditions (10% O_2, 5% CO_2, and 85% H_2) at 42 °C for 24 h and centrifuged at 5000 × *g* for 10 min at 22 °C. The supernatant was discarded and each pellet was placed on a 0.45 μm filter (up to three filters per dish) in plates with modified charcoal-cefoperazone-deoxycholate agar (mCCDA). From the 15th d of trial, 1 g of caecal content was placed on sterile 0.45 μm filters on mCCDA plates. They were incubated at 37 °C for 10 min. Subsequently, the filters were removed and plates were incubated at 42 °C in microaerophilic conditions (10% O_2, 5% CO_2, and 85% H_2) for 48 h. Presumptive *Campylobacter* colonies were observed on a contrast phase microscopy (curved bacilli with typical motility). Upon confirmation, colonies were subcultured to another mCCDA plate and incubated 48 h at 42 °C under microaerophilic conditions to obtain pure colonies. The last d of experiment, 40 cloacal swabs from 5 broilers per replicate were performed. Each swab was placed in a 9 ml tube containing Bolton broth (Oxoid, Basingstoke, United Kingdom), and transported to the laboratory. The procedures utilized were the same as the ones described for the first 14 d of study.

2.9. Performance parameters

Records of feed intake (FI) were kept per each replicate weekly, and broilers were weighted individually at d 0, and once a week until the end of the experiment; so that body weight gain (BWG) and feed conversion ratio (FCR) could be estimated. Mortality was recorded as it occurred and mortality rate was determined at the end of the study.

2.10. Statistical analysis

Crop and caecal microbiota, and performance parameters were analyzed with ANOVA and repeated measures by the general linear model using the software INFOSTAT version 2011 (InfoStat Group, FCA, Universidad Nacional de Córdoba, Argentina). Also, mortality and microbial translocation were scrutinized using Chi square test or Fisher's Exact Test. Treatment effects were considered significantly different at $P \le 0.05$. Results were expressed as the arithmetic mean ± standard deviation (SD).

3. Results

Once the bacteria were administered, LAB values in the crop and caecum of broilers were monitored. The administered bacteria were not present in the C-G broilers. Bacterial translocation to the liver was found in neither experimental group. Before strain administration on day 0, there were no rifampicin resistant bacteria detected from broilers' crop and caecum.

Counts of LAB, yeast, *Enterobacteriaceae*, and *E. coli* were evaluated in this study. Dietary treatment did not affect the level of LAB ($P = 0.162$), yeast ($P = 0.872$), *Enterobacteriaceae* ($P = 0.350$), and *E. coli* ($P = 0.827$) in the crop. Although there was no significant difference between the LAB populations of broilers fed diets with or without *L. salivarius* DSPV 001P, animals supplemented with *Lactobacillus* culture had slightly higher numbers of LAB in the crop (Table 2).

No significant differences in caecal LAB ($P = 0.377$), *Enterobacteriaceae* ($P = 0.748$), and *E. coli* ($P = 0.089$) concentrations were observed among the groups. When compared to the C-G, the probiotic decreased the number of yeast in the caecum ($P = 0.037$) in the P-G (Table 3).

There were no *Campylobacter* isolates from caecal samples and cloacal swabs in either experimental group.

Body weight at the beginning of the experiment (d 0) was not significantly different among groups ($P > 0.05$). Compared with the control, probiotic supplementation significantly improved final weight during the experimental period ($P < 0.001$). The group that received the probiotic weighed 2905 ± 365.4 g at the end of the trial, while the weight of C-G was 2724 ± 427 g (Fig. 1).

Consumption increased from 130 ± 0.01 g/week to 1665 ± 0.06 g/week in the P-G and 123 ± 0.01 g/week to 1645 ± 0.04 g/week in the C-G between the first and last week of the trial (Table 4). However, no statistical differences in FI ($P = 0.072$) were observed between treatments. Furthermore, in the current trial, there was no probiotic effect in the FCR of broilers ($P = 0.533$). Nevertheless, P-G animals were able to increase 1 kg body weight per 1.58 kg of feed consumed, while C-G broilers increased 1 kg per 1.63 kg of feed consumed.

At the end of the experiment, the mortality was 13.04% for supplemented broilers and 21.74% for broilers fed only a basal diet, but it was not possible to identify a statistically significant association ($P = 0.131$) (Fig. 2).

Table 2
Microbiota from broilers' crop supplemented and not supplemented with *L. salivarius* DSPV 001P.

Experimental group	Time (d)	Microbiological counts (Log CFU/g)				
		LAB	*L. salivarius* DSPV 001P	*Enterobacteriaceae*	*E. coli*	Yeast
P-G	0	5.41 ± 0.691	0.00	4.89 ± 1.065	2.82 ± 1.925	0.00
	7	7.64 ± 0.134	5.60 ± 0.723	4.23 ± 2.863	3.63 ± 1.509	2.05 ± 0.503
	14	8.45 ± 0.560	4.56 ± 0.702	6.02 ± 0.866	5.25 ± 1.243	0.62 ± 1.239
	21	8.13 ± 0.863	1.95 ± 2.363	3.77 ± 2.513	3.16 ± 2.396	3.16 ± 1.274
	28	8.20 ± 0.622	4.16 ± 1.128	5.41 ± 0.323	4.82 ± 0.810	1.65 ± 1.109
	35	7.86 ± 0.615	4.59 ± 0.775	6.23 ± 0.640	5.15 ± 0.842	1.77 ± 1.285
	42	8.28 ± 1.028	4.20 ± 1.086	5.74 ± 0.906	4.63 ± 1.531	1.32 ± 1.611
C-G	0	6.02 ± 1.052	0.00	5.11 ± 1.374	3.91 ± 2.611	0.67 ± 1.348
	7	7.63 ± 0.099	0.00	4.85 ± 1.635	3.74 ± 1.961	0.60 ± 1.191
	14	8.02 ± 0.555	0.00	5.46 ± 1.007	3.65 ± 0.927	0.00
	21	8.02 ± 0.183	0.00	6.19 ± 0.663	4.80 ± 0.836	3.14 ± 0.316
	28	6.91 ± 0.122	0.00	5.80 ± 1.121	4.77 ± 1.335	1.33 ± 1.557
	35	7.33 ± 0.322	0.00	5.58 ± 0.562	4.53 ± 0.350	1.29 ± 1.569
	42	8.20 ± 0.741	0.00	6.19 ± 2.163	4.88 ± 3.305	3.09 ± 2.201
P-value		0.162	0.000	0.350	0.827	0.872

4. Discussion

In the present study, lyophilized probiotic bacteria were added to the feed. However, spray drying is commonly used for the preservation of potential probiotic cultures (Corcoran et al., 2004). Spray-drying is an economical process for preparing industrial scale quantities of viable microorganisms. In spite of being economic and effective, the cells experienced both thermal and dehydration inactivation simultaneously during spray drying (Bigetti Guergoletto et al., 2012). Since lyophilization utilizes milder conditions, it is a preferred drying method for thermally sensitive bacteria as it keeps their survival at a reasonably high level. With regard to costs, lyophilization is generally seen as an expensive method of preservation (Peighambardoust et al., 2011). Nevertheless, studies indicate that, when an analysis is made of the total energy involved in the different processes, lyophilization is an advantageous technique. Furthermore, comparing the several steps involved, the added value and improvement in the quality of raw materials and the differentiated products, such as the case of probiotics is, lyophilization should not be considered prohibitive in terms of cost (Ratti, 2001).

After administration, *L. salivarius* DSPV 001P was re-isolated from the crop and caecum, thus indicating that it was established in GIT. At the dose used the lyophilized strain did not translocate to the broilers' internal environment, maintaining a behavior similar to the previous trial (Blajman et al., 2015). Hence, higher doses could be used in future experiences in order to achieve greater beneficial effects.

The results showed that there was a higher performance in broilers when *L. salivarius* DSPV 001P was included in the diets. In the present research, the addition of the probiotic significantly increased the BWG of broilers 42 days after feeding. Edens (2003) reported that probiotics improved digestion, absorption and efficiency of utilization of feed accompanied by positive effects on intestinal activity and increasing digestive enzymes. The beneficial effects of probiotics on broiler BWG were in agreement with a larger number of other studies using probiotics in broilers (Blajman et al., 2014), and; these findings provided valuable information about the relevant role that probiotics will play in broilers' breeding.

Probiotic group had no more efficient FCR than those of the control. In spite of what has previously been stated, there was a P-G tendency to consume more and have an increase feed efficiency. FCR values of different treated groups lacked significance in previous reports for poultry receiving probiotics (García-Hernández et al., 2016; Zhang et al., 2011). In contrast, Fajardo et al. (2012) and Liu et al. (2012) obtained improvements of feed efficiency. In future trials, a higher dose and a shorter test time could favour the expression of the probiotic effect of this strain.

It is known that the probiotic efficacy varies greatly and depends on many factors such as inocula composition, strain type, administration level (doses), method and frequency of application, animal health, overall diet, farm hygiene and environmental conditions (Patterson and Burkholder, 2003). In addition, the use of prebiotics may allow microorganisms to establish more efficiently within the GIT more

Table 3
Microbiota from broilers' caecum supplemented and not supplemented with *L. salivarius* DSPV 001P.

Experimental group	Time (d)	Microbiological counts (Log CFU/g)				
		LAB	*L. salivarius* DSPV 001P	*Enterobacteriaceae*	*E. coli*	Yeast
P-G	0	1.82 ± 3.630	0.00	0.00	0.00	0.00
	7	8.78 ± 0.323	4.85 ± 0.678	7.71 ± 0.651	6.85 ± 0.414	3.01 ± 2.809
	14	8.60 ± 0.653	4.22 ± 0.946	8.91 ± 0.563	7.68 ± 0.950	2.94 ± 2.394
	21	8.13 ± 0.496	3.24 ± 2.357	9.19 ± 0.500	7.57 ± 1.165	3.85 ± 0.749
	28	8.37 ± 0.285	1.90 ± 2.266	8.58 ± 0.598	6.90 ± 0.755	3.89 ± 1.516
	35	8.12 ± 0.095	3.86 ± 0.689	8.26 ± 0.507	7.22 ± 0.591	2.44 ± 1.895
	42	8.34 ± 0.701	3.14 ± 0.586	7.70 ± 1.404	6.73 ± 0.394	3.06 ± 2.402
C-G	0	5.99 ± 4.010	0.00	0.00	2.49 ± 2.897	0.00
	7	8.94 ± 0.575	0.00	7.99 ± 0.451	6.73 ± 0.599	2.36 ± 1.763
	14	8.26 ± 0.861	0.00	7.85 ± 0.258	7.36 ± 0.244	3.71 ± 0.296
	21	7.80 ± 0.746	0.00	8.53 ± 0.416	7.11 ± 0.420	4.87 ± 0.455
	28	7.62 ± 0.718	0.00	8.80 ± 0.570	7.90 ± 0.372	4.65 ± 0.326
	35	8.07 ± 0.656	0.00	9.33 ± 0.484	8.16 ± 0.569	3.77 ± 0.614
	42	8.77 ± 0.604	0.00	7.52 ± 0.425	6.64 ± 0.457	4.19 ± 0.489
P-value		0.377	0.000	0.748	0.089	0.037

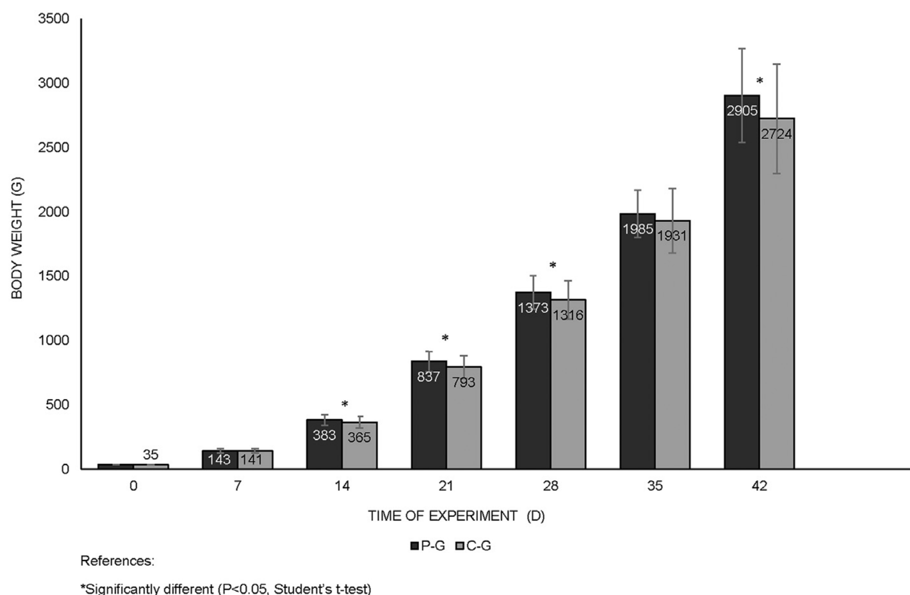

Fig. 1. Body weight from 0- to 42-day-old broilers after feeding with *L. salivarius* DSPV 001P.

Table 4
Feed intake from 0- to 42-day-old broilers after *L. salivarius* DSPV 001P supplementation.

Experimental group	Time (week)	Feed intake (g/week)
P-G	7	130 ± 0.01
	14	334 ± 0.01
	21	624 ± 0.02
	28	918 ± 0.04
	35	1199 ± 0.02
	42	1665 ± 0.06
C-G	7	123 ± 0.01
	14	347 ± 0.02
	21	591 ± 0.01
	28	907 ± 0.07
	35	1138 ± 0.04
	42	1645 ± 0.04
P-value		0.072

efficiently, and thus further improve the poultry productive parameters (Mookiah et al., 2014). Moreover, the probiotic effectiveness in broilers could be related to the presence of stress (Al-Fataftah and Abdelqader, 2014). Our study supports this theory since broilers were reared in a cold average temperature, considered stressful and harmful to broilers (Lyon, 1987).

There were 32 deaths in the course of the trial. Although there were no significant variations in mortality among treatments, 12 of those occurred in the P-G, and 20 in the C-G. A similar finding was presented by Trabelsi et al. (2016), who informed that mortality was different in the period of 21–35 days of age and was higher in the control group as compared with that of the broilers which were fed the probiotic product. In contrast, O'Dea et al. (2006) reported that there were no significant differences in broiler mortality between the probiotic *vs.* control treatments. In our study, no clinical cases were noted, and broilers were found lying on their backs. The factor that caused death could have been cold, since low ambient temperature is one of the main triggers for pulmonary hypertension syndrome (Pan et al., 2005), which causes ascites. The propensity for broilers to develop this syndrome increases as ambient temperatures decrease, because the broilers need more oxygen in order to maintain their body temperature (Wideman and Tackett, 2000). Growth rate, oxygen requirement, cardiac output, heat production and metabolic rate are closely linked (Julian, 2000). In accordance with our studies, Ipek and Sahan (2006) reported that broilers exhibited the highest ascites mortality during weeks 3 and 6. Moreover, in this research, body weight gain of broilers up to 3 weeks was significantly affected by cold stress. Broilers in the warm

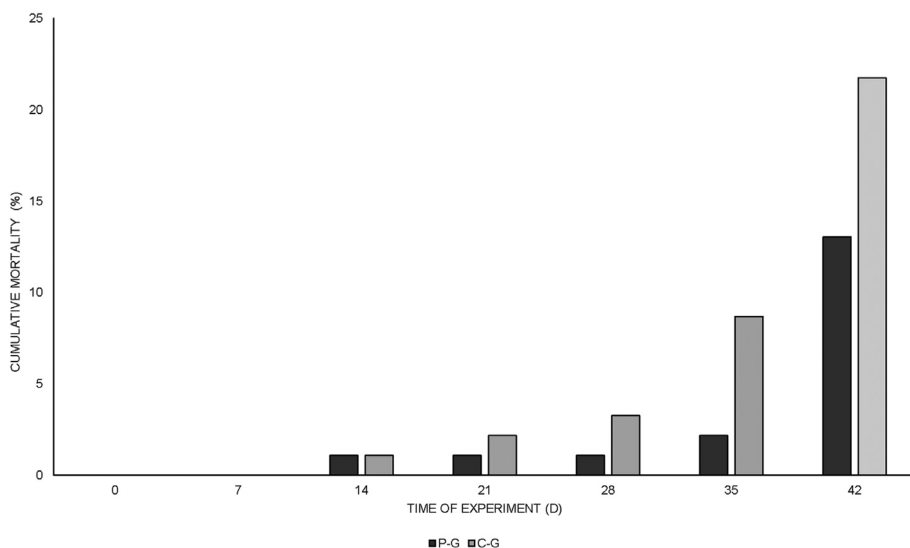

Fig. 2. Cumulative mortality from 0- to 42-day-old broilers after feeding with *L. salivarius* DSPV 001P.

environment had greater body weight gain compared with broilers in the cold stress treatment. If broilers are in a cool environment, a greater portion of their nutrient intake must be used to generate heat thus adversely affecting body weight gain (Bruzual et al., 2000). In our research, the use of a probiotic that improves efficiency of utilization of feed could have counteracted this lower weight gain in broilers.

In the current experiment, the inclusion of *L. salivarius* DSPV 001P did not affect counts of *Enterobacteriaceae* and *E. coli*. This result may be attributed to the following two factors: the animals' health was considered normal, and sanitary conditions were optimal throughout the trial. These results are in agreement with those of several studies which noted that addition of probiotics to broilers diet had no significant influence on intestinal microbiota (Mountzouris et al., 2007; Priyankarage et al., 2003). However, it should be noted that it is possible to detect differences in bacterial populations that were not analyzed in this essay (Jayaraman et al., 2013). Only the caecal yeast population was modified. In agreement with our studies but calf intestinal ecosystem, Ozawa et al. (1983) observed that the administration of LAB decreased yeast population in the P-G. Additionally, a previous report (Ozawa and Takeuchi, 1965) showed that *S. faecalis* BIO-4R strain prevents the over-colonization of yeasts in intestines of mice receiving oral streptomycin and that competition for fermentable carbon source was suggested to account for this antagonistic effect. However, Soto (2010) reported a higher number of yeast after supplementation with probiotic bacteria. It should be emphasized that different probiotic strains could have different properties and effects on yeasts; thus, it should be remarked that results from studies testing one strain should not be extrapolated to other strains.

The addition of *L. salivarius* DSPV 001P did not increase significantly total LAB in the crop and caecum. Similar LAB counts found in broilers could be due to the competition for nutrients by a large number of lactobacilli in the GIT of birds. However, the strain showed its competitive power, replacing at least in part the indigenous lactic microbiota in crop and caecum. Apparently, this behavior recurrently occurs when broilers have a balanced intestinal microbiota that allows them to develop properly.

A more promising approach seems to be related to the use of LAB in broilers with a disturbed intestinal microbiota. In our research, experimental broilers were not subjected to pathogenic bacteria. Hence, the inability to demonstrate any disturbance in the microbial composition may be due to the limited exposure of broilers to these challenges. Under challenging conditions, an imbalance in the intestinal microbiota is created, the body defense mechanisms decreased, and the multiplication of harmful bacteria increased. By the supplementation of probiotics, such problems would be minimized, evidencing differences between treatments (Ducatelle et al., 2015). Thus, it may be interesting to conduct new experiments where the strain effect in broilers is assessed in detail after being challenged with pathogens (Sadeghi et al., 2015; Ritzi et al., 2014).

It was also worth noting that, in this study, there were no *Campylobacter* isolates from samples and cloacal swabs in either group. This may be related to the biosecurity measures implemented to access the boxes. Generating and maintaining a clean farm environment is one way of reducing the risk of tracking *Campylobacter* into the poultry house (Newell et al., 2011). Human traffic into the house is a high risk that could be reduced by best hygiene practices and appropriate hygiene barriers. Consistent application of simple biosecurity measures, such as clothes disinfection, use of house-specific boots and overshoes and removal of dead broilers, among others, can reduce the risk of *Campylobacter* colonization by about 50% in intervention flocks (Gibbens et al., 2001). In addition, seasonality is an observed risk factor in many of the publications reviewed. The seasonal risk peak generally occurs in late summer/early autumn (Barrios et al., 2006), whereas our study was carried out in late autumn/early winter.

5. Conclusions

Our results have led us to hypothesize that *L. salivarius* DSPV 001P was non-pathogenic, safe and beneficial to broilers. Dietary inclusion of L. *salivarius* DSPV 001P displayed a positive effect in reducing mortality, and in promoting BWG compared with C-G. In summary, this strain has *in vivo* probiotic properties, thus making it a promising alternative to promote broilers' health. As the probiotic candidate will likely exert a more profound positive effect when there is a disturbed intestinal microbiota, future *in vivo* studies involving a challenge with pathogens should be conducted.

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References

Al-Fataftah, A.R., Abdelqader, A., 2014. Effects of dietary *Bacillus subtilis* on heat-stressed broilers performance, intestinal morphology and microflora composition. Anim. Feed Sci. Technol. 198, 279–285.

Babot, J.D., Argañaraz-Martínez, E., Saavedra, L., Apella, M.C., Perez Chaia, A., 2014. Selection of indigenous lactic acid bacteria to reinforce the intestinal microbiota of newly hatched chicken – relevance of in vitro and ex vivo methods for strains characterization. Res. Vet. Sci. 97, 8–17.

Barrios, P.R., Reiersen, J., Lowman, R., Bisaillon, J.R., Michel, P., Fridriksdóttir, V., Gunnarsson, E., Stern, N., Berke, O., McEwen, S., Martin, W., 2006. Risk factors for *Campylobacter* spp. colonization in broiler flocks in Iceland. Prev. Vet. Med. 74, 264–278.

Bigetti Guergoletto, K., Sivieri, K., Yuri Tsuruda, A., Peboni Martins, E., Bertuol de Souza, J., Massaguer Roig, S., Yoko Hirooka, E., Garcia, S., 2012. Dried probiotics for use in functional food applications. In: Valdez, Benjamin (Ed.), Food Industrial Processes - Methods and Equipment. InTech, USA, pp. 227–250.

Blajman, J.E., Frizzo, L.S., Zbrun, M.V., Astesana, D.M., Fusari, M.L., Soto, L.P., Rosmini, M.R., Signorini, M.L., 2014. Probiotics and broiler growth performance: a meta-analysis of randomised controlled trials. Br. Poult. Sci. 55, 483–494.

Blajman, J.E., Gaziano, C., Zbrun, M.V., Soto, L.P., Astesana, D.M., Berisvil, A.P., Romero Scharpen, A., Signorini, M.L., Frizzo, L.S., 2015. In vitro and in vivo screening of native lactic acid bacteria towards their selection as a probiotic in broiler chickens. Res. Vet. Sci. 101, 50–56.

Bruzual, J.J., Peak, S.D., Brake, J., Peeblest, E.D., 2000. Effect of relative humidity during the last five days of incubation and brooding temperature on performance of broiler chicks from young broiler breeders. Poult. Sci. 79, 1385–1391.

Corcoran, B.M., Ross, R.P., Fitzgerald, G.F., Stanton, C., 2004. Comparative survival of probiotic lactobacilli spray-dried in the presence of prebiotic substances. J. Appl. Microbiol. 96, 1024–1039.

De Man, J.C., Rogosa, M., Sharpe, M.E., 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23, 130–135.

Ducatelle, R., Eeckhaut, V., Haesebrouck, F., Van Immerseel, F., 2015. A review on prebiotics and probiotics for the control of dysbiosis: present status and future perspectives. Animal 9, 43–48.

Edens, F., 2003. An alternative for antibiotic se in poultry: probiotics. Braz. J. Poult. Sci. 5, 75–97.

Fajardo, P., Pastrana, L., Méndez, J., Rodríguez, I., Fuciños, C., Guerra, N.P., 2012. Effects of feeding of two potentially probiotic preparations from lactic acid bacteria on the performance and faecal microflora of broiler chickens. Sci. World J. http://dx.doi.org/10.1100/2012/562635.

FAO/WHO (Food and Agriculture Organization of the United Nations/World Health Organization), 2002. Guidelines for the Evaluation of Probiotics in Food. Report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food. April 30 and May 1. London Ontario, Canada. http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf.

Federation of Animal Sciences Societies (FASS), 1998. Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, First revised edition. pp. 129.

García-Hernández, Y., Pérez-Sánchez, T., Boucourt, R., Balcázar, J.L., Nicoli, J.R., Moreira-Silva, J., Rodríguez, Z., Fuertes, H., Nuñez, O., Albelo, N., Halaihel, N., 2016. Isolation, characterization and evaluation of probiotic lactic acid bacteria for potential use in animal production. Res. Vet. Sci. 108, 125–132.

Gibbens, J.C., Pascoe, S.J., Evans, S.J., Davies, R.H., Sayers, A.R., 2001. A trial of

biosecurity as a means to control *Campylobacter* infection of broiler chickens. Prev. Vet. Med. 48, 85–99.

Hardin, G., 1960. The competitive exclusion principle. Science 131, 1292–1297.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., Calder, P.C., Sanders, M.E., 2014. Expert consensus document: the International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat. Rev. Gastroenterol. Hepatol. 11, 506–514.

Ipek, A., Sahan, U., 2006. Effects of cold stress on broiler performance and ascites susceptibility. Asian Australas. J. Anim. Sci. 19 (5), 734–738.

Jayaraman, S., Thangavel, G., Kurian, H., Mani, R., Mukkalil, R., Chirakkal, H., 2013. *Bacillus subtilis* PB6 improves intestinal health of broiler chickens challenged with *Clostridium perfringens*-induced necrotic enteritis. Poult. Sci. 92, 370–374.

Julian, R.J., 2000. Physiological, management and environmental triggers of the ascites syndrome: a review. Avian Pathol. 6, 519–527.

Liu, X., Yan, H., Lv, L., Xu, Q., Yin, C., Zhang, K., Wang, P., Hu, J., 2012. Growth performance and meat quality of broiler chickens supplemented with *Bacillus licheniformis* in drinking water. Asian Australas. J. Anim. Sci. 25, 682–689.

Lyon, T.P., 1987. Probiotics: an alternative to antibiotics. Pig. News Info. 8, 157–164.

Marshall, B.M., Levy, S.B., 2011. Food animals and antimicrobials: impacts on human health. Clin. Microbiol. Rev. 24, 718–733.

Mookiah, S., Sieo, C.C., Ramasamy, K., Abdullah, N., Ho, Y.W., 2014. Effects of dietary prebiotics, probiotic and synbiotics on performance, caecal bacterial populations and caecal fermentation concentrations of broiler chickens. J. Sci. Food Agric. 94, 341–348.

Mountzouris, K.C., Tsirtsikos, P., Kalamara, E., Nitsch, S., Schatzmayr, G., Fegeros, K., 2007. Evaluation of the efficacy of a probiotic containing *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Pediococcus* strains in promoting broiler performance and modulating caecal microflora composition and metabolic activities. Poult. Sci. 86, 309–317.

Newell, D.G., Elvers, K.T., Dopfer, D., Hansson, I., Jones, P., James, S., Gittins, J., Stern, N.J., Davies, R., Connerton, I., Pearson, D., Salvat, G., Allen, V.M., 2011. Biosecurity-based interventions and strategies to reduce *Campylobacter* spp. on poultry farms. Appl. Environ. Microbiol. 77, 8605–8614.

O'Dea, E.E., Washington, D.C., Fasenko, G.M., Allison, G.E., Korver, D.R., Tannock, G.W., Guan, L.L., 2006. Investigating the effects of commercial probiotics on broiler chick quality and production efficiency. Poult. Sci. 85, 855–1863.

Olnood, C.G., Beski, S.S.M., Choct, M., Iji, P.A., 2015. Novel probiotics: their effects on growth performance, gut development, microbial community and activity of broiler chickens. Anim. Nutr. 1, 184–191.

Ozawa, K., Takeuchi, T., 1965. Research on drug resistant *Streptococcus faecalis*, II. The influence of artificial drug resistant *S. faecalis* on intestinal infection in mice treated with antibiotics. Jpn. J. Bacteriol. 20, 513–516.

Ozawa, K., Yabu-Uchi, K., Yamanaka, K., Yamashita, Y., Nomura, S., Oku, I., 1983. Effect of *Streptococcus faecalis* BIO-4R on intestinal flora of weanling piglets and calves. Int. J. Food Microbiol. 45, 1513–1518.

Pan, J.Q., Tan, X., Li, J.C., Sun, W.D., Wang, X.L., 2005. Effects of early feed restriction and cold temperature on lipid peroxidation, pulmonary vascular remodelling and ascites morbidity in broilers under normal and cold temperature. Br. Poult. Sci. 46, 374–381.

Patterson, J.A., Burkholder, K.M., 2003. Application of prebiotics and probiotics in poultry production. Poult. Sci. 82, 627–631.

Peighambardoust, S.H., Golshan, T., Hesari, J., 2011. Application of spray drying for preservation of lactic acid starter cultures: a review. Trends Food Sci. Technol. 22, 215–224.

Priyankarage, N., Silva, S.S.P., Gunaratne, S.P., Kothalawala, H., Palliyaguru, M.W.C.D., Gunawardana, G.A., 2003. Efficacy of probiotics and their effects on performance, carcase characteristics, intestinal microflora and *Salmonella* incidence in broilers. Br. Poult. Sci. 44, 26–27.

Ratti, C., 2001. Hot air freeze-drying of high-value foods: a review. J. Food Eng. 49, 319.

Ritzi, M.M., Abdelrahman, W., Mohnl, M., Dalloul, R.A., 2014. Effects of probiotics and application methods on performance and response of broiler chickens to an *Eimeria* challenge. Poult. Sci. 93, 2772–2778.

Rosmini, M.R., Sequeira, G., Guerrero Legarreta, I., Marti, L., Dalla Santina, R., Frizzo, L., Bonazza, J.C., 2004. Producción de probióticos para animales de abasto: importancia del uso de la microbiota intestinal indígena. Rev. Mex. Ing. Quim. 3, 187–197.

Sadeghi, A.A., Shawrang, P., Shakorzadeh, S., 2015. Immune response of *Salmonella* challenged broiler chickens fed diets containing Gallipro®, a *Bacillus subtilis* probiotic. Probiotics Antimicrob. Proteins 7, 24–30.

Salarmoini, M., Fooladi, M.H., 2011. Efficacy of *Lactobacillus acidophilus* as probiotic to improve broiler chicks performance. J. Agric. Sci. Technol. 13, 165–172.

Soto, L.P., 2010. Elección de un método de conservación que asegure la viabilidad de bacterias indígenas probióticas y mejore la efectividad de administración a terneros lactantes criados a campo. Tesis para la obtención del grado de Doctor en Ciencias Biológicas. Universidad Nacional del Litoral, Santa Fe, Argentina.

Trabelsi, I., Ktari, N., Ben Slima, S., Bouchaala, K., Ben Salah, R., 2016. Effects of supplementation with L. plantarum TN8 encapsulated in alginate-chitosan in broiler chickens. Int. J. Biol. Macromol. 89, 677–681.

Walter, J., 2008. Ecological role of lactobacilli in the gastrointestinal tract: implications for fundamental and biomedical research. Appl. Environ. Microbiol. 74 (16), 4985–4996.

Wang, L., Liu, C., Chen, M., Yac, T., Huang, W., Gao, P., Zhang, H., 2015. A novel *Lactobacillus plantarum* strain P-8 activates beneficial immune response of broiler chickens. Int. Immunopharmacol. 29, 901–907.

Wideman, R.F., Tackett, C., 2000. Cardio-pulmonary function in preascitic (hypoxemic) or normal broilers reared at warm or cold temperatures: effect of acute inhalation of 100% oxygen. Poult. Sci. 79, 257–264.

Zhang, B., Yang, X., Guo, Y.M., Long, F.Y., 2011. Effects of dietary lipids and *Clostridium butyricum* on the performance and the digestive tract of broiler chickens. Arch. Anim. Nutr. 65, 329–339.