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Feed supplementation with avian *Propionibacterium acidipropionici* contributes to mucosa development in early stages of rearing broiler chickens

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RESEARCH ARTICLE

Abstract

Different studies in animal rearing claim the probiotic potential of species of the genus *Propionibacterium*. The effects of strains of *Propionibacterium acidipropionici* isolated from poultry intestine on microbiota activity and intestinal mucosa development were investigated in the early stage of rearing chicks and the safety of the dose used was investigated. The strains *P. acidipropionici* LET105 and LET107, administered as monoculture to chicks from the 1st to 14th day of life in a daily dose of 10^6 cfu/ml administered in the drinking water resulted harmless. The animals arrived at the expected weight for age and no differences were observed with respect to the food intake and water consumption related to control without bacteria administration. The analysis of microbiota composition revealed the presence of propionibacteria at the middle and end of the trial only in treated groups. Normal development of lactic acid bacteria and bifidobacteria, and slow colonisation by *Bacteroides* at the 7th day of the study was observed in the same groups. Analysis of the organic acids concentrations in the caecal content of birds revealed higher lactic acid and lower butyric acid production. Lower short chain fatty acids total concentration than expected during treatment was related to a better development of the gut mucosa. Increase in length of villus-crypt units, goblet cells counts and neutral mucins production were evidenced. Higher mucus secretion produced by dietary supplementation with propionibacteria could provide increased protection against pathogens.

Keywords: dairy propionibacteria, chicken, probiotic, microbiota, gut

1. Introduction

The genus *Propionibacterium* is represented by Gram positive, non-spore forming organisms, with fermentative metabolism, that inhabit environments either anaerobic or with low oxygen content. Based on their natural habitat, they are grouped as 'cutaneous propionibacteria', usually found in the skin and gastrointestinal tract of humans and animals, and 'classical or dairy propionibacteria', frequently isolated from raw milk, cheese, fermented vegetables and silage (Cummins and Johnson 1992; Von Freudenreich, and Orla-Jensen 1906). The 'classical group' species most frequently found in dairy and vegetal products are *Propionibacterium freudenreichii*, *Propionibacterium acidipropionici* and *Propionibacterium jensenii.*

Physiological characteristics of these propionibacteria have been extensively exploited for different industrial purposes, like Swiss type cheese elaboration, propionic acid production and biopreservation (Zárate *et al.*, 2011). Due to their long history of safe use in foodstuff for human consumption, classical or dairy propionibacteria have the status of Generally Recognised as Safe (GRAS) and, some species, the EFSA Qualified Presumption of Safety (QPS) (EFSA, 2012; Meile *et al.*, 2008).

Since long time ago, many studies in animal feeding have claimed the probiotic potential of species of this genus. At this respect, cultures of propionibacteria were used to improve the health and production of cattle beef and

pigs (Adams *et al.*, 2008; Cousin *et al.*, 2012; Vasconcelos *et al.*, 2008).

In the poultry industry, live bacteria administration has been a frequent rearing strategy to ensure the establishment of a safe intestinal microbiota in newly hatched chickens. However, the inclusion of dairy propionibacteria in protective cultures (Oyarzabal and Conner, 1996) has been limited and its contribution to the poultry health status has not fully characterised in monocultures. Only recently, Waititu *et al.* (2014) have demonstrated enhancement of the immunological status in chicken during administration of a strain of *P. acidipropionici* as a direct-fed microbial (DFMP).

Recently, *P. acidipropionici* and *Propionibacterium avidum* strains, the last belonging to the cutaneous group, were identified among isolates obtained from the large intestine and caecum of healthy hens and their physiological and functional features were studied (Argañaraz-Martínez *et al.*, 2013). They were characterised by their ability to resist the gastrointestinal digestion, produce propionic and acetic acid in the gut content and adhere *ex vivo* to the intestinal epithelium. In addition, one of the *P. acidipropionici* strains was able to exclude *Salmonella* Enteritidis in an *ex vivo* assay. Based on these findings and the advantage of the host specificity for the bacterial establishment in the intestine, we inferred that these strains of *P. acidipropionici* could be used as probiotics in the rearing of chickens.

Although the probiotic potential of autochthonous dairy propionibacteria is very promising, the safety of dose and effect of monocultures on intestinal colonisation by other microorganisms must be studied. The main goals of the current study was to evaluate the safety and effects on weight gain, gut maturation, organic acids production and microbiota behaviour at the minimum dose previously used with other probiotics for poultry.

2. Materials and methods

Experimental animals

Commercial Cobb broiler chicks (Indacor S.A., Córdoba, Argentina), vaccinated at hatch for Marek's disease, were used in this study. They were received 30 h after hatching in the animal facilities of Facultad de Bioquímica, Química y Farmacia – UNT. Upon arrival, chicks were weighed and housed in cages with 10 animals each in a separated room for birds. The room temperature was fixed at 30±2 °C and cycles of 14 h light and 10 h darkness were maintained during all the experiments. All chicks were fed a maize and soybean meal-based starter diet formulated according to NRC requirements (NRC, 1994) (Table 1), which was provided by 'El Colmenar' fodder (Tucumán, Argentina). The solid diet and drinking water were available *ad libitum*. The procedures after the chicks's reception, provision of feed and water, and daily care were similar for all the birds in every assay. Microbiological controls were regularly performed from feeds, drinkers and samples of cloaca swabs, by plate counting in Salmonella-Shigella agar (SSA; Britania, Buenos Aires, Argentina) and Mac Conkey agar media (Britania). The animal handling protocols of this investigation were adjusted to the 'Marco Ético de Referencia para las Investigaciones Biomédicas en Animales de Laboratorio, de Granja y Obtenidos de la Naturaleza' (Ethical Framework of Reference for Biomedical Research in Laboratory Animals, from Farm and Obtained from Nature), Resolution no. 1047/05 – Annex II of CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) – Argentina. All animal experimental procedures were approved by the Ethics Committee for Animal Studies of CERELA – CCT Tucumán (CONICET).

Table 1. Composition of conventional solid diet (g/kg).[1]

Ingredient	g/kg
Maize	630
Soybean pellet	204
Deactivated soybean meal	100
Meat meal	44
Calcium carbonate	10
NaCl	2.4
Vitamin-Mineral Nucleo[2]	2.0
DL-methionine	2.7
Choline chloride	2.3
L-Lysine HCl	2.6

[1] Nutritional composition (w/w %): crude protein 23; crude fibre 4; ether extract 3; calcium 1; phosphorus 0.40; minerals 3; humidity 12. Food provided by 'El Colmenar' fodder (Tucumán, Argentina).
[2] A mixture of vitamins and minerals from Rovimix Premix DSM Nutritional Products Argentina S.A. (Buenos Aires, Argentina) including: vitamins A, E, D, B, K, H; panthothenic acid, nicotinic acid, biotin, copper, cobalt, iodine, iron, manganese, selenium and zinc.

Bacterial strains and culture conditions

Two strains isolated from hens (Argañaraz-Martínez *et al.*, 2013), *P. acidipropionici* LET105 (accession number FN824485) and LET107 (FN824487), were selected for the present investigation. The strains were stored at -20 °C in 10% (w/v) reconstituted non-fat milk (NFM) supplemented with 20% (v/v) glycerol. Prior to use, the cultures were transferred to LAPTg broth (Raibaud, 1961) and activated by three successive transferences to fresh medium after incubation of 24 h at 37 °C. Drinking water was used as vehicle for strain administration in this study. 24-h cultures of each strain in LAPTg broth, were centrifuged, washed

twice with sterile saline solution and finally suspended in sterile tap water to 1-5×10^6 cfu/ml.

Trial 1. Safety assessment of propionibacteria cultures

Sixty chicks were separated in three equal groups of 20 birds each and fed with the same solid diet during 14 days. Chicks in Group 1 were provided of drinking water without bacterial supplementation (control group) while Groups 2 and 3 received water containing 1-5×10^6 cfu/ml *P. acidipropionici* LET105 or LET107, respectively. The drinking water was daily renewed in order to guarantee the viability of the suspended bacteria. It was replaced for sterile water 12 h before assessing intestinal parameters in the test groups. The safety of the dose used was assessed by studying the bacterial translocation to spleen and liver. On the 1st, 3rd, 7th and 14th day of the treatments, five animals of each group were sacrificed. Spleen and liver of each animal were removed aseptically, weighed and then disrupted in sterile saline solution. Samples were seeded in Mac Conkey agar to assess counts of enterobacteria after incubation at 37 °C for 24-48 h. The presence of intestinal anaerobic bacteria was investigated in Brain Heart Infusion agar (Britania) and modified lactate agar medium (Argañaraz-Martínez *et al.*, 2013) after incubation at 37 °C for 5 days in anoxic conditions (Anaerocult A, Merk KGaA, Germany) in an anaerobic jar (Oxoid, Basingstoke, UK). The gross appearance of the intestine, regression of the yolk sac, consistence of the gut content and presence of undigested food in the distal small intestine, were evaluated at naked eye.

Trial 2. Effects of the dietary supplementation on healthy status and intestinal development

Ninety chicks were weighed and randomly separated into three equal groups of 30 birds each, which were assigned to the same treatments described in the Trial 1. The groups were then divided into three subgroups of 10 animals each and used as replicate of each treatment. Body weight (BW) was assessed at days 0, 5, 7, 11 and 14 of the trial. Feed and water consumption and bird's mortality were registered daily for each group. Feeding efficiency was calculated as weight gain : intake ratio × 100. Five chicks randomly selected from each treatment group were sacrificed at the beginning of trial and at the 7th and 14th days. The water with the assigned probiotic suspension was withdrawn and changed by sterile water 12-h before the chicks were slaughtered at the 7th and 14th day. Caeca of each bird were collected aseptically, placed in a sterile Petri dish on ice, and immediately transported to the laboratory for counting microbial populations and assessing concentrations of metabolic products. Segments of ileum adjacent to caecal tonsils were dissected, flushed with cold sterile saline solution, opened longitudinally, and placed, mucosa side up, onto small pieces of blotting paper. The intestinal specimens were then fixed overnight at 4 °C with 10% (w/v) formaldehyde solution in phosphate-buffered saline (PBS) pH 7. After fixation, samples were dehydrated using a graded series of ethanol and xylene and then embedded in paraffin according to standard histological methods. At least 5 serial sections of 4 µm in thickness were cut from each block (Hyrax micrometer, Carl Zeiss, Oberkochen, Germany) and placed individually onto slides. After deparaffinisation with xylene and rehydration in a decreasing gradient of ethanol, samples were stained with haematoxylin/eosin and periodic acid-Schiff (PAS) (Biopur Diagnostics, Argentina) for histopathology and morphometric analysis. The stained samples were observed at 400× and 1000× magnification under light microscope (Axio Scope A1 microscope, Carl Zeiss). Ten well-oriented villus-crypt units were selected from different sections of each ileum sample. The number of epithelial cells and the number of goblet cells per villus-crypt unit were counted from a crypt to another. Length of the villus-crypt units was measured from the villus tip to the lamina propria by using the AxioVision Release 4.8 program of Carl Zeiss Imaging Systems. Mean values for the ileum sample of each animal were obtained and used to calculate the means ± standard deviation for each specific parameter in each group.

Enumeration of bacterial populations in the caecum

The caecal contents were squeezed into a pre-weighted sterile tube under a laminar flow cabin and diluted in pre-reduced sterile saline solution to obtain samples of 5% (w/v) concentration (Argañaraz-Martínez *et al.*, 2013). Half volume of these samples was used for enumeration of bacterial populations and the remnant was processed for determination of fermentation products. Samples for microbial enumeration were prepared following the protocol described by Lorenzo-Pisarello *et al.* (2010). Briefly an aliquot of 300 µl of caecal slurry was diluted in 900 µl of a cold solution of paraformaldehyde 4% (w/v) in PBS pH 7.2 and fixed for 16 h at 4 °C. After centrifugation (10,000×*g*, 10 min), samples were washed, suspended in 500 µl of a mixture of ethanol 96°: PBS (1:1) and finally stored at -20 °C until use. Total bacterial count per ml of caecal slurry was obtained by staining 50 µl of the appropriated dilution of fixed cells with 5 µl of DAPI solution (10 µg/µl) for 5 min in darkness. A standardised volume of each sample stained (15 µl) was placed on a slide, covered with a coverslip and observed at 1000× magnification in a fluorescence microscope (Axio Scope A1) using the appropriated filter for DAPI. The number of fluorescent cells was determined according to Lorenzo-Pisarello *et al.* (2010).

Fluorescent probes used for this study are listed in Table 2. Aliquots of 10 µl of cell fixed suspension of each sample were placed on previously defined positions of 10 mm in diameter in slides previously coated with gelatin ($KCr(SO_4)_2$ 0.01% (w/v), 0.1% (w/v) gelatin). They were dried at room

temperature for 30 min and dehydrated sequentially in 50°, 80° and 96° ethanol for 3 min. Slides for each probe and the corresponding positive and negative hybridisation controls (probes Eub 338 and Non 338, respectively) were prepared for the different samples. Protocols for hybridisation with probes Chis150, Lab158, Bif164, Bac303 and Pap446 were similar to those previously reported (Babot *et al.*, 2011; Depeint *et al.*, 2008; Franks *et al.*, 1998; Harmsen *et al.*, 2000; Langendijk *et al.*, 1995). After hybridisation, slides were observed at 1000× magnification with filters for 6-FAM and DAPI. At least 20 fields of each slide were randomly chosen for cells counting. The mean values obtained with specific and Eub probes were used to obtain the percentage of each population in the caecal microbiota. Total bacteria per gram and the percentage of each population were used to calculate the number of bacteria per gram of caecal content (Lorenzo-Pisarello *et al.*, 2010). Results were reported as log cells number/g of caecal content.

Caecal fermentation products

Samples from caecal slurries were centrifuged at 10,000×*g* for 10 min at 4 °C. One ml aliquots were deproteinised with H_2SO_4 (final concentration 0.1 M) at 4 °C for 15 min and centrifuged at 10,000×*g* for 10 min. Twenty µl of these samples were injected into HPLC system (Knauer, Berlin, Germany), equipped with Smartline pump 100, refractive index detector (K-2301; Knauer), smart line auto sampler AS 3800 plus and the ion-exclusion column BioRad Aminex HPX-87H (300×7.8 mm) (BioRad, Hercules, CA, USA).The samples were eluted with sulfuric acid 5 mM at flow rate of 0.6 ml/min. Acetic, propionic, butyric and lactic acids, and ethanol were used as standard solutions. Products concentrations were reported as µmol/g of caecal content.

Lectin binding on sugar residues in the gut mucin

Sections obtained as described above were prepared for lectins binding as reported by Gheri *et al.* (1999), modified. Briefly, slides were put on PBS/bovine serum albumin (BSA) 2.5% (w/v) overnight followed incubation with fluorescein-5-isothiocyanate (FITC) labelled wheat germ agglutinin (WGA) (*Triticum vulgare*, binding specificity [α-D-GlcNAc] n, 5 µg/ml) or *Ulex europaeus* agglutinin I (UEA I) (binding specificity [α (1,2)-fucose], 10 µg/ml) for 3 h in the dark at room temperature. The slides were washed three times with PBS/BSA 2.5% (w/v). The stained samples were observed at 400× magnification in a fluorescence microscope (Axio Scope A1) using the appropriated filter for FITC. Three sections obtained from each animal intestine were incubated with FITC labelled lectins. The fluorescence intensity was determined semiquantitatively by three different operators who rated the fluorescence and arrangement on tissue as follow: (-) no signal, (+) weak [outline of the villi and a few goblet cells stained], (++) moderate [outline of the villi and most goblet cells stained] and (+++) intense signals [outline of the villi and majority of goblet cells intensely stained]. Images were process by Software Release 4.8 AxioVision.

Statistical analysis

Results were expressed as mean ± standard deviation and were compared by one-way analysis of variance (ANOVA). Tukey's test was used to identify statistically significant differences ($P<0.05$). These analyses were carried out using statistical software (Origin Pro 8.0; OriginLab, Northampton, MA, USA).

3. Results

Safety of *Propionibacterium* strains

Agar plates seeded with liver and spleen homogenates were compared after incubation. As these organs are naturally sterile, absence of colonies in agar plates was the expected result for animals analysed from the control group. The same result in agar plates obtained from control and treated groups was assumed as indicative of negative translocation in the test groups of birds. Chicks of groups treated with propionibacteria evidenced negative translocation at the dose used in this study. The intestinal mucosa was observed by naked eye after necropsy. No changes pathognomonic of disease or damage in different segments of intestine and bursa were observed. The regression of the yolk sac was the expected for the age of the animal. The intestinal contents showed the normal consistence.

Table 2. 16S rRNA-targeted oligonucleotide probes.

Probe	Sequence (5'à3')	Target organisms	Reference
Eub338	GCTGCCTCCCGTAGGAGT	*Bacteria*	Amann *et al.*, 1990
Non338	ACATCCTACGGGAGGC	Negative control	Wallner *et al.*, 1993
Bac303	CCAATGTGGGGGACCTT	*Bacteroides* spp., *Prevotella* spp.	Jansen *et al.*, 1999
Chis150	TTATGCGGTATTAATCTYCCTTT	*Clostridium histolyticum* subgroup	Franks *et al.*, 1998
Lab158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus* spp., *Enterococcus* spp.	Harmsen *et al.*, 2000
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.	Langendijk *et al.*, 1995
Pap446	ACACCCCAAAACGATGCCTTCGCC	*Propionibacterium acidipropionici*	Lorenzo-Pisarello *et al.*, 2010

Chicken weight and feed intake

In Trial 2, the live BW of each chick was measured at the days 0, 5, 7, 11 and 14 of the trial (Table 3). The mean values of weight at the end of feeding were 413.78±21.96, 387.33±4.72 and 380.18±19.90 for animals of groups control and groups receiving the strains LET105 and LET107, respectively. Moreover, weight gain, feed intake and efficiency values (weight gain : feed intake ratio %) were similar to control group at the end of the trial (Table 3). The death of only one animal from the control group was observed at the 7th day of the trial.

Changes on the main populations of the caecum during the feeding trial

The study of the caecal microbiota was conducted by the fluorescence *in situ* hybridisation technique. At 30 h of life the animals showed a microbiota predominantly of lactic acid bacteria, with counts of lactobacilli and enterococci of 8.41±0.39 and 9.13±0.38 log cells number/g, respectively (Figure 1A). Regarding the anaerobic population, counts of 7.71±0.38, 8.20±0.32 and 7.92±0.14 log cells number/g were obtained for clostridia, *Bacteroides* and bifidobacteria, respectively.

At the end of the first week of the assay, there was a shift in lactobacilli and enterococci populations, as their relationship was reversed respect to the beginning of treatment; they reached counts of 9.32±0.39 and 8.15±0.46 log cells number/g, respectively (Figure 1A). Clostridia increased in all groups in an average of 0.78 log units, in comparing to the initial day (30 h post-hatch). *Bacteroides* population remained close to 8 log cells number/g, with slightly lower counts in the treated groups than in control. Bifidobacteria exhibited an average increase of 0.41 log units in comparing to the initial microbiota both in the control and treated group with *P. acidipropionici* LET105. Groups receiving supplements of *Propionibacterium* showed counts over than 7.5 log cells number/g for this genus. Propionibacteria were not detected neither at the beginning of the treatment nor in control group.

At the end of treatments, lactobacilli and enterococci were in lower concentration than in the 7th day (Figure 1B) in all groups. Counts of these populations in treated groups were not different from control, which reached mean values of 8.4 and 7.8 log cells number/g for lactobacilli and enterococci, respectively. There was also a decrease in counts of clostridia on the 14th day with respect to the 7th day in all the feeding groups. *Bacteroides* counts were in the same level than the recorded on the 7th day in groups treated with *P. acidipropionici* LET105 and LET107, and differences between treated groups and control were not significant. Bifidobacteria increased on 14th day respect to 7th day in animals treated with *P. acidipropionici* LET107 to levels of 7.95±0.93 log cells number/g, while the others groups showed similar values each other with a marked decrease with respect to day 7 (Figure 1B). Propionibacteria were only evidenced and counted in the treated groups, as in the 7th day, and maintained in a level over than 7.3 log cells number/g.

Table 3. Body weight (BW) and feed intake in control and treated groups.

	Day	Control	*Propionibacterium acidipropionici* strains	
			LET 105	LET 107
BW[1] (g/bird)	0	42.53±2.52	43.09±4.15	41.46±4.95
	5	108.68±7.78	105.01±1.04	104.53±6.38
	7	163.45±4.76	144.76±9.95	148.88±9.71
	11	302.17±16.93	269.67±8.01	279.50±18.38
	14	413.78±21.96	387.33±4.72	380.18±19.90
BW gain[2] (g/bird)	14	371.25±19.31	344.92±3.34	341.54±20.89
Feed intake[3] (g/bird)	14	612.99±62.80	567.36±8.13	551.18±52.32
Efficiency[4] (%)	14	60.80±3.72	60.80±0.63	62.07±2.10

[1] BW of all the birds was assessed at days 0, 5, 7, 11 and 14 and values of weight per bird were calculated for each replicate of the treatment groups. Results are mean values ± standard deviation of replicates (n=3).

[2] BW gain was calculated at the 14th day as the difference of final and initial BW per bird in each replicate of the treatment groups. Results are mean values ± standard deviation of replicates (n=3).

[3] The consumed feed was determined daily in each cage and was registered as gram of consumed feed per bird. The cumulative intake per bird for each replicate was calculated at the 14th day. Results are mean values ± SD of replicates (n=3). The same procedure was used for all the groups of treatment.

[4] Efficiency (%) was assessed as the BW gain (g) : cumulative feed intake (g) ratio × 100 in each group of treatment.

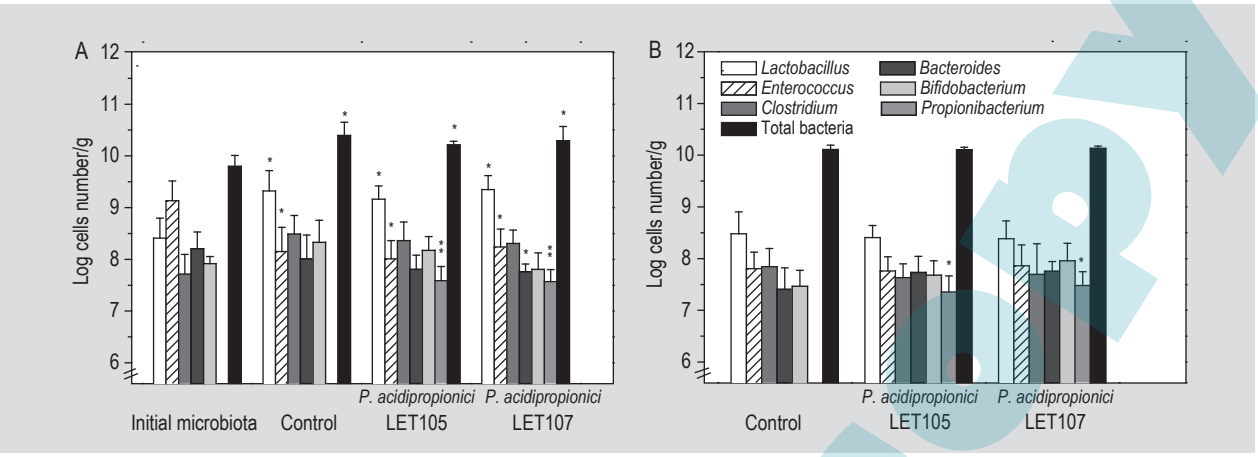

Figure 1. Caecal microbiota of animals fed with a conventional diet (control) and animals receiving a daily dose of 10^6 cfu/ml of *Propionibacterium acidipropionici* LET105 or LET107 in the drinking water for 7 days, compared with the initial microbiota at 30 h after hatching (A) and caecal microbiota of the same groups at 14 days (B) using genus specific probes. Results of the log cells number per gram of caecal content are expressed as means ± standard deviation. One asterisk indicates significant differences with respect to counts at the beginning of the trial and two asterisks represent differences with respect to Control group ($P \leq 0.05$).

Fermentation products in the caecal contents

Caecal homogenates of animals sacrificed at the beginning of trial (day 0) were used to establish baseline levels of organic acids. Lactic acid was in a value of 8.04±2.68 µmol/g and only trace amount of other acids was detected (data not shown). At 7th day of assay, the mean of short chain fatty acids (SCFA) concentration was lower in treated groups than in control but the difference was significant only in the group that received strain LET107 (Figure 2A). No statistical difference in the level of acetic acid among the three groups was observed although its concentration was moderately lower in the treated groups. Propionic acid was significantly higher in the group receiving strain LET105 (2.05±1.24 µmol/g) while butyric acid was in higher concentration in the control group (8.22±0.11 µmol/g) than in the others. Lactic acid showed values higher than those recorded at the start of trial in all groups, mainly in treated groups, but differences were not significant considering the high variability between animals of a same group. Ethanol production by caecal microbiota was higher in the control group (21.08±7.95 µmol/g) than in other ones (Figure 2A).

On the 14th day of feeding, total SCFA levels increased in all groups related to the 7th day (Figure 2B). SCFA were moderately higher in the group fed with strain LET105 than in the control group, but significantly higher than in the group treated with strain LET107. The main SCFA in all groups was acetic acid; its concentration was the highest in the group that received strain LET105 than in the others, but only the group treated with LET107 showed significantly lower concentration of acetic acid. Notable reduction in ethanol and lactic acid concentration was observed in the control group of chicken in the 14th related to the 7th day. On the contrary, total SCFA and acetic acid concentrations were higher at the end of the trial.

The groups receiving propionibacteria as dietary supplement, showed a decline in lactic acid concentration with respect to the 7th day but the values obtained were higher than in the control group (Figure 2B). Consistent with these reductions was an increment in SCFA and acetic acid at the 14th day, although this was less noticeable in chickens receiving strain LET107.

Development of gut mucosa

The villus-crypt unit of control samples reached 290.4±28.4 µm of length at the 7th day of trial (Table 4). The group treated with *P. acidipropionici* LET105 showed a significantly longer crypt-villus unit than control (349.1±30.8 µm) while in the group treated with *P. acidipropionici* LET107 there was a moderate increase in the length. Accordingly, the total number of cells in the crypt-villus unit showed significant differences in the group receiving the LET105 strain. Besides, both treated groups showed increase in the number of goblet cells compared to the control, being the highest in the group treated with LET105.

At the 14th day of the experiment, the length of the crypt-villus unit in all groups reached mean values higher than at day 7 (Table 4). Values in both treated groups (473.37±53.76 and 493.52±30.40 µm for LET105 and LET107, respectively) were higher than control (410.53±45.27 µm); the group receiving *P. acidipropionici* LET 107 being significantly different. The number of goblet cells increased significantly related to control only in the group treated with *P. acidipropionici* LET105.

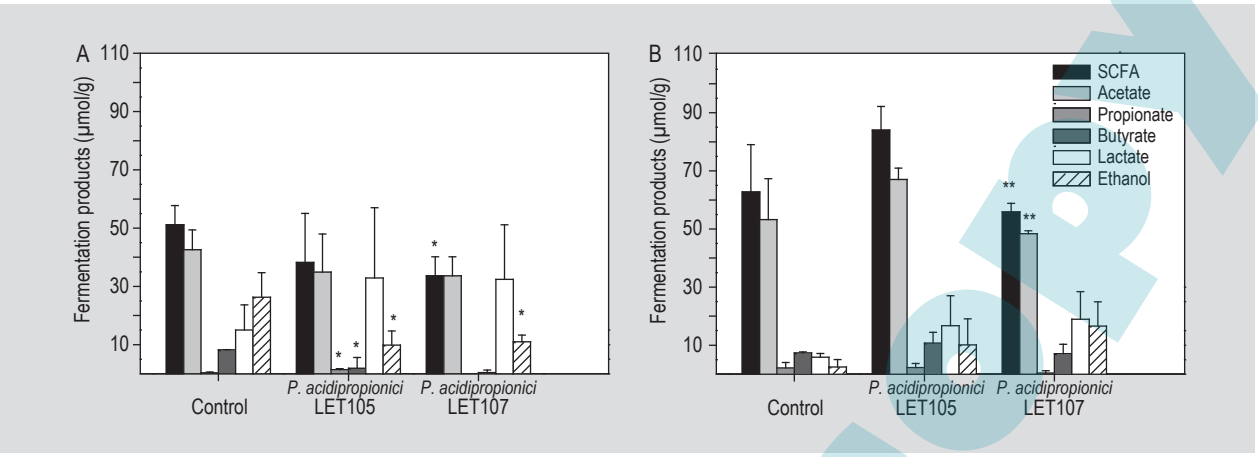

Figure 2. Caecal fermentation products from animals receiving a daily dose of 10^6 cfu/ml of *Propionibacterium acidipropionici* LET105 or LET107 in the drinking water for 7 (A) and 14 (B) days. Results of µmol/g caecal content of total short chain fatty acids (SCFA), acetic, propionic, butyric and lactic acid and ethanol are expressed as means ± standard deviation. One asterisk indicates significant differences with respect to Control and two asterisks represent differences between treated groups ($P \leq 0.05$).

Table 4. Villus-crypt unit length, total cells and goblet cells numbers during treatments[1].

	Control	*P. acidipropionici* LET 105	*P. acidipropionici* LET 107
Broilers at the 7th day			
Villus-crypt unit length (µm)	290.35±28.35	349.09±30.77 [a]	308.13±22.31
Total cells number	92.20±10.09	126.80±8.75 [a]	106.30±12.86
Goblet cells number	8.90±2.13	25.00±5.81 [a]	18.10±2.73 [a]
Broilers at the 14th day			
Villus-crypt unit length (µm)	410.53±45.27 [b]	473.37±53.76 [a b]	493.52±30.40 [a]
Total cells number	173.60±35.51	180.20±27.14	196.90±14.96
Goblet cells number	27.60±5.40 [b]	38.10±9.69 [a]	35.70±10.71 [a b]

[1] Means values with different superscript letters within the same row differ significantly ($P \leq 0.05$).

Lectin assay

To determine if *P. acidipropionici* strains influence the expression of glycoconjugates in the gut mucus, labelling with WGA and UEA I-FITC lectins was assayed. UEA I agglutinin was detected in all groups at 7th and 14th day of the trial. Similar intensity and pattern, with a weak signal in the outline of the intestinal villi and in some cases within the goblet cells, was observed at both sampling times in all feeding groups (Table 5). On the other hand, different patterns of binding WGA in control and treated groups were observed. Animals that received *P. acidipropionici* strains showed fluorescence of moderate intensity in the outline of villi and within goblet cells at 7th day, while a weak and scarce signal on villi and goblet cells was observed in the control group. At day 14 in the treated groups, mucus on the outline of the intestinal villi and filled and intensely stained goblet cells were observed (Figure 3). This was not the case of control group where a weak signal pattern similar to that of UEA was evidenced.

Table 5. Lectin binding on goblet cell sugar residues in the gut of broilers fed *Propionibacterium acidipropionici*.[1,2]

	Control	*P. acidipropionici* LET 105	*P. acidipropionici* LET 107
Broilers at the 7th day			
UEA I [α(1,2)-fucose]	+	+	+
WGA [α-D-GlcNAc]	+	++	++
Broilers at the 14th day			
UEA I [α(1,2)-fucose]	+	+	+
WGA [GlcNac]	++	+++	+++

[1] Relative intensity of fluorescent: +, weak; ++, moderate; +++, intense signals. Carbohydrates binding are indicated below each lectin.
[2] WGA = wheat germ agglutinin; UAE 1 = *Ulex europaeus* agglutinin I.

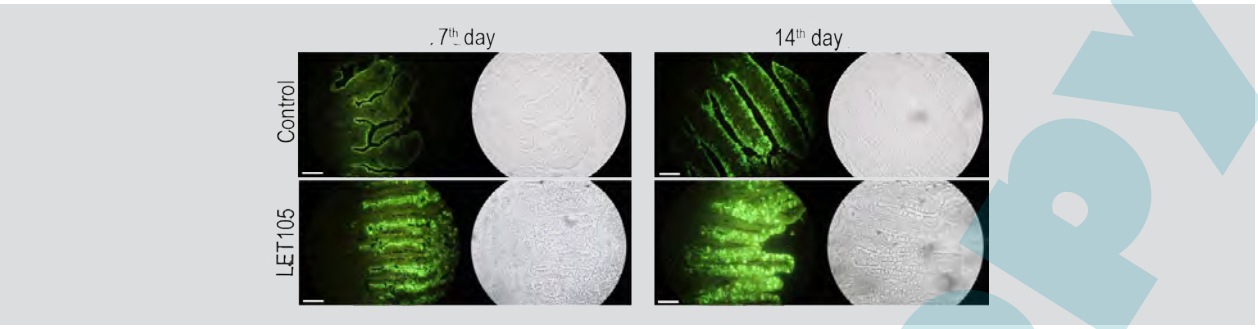

Figure 3. Epifluorescence microscope images (magnification: 400×) of the intestinal ileal mucosa of *Propionibacterium acidipropionici* LET105 and control groups at 7th and 14th days staining by FITC-labelled wheat germ agglutinin, as representative experiment, are shown. Note the difference in the signal intensity through the days on outline villi and goblet cells. Scale bar = 100 µm.

4. Discussion

The gut microbiota of poultry plays a critical role in the performance and health of the host and is implicitly associated with feed conversion rate, weight gain and vulnerability to infection by pathogens microorganisms. The establishment of a stable and safe microbiota, as well as suitable rearing conditions, is needed to reach an upgrade in the poultry production.

Recently, we have focused the study of poultry microbial populations on the species of propionibacteria present in laying hens. Propionibacteria were found in the intestine of hens in counts of 4-5 log cfu/g caecal content and *P. acidipropionici* and *P. avidum* were the only species identified (Argañaraz-Martínez *et al.*, 2013). Some characteristics desirable in probiotic strains were attributed to strains of *P. acidipropionici* isolated in that study, and assays to determine safety of the dose and behaviour of these bacteria in chicks intestine when used as bacterial supplements were carried out in the present investigation.

The response to probiotics administration has demonstrated to be dose dependent (Stein *et al.*, 2006). In the present work two autochthonous strains of *P. acidipropionici* were tested at a dose of 1-5×10^6 cfu/ml in the drinking water to assess their safety and effects on intestinal microbial populations, organic acids production and gut maturation, from birth until 2 weeks of life. This level of bacteria administration was near to the minimum used with other probiotic strains.

Food safety is the most important criterion to consider in developing new foods and supplements (AAFCO, 1999); so the microorganisms to be used as DFM or probiotics must be able to meet safety standards (Saarela *et al.*, 2002). Bacterial translocation is the first step in pathogenesis processes for opportunistic microorganisms found in the intestinal lumen and the result of translocation could provide information on the ability to infect or developing a disease (Steffen and Berg, 1983). Therefore, bacterial translocation is currently recommended as indicator of the security degree of any microorganism with potential probiotics characteristics (Zhou *et al.*, 2000). In this work, the strains *P. acidipropionici* LET105 and LET107 were not able to leave from the intestinal lumen to other organs, to induce translocation of other intestinal bacteria, or produce pathognomonic signs associated to diseases. Furthermore, no damages were observed in the intestine or bursa and the yolk sac was in regression as expected for chicks of 14 days after feeding with LET105 and LET107 strains (Uni *et al.*, 1998). These last parameters are of importance because they are responsible for death during poultry rearing. Moreover, no significant difference was observed in weight gain, food intake or feeding efficiency among groups with *Propionibacterium* supplementation and the control one during the first 14 days of rearing. Hence, we considered that both strains of *P. acidipropionici* are safe for use in the dose tested. Improvement of the growth performance by probiotics administration has been observed after longer feeding periods than the used in our investigations. Indeed, Mountzouris *et al.* (2010) reported increased broiler body weight during the grower (15 to 21 days) and finisher (22 to 42 days) phase of growth but not during the starter phase (1 to 14 days) in birds that ingested a 5-species probiotic product. Recently, Peng *et al.* (2016) reported improved average daily weight and feed conversion ratio during the finisher or the entire growth period compared to the starter phase, by the dietary supplementation with *Lactobacillus plantarum* B1. Our results were in agreement with these reports, although greater number of birds and extended periods of feeding will be required to investigate the effect of propionibacteria on the growth performance of poultry. Both strains used in the present investigation exhibited a remarkable tolerance to *in vitro* gastrointestinal digestion and ability to develop in a natural medium of caecal water in our previous studies (Argañaraz-Martínez *et al.*, 2013). Therefore, it is expected that strains reaching the ileum and cecum remain alive to interact with the resident microbiota and the intestinal epithelium. Counts of propionibacteria in the caecum during the experiments confirmed the viability of both strains during the feeding period evaluated.

Previous reports on the intestinal colonisation of chicks indicated that microbiota reached 8 and 10 log cfu/g in the ileum and caecum at 24 h, respectively. It increased to 9 and 11 log cfu/g at 72 h and remained relatively stable during the following 30 days (Apajalahti *et al.*, 2004). In agreement with these reports, in the present investigation the initial microbiota reached values higher than 9.5 log number of cells/g with lactobacilli and enterococci being the predominant bacteria. Simultaneously, lactic acid was detected in the caecum of these animals as it is the main acid product of these genera.

SCFA production is particularly important for maintaining bird health, not only to attend the strong demand for energy by the gut for its development, but also participates in the recycling of inorganic ions through the improvement of transport in the caecum (Rice and Skadhauge, 1982). On 7th day of trial, the concentrations of SCFA increased in the control and to a lesser extent in the experimental groups, but were lower than the levels reported for adult animal (Pourabedin *et al.,* 2015; Van der Wielen *et al.*, 2000). Propionic and butyric acids are products of a strictly anaerobic microbiota that began to colonise the gut at this stage. Therefore, it is possible to infer that the competition between anaerobic populations during the establishment of propionibacteria delayed the production of these organic acids as Józefiak *et al.* (2004) reported. Moreover, sharp increase was also observed for lactic acid and ethanol, fermentation products of lactic acid bacteria that remained in high counts. These data are coincident with Van der Wielen *et al.* (2000) that showed that lactobacilli and enterococci are present in large numbers during the first days of life. Interestingly, propionibacteria were found in low number in the intestine of adult hens without probiotics administration (Argañaraz Martínez *et al.*, 2013) and detected only after an enrichment of faecal shedding, during supplementation with *P. jensenii* 702 (Luo *et al.*, 2010), suggesting a strong competition with other anaerobes that limits the development of this genus in adult birds. This could be overcome by early administration of propionibacteria during colonisation of the anaerobic populations. Indeed, *P. acidipropionici* was detected in treatment groups of this investigation in over than 7 log number cells/g during the first two weeks of life, reaching comparable values to other anaerobic bacteria studied in the trial.

Changes in SCFA profiles were observed at the end of the experiment. A significant reduction in lactic acid in the treated groups compared to the 7th day was observed, apparently due to consumption by propionibacteria and other anaerobic bacteria. However, no accumulation of propionic acid was observed. This could be due to the constant absorption of this acid by the intestinal mucosa as was informed by some researchers (Kripke *et al.*, 1989) and the strong competition established among anaerobic populations producers of propionic acid like propionibacteria, clostridia and *Bacteroides* which are not fully established in the ecosystem until 14th day (Lumpkins *et al.*, 2010). During the partial replaced of these populations, it is not expected a major change in propionic acid concentration in caecum. Lower concentrations of butyric acid, produced mainly by clostridia and not by propionibacteria, were observed in the treated groups than in control, indicating a partial displacement the clostridia by propionibacteria in these groups of feeding. This was confirmed for higher counts of clostridia in the control with respect to the groups treated with propionibacteria found in the assay. All these changes on the intestinal microbiota composition tend to maintain a dynamic equilibrium while the main functions remain unalterable (Pan and Yu, 2014). This was consistent with the count of the total microbiota that remained constant.

The gastrointestinal tract of poultry is an organ that develops rapidly in constant interaction with the resident microbiota. Fermentation products as SCFA are source of energy for enterocytes and responsible in part of mucosa growth (Fukunaga *et al.,* 2003). In the present study, supplementation with dairy propionibacteria during poultry rearing induced changes in the gastrointestinal tract of chickens. At the first week of treatment length of the crypt-villus units and epithelial and goblet cells numbers differed from the control. This was mainly observed in the group treated with *P. acidipropionici* LET105, and was coincident with the lower concentration of SCFA showed in the caecal content of treated groups, suggesting acids absorption from lumen to contribute to the nutrition of the gut. On the 14th day the length of the villus-crypt units of control birds increased and reached values similar to the reported by Forder *et al.* (2007), although it was significantly lower than in the group treated with *P. acidipropionici* LET107.

No difference in total cell number was observed between groups, while the number of goblet cells was higher in birds treated with *P. acidipropionici* LET105. Comparable effects were obtained by Awad *et al.* (2009) in feeding broilers with a direct fed microbial which contained *Lactobacillus* sp., and by Chae *et al.* (2012) when administered a supplemented diet with *Lactobacillus acidophilus*, *Bacillus subtilis* or *Saccharomyces cerevisiae.* Pan and Yu (2014) attributed these morphological changes to the microbiota composition induced by the feed supplements.

On the other hand, the effects of microbiota on gut maturation in poultry were studied by Uni *et al.* (2003) and Forder *et al.* (2007). They found that neutral mucins in goblet cells are present from the first day of life and increase over time in the ileum. This differs from mammalian models, where the number of goblet cells with neutral mucin in ileum is very low or scarce at birth (Deplancke and Gaskins, 2001). Neutral mucin

production by goblet cells and subsequent release for the mucus layer formation, acts as a protective mechanism against invasion of pathogens (Dean-Nystrom and Samuel, 1994; Runnels *et al.*, 1980). As shown by us, more neutral mucins, PAS stained, were produced by goblet cells during propionibacteria administration. This leads us to believe that birds consuming *P. acidipropionici* strains are more protected against opportunistic infections than chicks of the control group.

To understand the role of neutral mucin, FITC-labelled lectins were used to detect the type of glycosylation in the mucin secreted by goblet cells during the trial. Mucins are glycoproteins composed of backbone peptide domains containing alternate glycosylated and unglycosylated regions. Glycosylated regions comprise 60 to 80% of the polymer. *N*-acetyl-glucosamine (GLcNAc), *N*-acetyl-galactosamine, galactose and fucose (Fuc) are the four major components of oligosaccharides of neutral mucin (Deplancke *et al.,* 2001). In this work, we focus on the residues of higher and lesser proportion in the mucin from ileum using WGA and UEA I-FITC labelled lectins to bind GlcNac and Fuc, respectively. In a feeding trial with probiotics, Tsirtsikos *et al.* (2012) revealed that molar ratios of the monosaccharides GlcNAc and Fuc on ileal mucin depends on the inclusion level of probiotics.

In this work the administration of propionibacteria induced or improved the expression of GlcNAc, being remarkable in the *P. acidipropionici* LET105 group. On the other hand, despite that Fuc residues are considered abundant in mucus they are in low molar rate which is not influenced by bacterial colonisation (Tsirtsikos *et al.,* 2012), which is also evidenced in the present investigation. Different researchers informed the effect of microbiota and probiotic on mucin expression in poultry gut (Forder *et al.,* 2007; Smirnov *et al.,* 2005; Struwe *et al.,* 2015; Tsirtsikos *et al.,* 2012), however to the best our knowledge, this is the first report of dairy propionibacteria action on the growth of the intestinal mucosa, mucus production and mucins glycosylation. Future research may reveal the mechanism by which dairy propionibacteria interact with the poultry gut mucosa inducing changes in mucus composition.

5. Conclusions

The results obtained in this investigation highlight the potential of dairy *Propionibacterium* strains isolated from poultry as probiotic cultures intended for rearing broilers chickens. Feeding with these bacterial supplements was safe for newly hatched chickens, and contributed to the microbiota modulation, early epithelial development, mucus production and neutral mucins expression in the intestine. Considering the role of mucins in the intestinal barrier function and their enhanced expression during the propionibacteria supplementation, it may be expected an increased protection against enteric pathogens in the rearing of birds. However, further investigations are necessary to elucidate the ability of this genus to upgrade the productive parameters for the poultry industry.

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