Dietary *Lactobacillus acidophilus* positively influences growth performance, gut morphology, and gut microbiology in rurally reared chickens

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ABSTRACT In a market undergoing constant evolution, the production of chicken meat that consumers would perceive as "natural" and "animal friendly" is crucial. The use of probiotics in rurally reared chickens could represent a major opportunity to achieve mutual benefit for both the industry and consumers. A total of 264 male Kabir chicks were randomly distributed to one of 2 dietary treatments: the L group received a commercial feed supplemented with 2.0 g/100 kg of *Lactobacillus acidophilus* D2/CSL, while the C group received the same basal diet without the additive. To assess the effects of probiotic supplementation in the chickens' diet, productive performance was evaluated at d 21 and 42, whereas microbiological analyses of the intestinal content and intestinal histology and morphometry were performed at the end of the trial (d 42). At d 21 and 42, L birds showed better ($P < 0.001$) performance in terms of body weight, average daily gain, and feed conversion ratio. Enterococci, staphylococci, and *Escherichia coli* populations were not influenced by dietary treatment. On the contrary, *Lactobacillus* population increased ($P = 0.032$) in the L group. Furthermore, a tendency ($P = 0.069$) was observed for the coliforms to be influenced by diet, with lower values in the L group in comparison to the C group. Histological techniques revealed that the number of goblet cell containing neutral mucins was lower in the C group. Morphometric evaluations demonstrated that the probiotic supplementation increased the height of the mucosal layer by improving ($P = 0.040$) villus height, while crypt depth was unaffected. In conclusion, the results obtained in this study demonstrate that it is possible to use *Lactobacillus acidophilus* D2/CSL (CECT 4529) in rurally reared chicken breeds with positive effects on performance and gut health.

Key words: Feed Additive, Intestinal Histology, Microbiota, Probiotics, Slow-growing Chicken Genotype

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INTRODUCTION

The chicken meat market is undergoing constant evolution. Consumers are now showing a greater awareness and paying more attention to products that are perceived to be obtained in a "more natural" way (Samant and Seo, 2016). Various slow-growing chicken genotypes are available in Europe, and researchers have suggested that the quality of their meat is appropriate for a market characterized by an increased consumer awareness regarding animal husbandry and welfare (Castellini et al., 2002; Gordon and Charles, 2002).

In Italy, 85% of the total poultry industry is managed solely by a few integrated companies that cover the entire food chain of chicken meat, from the egg to the consumer (Nomisma, 2016). No statistics are available on the exact numbers of alternative broiler genotypes (slow-growing or certified broilers) in the EU; however, industry experts estimate the market share to be between 5 and 10% of the total production (EU Commission, 2016). Since the total economic balance of the poultry industry (2,850 million euro) gained more than 31% in 2015, in comparison to 2009, the importance of conducting studies in this field could be crucial.

Considering the economic relevance of the poultry meat industry and the increasing consumer demand for "natural" products, the use of probiotics could represent a major opportunity to achieve mutual benefit for both the industry and consumers. Different kinds of microorganisms can exert a probiotic effect when included in poultry diets. In the literature, data are available for

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Lactobacillus spp., *Streptococcus* spp., *Bacillus* spp., *Bifidobacterium* spp., *Enterococcus* spp., *Aspergillus* spp., *Candida* spp., *Saccharomyces* spp., and other microbial species. It is claimed that these strains positively affect growth performance (Smith, 2014), egg production and quality (Forte et al., 2016a), modulation of intestinal microflora and pathogen inhibition (Patterson and Burkholder, 2003), immunomodulation, and chicken meat quality (Mountzouris et al., 2007).

Lactobacilli are often considered in the formulation of probiotics. *Lactobacillus* is one of the predominant bacterial genera in the gastrointestinal tract of both humans and animals (Amit-Romach et al., 2004). Lactobacilli can be roughly divided into 2 metabolic groups: homofermentative, converting glucose to lactic acid, and heterofermentative, converting glucose to lactic acid, acetic acid, ethanol, and CO_2. These metabolites reduce intestinal lumen pH, creating an unfavorable environment for potential pathogenic bacteria (Axelsson, 2004; Menconi et al., 2011). *Lactobacillus* has been proven to exert a competitive exclusion effect on enterobacteria such as *Salmonella enterica* serovar Enteritidis in chickens (Penha Filho et al., 2015). Moreover, it positively affects the equilibrium of the gastrointestinal microbiota, increasing the presence of beneficial bacteria such as *Bifidobacterium* spp., and reducing potentially harmful bacteria such as *Escherichia coli*, clostridia, and staphylococci (Forte et al., 2016b).

The *Lactobacillus* genus includes about 200 species (Foschi et al., 2017) and is continuously evolving. Among these, *Lactobacillus acidophilus* D2/CSL is a bacterium isolated from the intestinal content of broilers (De Cesare et al., 2017), which is currently used as a probiotic in the egg production industry. Studies have demonstrated the efficacy of this particular probiotic in increasing antibody production against viruses such as Newcastle disease (Forte et al., 2016b). In broilers treated with *Lactobacillus acidophilus* D2/CSL, a positive effect was observed on productive performance and metabolic function, implying improved animal health (De Cesare et al., 2017).

To our knowledge, no studies have been previously performed to investigate the effects of *Lactobacillus acidophilus* D2/CSL on rurally reared chickens. The purpose of this study was to evaluate the effects of the dietary supplementation of *Lactobacillus acidophilus* D2/CSL (CECT 4529) on the productive performance of male chickens reared in conditions simulating small rural farming systems.

MATERIALS AND METHODS

Experimental Design

The experiment was conducted in a small farm of Umbria, Central Italy. A total of 264 day-old male Kabir chicks, obtained from the same hatching session, were used. At housing, all chicks were individually weighed and randomly distributed to one of the 2 dietary treatments. The chickens belonging to the L group received a commercial feed supplemented with 2.0 g/100 kg (20 g/ton) of *Lactobacillus acidophilus* D2/CSL (CECT 4529 - freeze-dried live cells), corresponding to a calculated dose of $1^{*}10^{9}$ CFU*kg^{-1}. The animals of the C group received the same basal diet without the additive. A starter diet (Diet 1) was administered until the chicks were 21 d old, whereas a grower-finisher diet (Diet 2) was given from 22 to 42 d of age. The 2 treatment groups (C and L), which consisted of 132 individuals per group, were divided into 6 replicates (pens-experimental units), each housing 22 birds. Only birds without any sign of illness and with a normal behavioral pattern were included in the trial. All the birds were vaccinated against IBV, Marek's and Newcastle diseases and coccidiosis in the hatchery. Pens (n = 12) were equipped with one plastic feeder and one plastic waterer each of identical manufacture, type, size, color, and any other notable physical feature. Fresh wood shavings were used as litter. Feed and water were provided ad libitum.

Observations for general flock condition, temperature, lighting, water, feed and mortality were recorded twice daily. The experimental protocol was in accordance with Guide for the Care and Use of Agricultural Animals in Research and Teaching (McGlone, 2010) and EU Directive 2010/63 (European Commission, 2010), and was approved by the Ethical Council of Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche.

Feed Analyses and Productive Performance

Samples of feed were collected twice per week for each dietary treatment (n = 12) and analyzed for chemical determination (Table 1) according to A.O.A.C. methods (1990), while EN 15787:2009 (Standards Centre, 2009) methods were applied for lactobacilli counts. The suggested dosage of the probiotic is 1×10^{9} CFU/kg feed and the commercial product contains 5×10^{10} CFU/g. The dosage that was used in this trial is 20 g/ton feed. The feed was supplied in mash form and ad libitum throughout the experiment.

Chicks were weighed at housing (d 1) and at d 21 and 42. Productive performance, average daily gain (**ADG**), average daily feed intake (**ADFI**) and feed conversion ratio (**FCR**) were calculated for each period (d 1 to 21 and d 21 to 42) as well as for the overall experiment (d 1 to slaughter), and were corrected for mortality.

Microbiological Analyses

At the end of the trial, samples of intestinal content (ileum, from Merkel's diverticulum to a point 40 mm proximal to the ileocecal junction) were collected from 3 different subjects for each replicate (n = 36). Analyses were conducted according to Forte et al. (2016b) with

Table 1. Ingredients and composition of the experimental diets.

	Starter (0–21 d)	Grower-finisher (21–42 d)
INGREDIENTS (g/100g)		
Corn	36.00	46.90
Soybean Meal	27.00	25.00
Corn Gluten Meal	19.60	15.10
Rice Middlings	–	5.10
Maize Distillers	9.00	–
Bran	–	4.00
Rice Bran	4.20	–
Soybean Oil	1.30	1.5
Calcium Carbonate	1.29	1.37
Dicalcium Phosphate	0.40	–
Salt	0.30	0.30
Sodium Bicarbonate	0.15	–
Vit-Min Premix[1]	0.76	0.73
CHEMICAL COMPOSITION (AS FED)		
Analyzed (%)		
Dry Matter	85.87	85.87
Protein	22.00	18.00
Lipid	5.00	5.00
Fiber	4.00	4.50
Ash	6.40	6.20
Calcium	1.10	1.00
Phosphorus	0.77	0.67
Lysine	1.16	0.79
Methionine	0.50	0.35
Calculated		
Metabolizable Energy (kcal/kg)	2837	2771

[1]Supplied per kilogram of diet: vitamin A, 12,500 I.U. (retinol); vitamin D3, 3,000 I.U.; vitamin E (tocopheryl acetate), 50 mg; vitamin K3, 2 mg; thiamine, 2 mg; riboflavin, 4 mg; pyridoxine, 1 mg; cyanocobalamin, 0.015 mg; pantothenic acid 15 mg; folic acid, 50 mg; biotin, 10 mg; choline chloride, 60; iodine, 3 mg; selenium, 20 mg; iron, 3 mg; manganese, 12, mg; copper, 1,5 mg; zinc, 5 mg.

some modifications. Replicate associated pool samples (n = 12) were obtained by mixing the samples collected from subjects belonging to the same replicate. An aliquot of 1 g was collected from each pool and diluted in tubes containing 2 mL of 0.9% sterile saline solution. Tubes were brought to volume (10 mL) with the same solution. Specimens were 10-fold serially diluted in 0.9% sterile saline solution, thus obtaining dilutions from 10^{-1} to 10^{-10} according to UNI EN ISO 6887–1. Chromogen Rapid'E.coli2/Agar (Bio-Rad Laboratories, Redmond, WA) and Slanetz-Bartley Agar (Oxoid Basingstoke, UK) were used for the enumeration of coliforms and enterococci, respectively. Mannitol salt agar (Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Perugia, Italy) was used for the enumeration of staphylococci. Plates were incubated aerobically at 37°C for 24–48 h and the colonies were then counted.

For the enumeration of sulfate-reducing anaerobic bacteria, TSC Agar (Tryptose Sulfite Cycloserine Agar—Oxoid Basingstoke, UK) enriched with egg yolk was used. Plates were incubated at 37°C for 24–48 h in anaerobic condition. Enumeration of lactobacilli was performed using Man Rogosa Sharpe (**MRS**) agar (Thermo Fisher Scientific, Cleveland, OH). Plates were incubated in microaerophilic conditions at 35°C for 72 hours. Results were expressed as log10 cfu/g.

Intestinal Histology and Morphometry

Samples of ileum (as described above, from Merkel's diverticulum to a point 40 mm proximal to the ileocecal junction) collected from 3 subjects per replicate (n = 36) were fixed in 10% neutral buffered formalin solution and processed for histology in accordance with standard procedures. Serial histological sections measuring 4 to 5 μm were subjected to hematoxylin and eosin (**HE**) and periodic acid-Schiff (**PAS**) stains. The criterion for villus selection was based on the presence of intact *lamina propria*; for each sample 5 intact villi, selected in triplicate for each intestinal cross-section, were included in the study. For the histochemical analysis, goblet cells taken from 5 randomly selected fields (40×) were counted. For morphometric evaluations, HE stained intestinal sections were used to evaluate villus height (**VH**, measured from the tip of the villus to the crypt-villus junction) and crypt depth (**CD**, measured from its base up to the crypt-villus transition region) as reported by Aliakbarpour et al. (2012). Muscular wall thickness was also recorded and analyzed. Images were digitalized using a Nikon DS-Fi1 digital camera (Nikon Corporation, Tokyo, Japan) connected to a Leica DMR microscope (Leica Microsystems, Milan, Italy), using NIS-Elements Br-2 as software as reported in Forte et al. (2016b).

Statistical Analysis

Data were analyzed using the General Lineal Model (**GLM**) procedure of SAS (JMP 9; SAS Institute, 2010). An analysis of variance (**ANOVA**) model was used with diet (C and L) as the fixed factor. The replicate effect did not prove to be statistically significant and was thus removed from the model. The differences of the means were defined using the Tukey test and considered significant when $P < 0.05$. Tendencies were discussed for P values between 0.090 and 0.051.

RESULTS

Lactobacilli count in feed was in line with expectations, resulting 1×10^6 CFU/g in L feed and 4.2×10^2 CFU/g in C feed. Mortality for the overall duration of the trial was always below values of 0.5% per pen. Post mortem examination did not evidence any significant result, as no pathological signs were found.

Results regarding performance are reported in Table 2. At 21 d of age L birds showed higher BW ($P < 0.001$) than C birds and showed better ($P < 0.05$) ADG and FCR (Table 2). Consistently with the initial results, at the end of trial (42 d) L birds showed higher ($P < 0.001$) BW in comparison to the C birds, as well as better ($P < 0.05$) ADG and FCR. The overall productive performance from 0 to 42 d (Table 2) revealed that the probiotic supplementation positively affected BW, average daily gain and feed conversion ratio of the birds ($P < 0.05$).

Table 2. Effect of dietary treatment on body weight (BW), average daily gain (ADG) and feed conversion efficiency (FCE).

Item	Sampling time	C	L	SEM	P
BW	T1	45.18	45.13	0.396	0.930
	T2	511.67 b	550.66 a	8.159	<0.001
	T3	1444.03 b	1636.40 a	18.845	<0.001
ADG	T1-T2	22.21 b	24.07 a	0.549	0.038
	T2-T3	44.39 b	51.70 a	1.082	<0.001
	OVERALL	34.97 b	39.78 a	0.0591	<0.001
FCE	T1-T2	2.57 a	2.31 b	0.052	0.018
	T2-T3	3.13 a	2.68 b	0.118	0.028
	OVERALL	2.94 a	2.56 b	0.079	<0.001

T1: d1; T2: d21; T3: d42

C: basal diet; L: basal diet supplemented with 2.0 g/100 kg (20 g/ton) of *Lactobacillus acidophilus* D2/CSL.

Different letters in the same row denote significant difference.

Table 3. Effect of *Lactobacillus acidophilus* supplemented diet on intestinal *Enterococcus* spp., *Staphylococcus* spp., *Escherichia coli*, Coliforms and *Lactobacillus* spp. populations (log10 cfu/g).

	Enterococcus spp.	*Staphylococcus* spp.	*Escherichia coli*	Coliforms	*Lactobacillus* spp.
C	6.310	5.923	6.330	5.783	5.820 b
L	6.005	5.657	6.447	5.080	6.438 a
SEM	0.183	0.231	0.274	0.244	0.176
P	0.255	0.434	0.769	0.069	0.032

C: basal diet; L: basal diet supplemented with 2.0 g/100 kg (20 g/ton) of *Lactobacillus acidophilus* D2/CSL.

Samples (n = 36) were collected from Merkel's diverticulum to a point 40 mm proximal to the ileocecal junction.

Different letters in the same column denote significant difference.

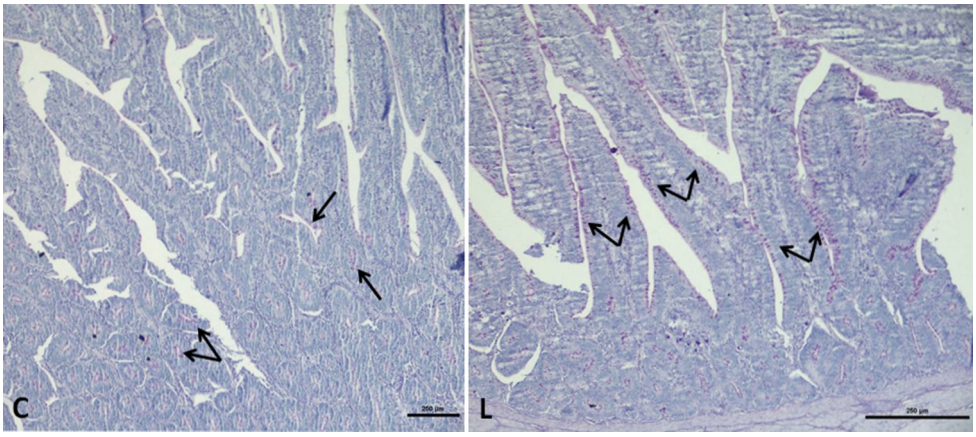

Figure 1. Duodenal sections from control (C) and treated (L) group. Goblet cells (arrows) appear as fuchsia spots (PAS stain. Bars 250 μm).

As for the microbiological evaluations, results are as shown in Table 3. Enterococci, staphylococci and *Escherichia coli* were not influenced by dietary treatment. On the contrary, *Lactobacillus* population increased ($P = 0.032$) in the L group. A tendency ($P = 0.069$) was observed for the coliforms to be influenced by diet, with lower values in the L group in comparison to the C group.

The PAS-stained ileal sections were assessed with histochemical methods, and the number of goblet cells containing neutral mucins was evaluated both in crypts and columnar epithelium on the ileum tract (Fig. 1). The number of these cells resulted lower in the control group ($P = 0.005$) compared to L group, as reported in Table 4. Morphometric evaluations of the ileum (Table 4) revealed that that the probiotic supplementation increased ($P = 0.041$) the height of the mucosal layer by improving ($P = 0.040$) villus height, while crypt depth was unaffected.

DISCUSSION

The supplementation of *Lactobacillus acidophilus* D2/CSL (CECT 4529) at the recommended dietary dosage of 1.0×10^9 CFU/kg of feed in rurally

Table 4. Effect of *Lactobacillus acidophilus* supplemented diets on intestinal histology and morphometry.

	Muscular wall thickness	Villus height	Crypt depth	Mucosal layer height	Goblet Cells Pas$^+$
C	433.27	2021.98 b	588.28	2610.27 b	66.62 b
L	491.54	2471.52 a	777.58	3249.11 a	84.18 a
SEM	48.801	146.61	80.087	209.25	4.044
P	0.406	0.040	0.107	0.041	0.005

C: basal diet; L: basal diet supplemented with 2.0 g/100 kg (20 g/ton) of *Lactobacillus acidophilus* D2/CSL.
Samples (n = 36) were collected from Merkel's diverticulum to a point 40 mm proximal to the ileocecal junction.
Pas$^+$: Periodic Acid Schiff-stain positive goblet cells.
Different letters in the same column denote significant difference.

reared chickens significantly improved performance (BW, weight increments and FCR) for the full length of the trial. The results are in line with those obtained in other studies that used *Lactobacillus acidophilus* alone (Salarmoiniand and Fooladi, 2011) or in combination with other *Lactobacillus* strains (Kalavathy et al., 2003; Smirnov et al., 2005; Pour and Kermanshahi, 2010; Shim et al., 2012; Zhang and Kim, 2014; Hossain et al., 2015). In particular, results acquired in the present study are in accordance with those obtained by Khan et al. (2007) using *Lactobacillus* strains in Kabir chickens. The positive effects observed in the performance of chickens included in the study are supported by the results obtained from microbiological, histological, and histochemical evaluations.

In 2017, De Cesare et al. observed comparable amounts of *Lactobacillus acidophilus* in the cecal contents of broilers fed either a control diet or a diet supplemented with the same probiotic strain used in the present study. The authors investigated the cecal microbiome, hypothesizing a probiotic effect resulting from changes in the environmental conditions of the intestinal lumen and a cross-feeding mechanism. Lu et al. (2003) demonstrated that chickens' gut microbiome varies from one intestinal segment to another; in particular, *Lactobacillus* species are abundant in the ileum, while in the ceca *Clostridiaceae* species are the most represented. *Lactobacillus acidophilus* D2/CSL is a probiotic isolated from the gastro-intestinal tract of healthy chickens (Bianchi Salvadori et al., 1985), therefore it has effective adhesive and multiplicative capacities in the chickens' enteric environment. It is possible to assume that the increase of the lactobacilli population observed in the present study, which takes into account all the *Lactobacillus* species of the ileum tract, could be the combined result of both an increase of the chickens' native intestinal lactobacilli and of the colonization of *Lactobacillus acidophilus* D2/CSL supplemented in feed.

When comparing studies regarding probiotics, it is essential to consider that mechanisms of action and beneficial effects are suggested to be specific for genus, species, and strain of the examined microorganisms (Timmerman et al., 2004). Furthermore, the variation of a probiotic's efficacy could be due to external experimental conditions, other than to the differences in the preparation itself (Bomba et al., 2002). Studies that employed *Lactobacillus acidophilus* as a monostrain probiotic are not abundant in literature. Results acquired from the microbiological evaluations of the present study are in accordance with Li et al. (2014). These authors, as well as Mookiah et al. (2014), hypothesize that, being part of the chickens' healthy gut microflora, *Lactobacillus acidophilus* supplemented via feed efficiently colonises the intestinal tract and exerts a competitive exclusion effect on pathogenic bacteria. It also creates a lower pH gut environment that inhibits the growth of pathogenic bacteria, yeast, and fungi. However, other studies conducted solely on *Lactobacillus acidophilus* did not report any significant effect on the lactobacilli population, and only partial effects on coliforms (Jin et al., 1998; Salarmoini and Fooladi, 2011). This demonstrates the complexity of probiotic mechanisms of action and interactions with the host. Olnood et al. (2015) investigated the effects of 4 different strains of *Lactobacillus* on the intestinal tract of broilers. They observed an increase in the lactobacilli population and a reduction of the population of *Enterobacteria* in the ileum and ceca of the treated subjects, which the authors attribute to the lactobacilli's production of antimicrobial substances such as volatile fatty acids, other organic acids, and bacteriocins. Watkins and Kratzer (1983; 1984) performed similar studies with *Lactobacillus* spp. based multistrain probiotics, but did not report significant variations in the lactobacilli and coliform populations. Likewise, studies employing multistrain probiotics containing *Lactobacillus acidophilus* and other microorganisms, showed mixed results. Some are in accordance with the present study (Smirnov et al., 2005; Shim et al., 2012, Zhang and Kim, 2014; Hossain et al., 2015), and others show no significant results regarding lactobacilli and coliform intestinal populations (Daskiran et al., 2012). The reason for the discrepancy between the results observed in the aforementioned studies could be related to the diversity of probiotic formulations (mono-species/mono-strain, or mono-species/multistrains, or multispecies, or even multigenera), administration methods (specific dosages in feed and/or in water), chicken genotypes and rearing systems taken into consideration, all of which may affect and thus make it difficult to compare the efficacy of different probiotic products (Mountzouris et al., 2007). Further studies characterized by a systematic approach and the use of advanced technologies will be needed in

order to fully comprehend the mechanisms of action of different probiotic strains and to better assess their use in poultry nutrition.

Histological methods demonstrated how *Lactobacillus acidophilus* supplementation can influence villus height, thus inducing small intestinal goblet cell hyperplasia. The present study shows changes in the mucosal architecture in terms of increased duodenal villus height and goblet cell number in the L group. The increase of villus height suggests the development of an increased surface area, capable of greater absorption of available nutrients (Chichlowski et al., 2007). Furthermore, probiotic supplementation induced goblet cell hyperplasia and mucin production. Mucins are synthetized in goblet cells and are then packaged into granules, transported to the cell surface and secreted into the lumen, hence contributing to the production of the mucous intestinal barrier, which acts as a dynamic protective surface (Kim and Khan, 2013). The results obtained in the present research are in line with studies in which intestinal morphology and mucin dynamics were evaluated (Smirnov et al., 2005; Awad et al., 2010; Wang et al., 2012), thus confirming the positive effects of *Lactobacillus* based probiotics on the intestinal ecosystem. It is important to take into consideration the fact that mucin synthesis might be challenging for the chickens in terms of amino acids consumption, but it is possible to hypothesize that the increased nutrient absorbent surface of the ileum may contribute to an increase of amino acids absorption. This would justify the increase of both mucin production and BW of chickens fed the *Lactobacillus acidophilus* D2/CSL supplemented diet.

In conclusion, the results obtained in this study demonstrate that it is possible to successfully use *Lactobacillus acidophilus* D2/CSL (CECT 4529) in rurally reared chicken breeds. The adaptability of *Lactobacillus acidophilus* D2/CSL to alternative rearing systems could provide the means to offer an innovative product that meets the demands of the new generation of environmentally aware consumers.

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