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Physiological and functional characteristics of *Propionibacterium* strains of the poultry microbiota and relevance for the development of probiotic products

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ABSTRACT

The prevention and control of pathogens colonization through probiotics administration in poultry feeding is of increasing interest. The genus *Propionibacterium* is an attractive candidate for the development of probiotic cultures as they produce short chain fatty acids (SCFA) by carbohydrates fermentation. The presence of strains of this genus in hens of conventional production systems and backyard hens was investigated. Propionibacteria were isolated from the intestine and identified by physiological and biochemical tests. PCR amplification of the 16S rRNA gene of the isolates was performed and products were compared with sequences from databases. The presence of the genus *Propionibacterium* was demonstrated in 26% of hens and *Propionibacterium acidipropionici* and *Propionibacterium avidum* were the identified species. A comparative study of their physiological and functional characteristics was performed. *P. acidipropionici* strains were the most resistant to *in vitro* gastrointestinal digestion, but the adhesion to intestinal tissue was strain dependent. Some differences were found between both species with respect to their growth and SCFA production in an *in vitro* cecal water model, but all the strains were metabolically active. The production of SCFA in cecal slurries inoculated with the strain *P. acidipropionici* LET 105 was 30% higher than in non-inoculated samples. SCFA concentrations obtained were high enough to inhibit *Salmonella enterica* serovar Enteritidis when assayed in a cecal water model. *P. acidipropionici* LET 105 was also able to compete with *Salmonella* for adhesion sites on the intestinal mucosa in *ex vivo* assays. Results contribute to the knowledge of the species diversity of the genus *Propionibacterium* in the intestine of poultry and provide evidence of their potential for probiotics products development.

1. Introduction

The complex microbial community that colonizes the gastrointestinal tract of newly hatched chicks influences the maturation of the gut mucosa, contributes to breakdown of complex nutrients and protects against colonization of the intestine by pathogens [1]. Chicks hatched in a sterile environment of poultry husbandry may develop slowly the intestinal microbial community with negative effects on food efficiency, weight gain and enteric infections resistance. Animals also become more susceptible to infections after changes in feed, housing conditions or displacements to rearing farms, which disturb the intestinal microbial composition [2]. Enteric pathogens may contaminate hens' eggs during the process of formation or contaminate meat of broilers during the evisceration. This is a matter of ongoing concern for the food industry because pathogenic bacteria may be transmitted to humans through the ingestion of contaminated foods of avian origin.

Different strategies have been used to guarantee the intestinal colonization of newly hatched chicks with a safe microbiota and prevent pathogenic bacteria colonization, like the inoculation with live microbial supplements named competitive exclusion cultures (CE) or Nurmi type cultures (NTC). These are undefined or semi-defined cultures which contain complex mixtures of facultative and strict anaerobes derived from the cecal content of healthy adult birds [1]. Antimicrobial agents in feed have also been used by several decades to modulate the intestinal microbiota composition and activity, and to improve health and performance in animal

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production [3], but this practice is at present banned in some countries due to the concern about transmission of antimicrobial resistances. Other alternative to ensure the establishment of beneficial bacteria in the intestine of poultry is the administration of probiotic cultures. These are source of live microorganisms, identified by molecular tools, which may exert beneficial effects in the intestinal environment by one or more mechanisms. *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Bacillus*, *Saccharomyces*, among others, are used for this purpose [4,5]. Some mechanisms by which probiotic bacteria may protect against infections include competition for adhesion sites of pathogens to the mucosa, competition for nutrients, production of antimicrobial compounds and stimulation of the immune system. Several organic acids like short chain fatty acids (SCFA) and their salts are also used in poultry production as drinking water supplements or feed additives due to their antimicrobial activity [6,7]. By contrast, the production of SCFA in the poultry intestine by probiotic bacteria has not been enough studied.

The genus *Propionibacterium* describes anaerobic to aerotolerant gram-positive organisms, non-spore forming, that produce acetic and propionic acids by carbohydrates fermentation. They are grouped as “cutaneous” and “classical or dairy” propionibacteria. Species of the cutaneous group are usually isolated from the skin and the gastrointestinal tract of humans and animals, while the species of the classical group have been mainly isolated from raw milk, fermented dairy products, fermented vegetables and silage [8]. Currently, some classical propionibacteria are used as human and animal probiotics [9,10].

The presence of the genus *Propionibacterium* as autochthonous population of the poultry intestine has been previously reported by Barnes and Impey [11], Salanitro et al. [12,13] and Apajalahti et al. [14] and described in CE and NTC cultures by Corrier et al. [15] and Waters et al. [16] respectively. Propionibacteria of cutaneous group, *Propionibacterium acnes* [11,12] and *Propionibacterium propionicum* [16], have been identified in poultry by biochemical methods while no species of the classical or dairy group was reported as members of the indigenous microbiota of these animals. However, the species identification by molecular methods was not successful or revealed discrepancies with previous identification by physiological and biochemical tests in CE and NTCs [16,17].

The isolation of propionibacteria from the intestinal microbiota of adult animals may be of interest for the development of probiotic cultures intended to reinforce the microbiota of young chickens; the ability of propionibacteria to produce propionic and acetic acids by carbohydrates fermentation would be useful for controlling pathogens in large-scale rearing facilities. However, the available information about the species identity of *Propionibacterium* from poultry is still scarce and the behavior of autochthonous strains of this genus in the intestine and their influence on avian health remains unexplored.

With the aim to contribute to the knowledge of these subjects, our investigation was focused on the isolation and molecular identification of *Propionibacterium* strains from avian intestine and further assessing of their physiological and functional features, particularly the production of SCFA in the intestinal content and adhesion to mucosa as properties of interest to pathogens control.

2. Materials and methods

2.1. Animals and samples

Laying and reproductive hens ($n = 24$) from different flocks, housed at two commercial farms of intensive production, were randomly selected for this study in three different sampling times. They were sacrificed by cervical dislocation and the gastrointestinal tract was aseptically removed. Lower ileum, ceca and large intestine were emptied by gentle squeezing and their contents received into sterile containers. Contents were suspended at 10% *w*/*v* in peptone water (10 g/L peptone in water), homogenized and kept on ice until use. Samples of rations used for the flocks feeding in the commercial farms were also taken and processed as described. In addition, cloacal swabs were carried out in backyard hens ($n = 10$) provided by three rural poultry keepers and transported to the laboratory with refrigeration. Experimental procedures were approved by The Committee of Ethics for Animal Studies (CERELA-CONICET).

2.2. Isolation and culture conditions

Successive 1/10 dilutions in peptone water were performed on the samples. The isolations were made on modified Lactate agar [18] with the following composition: 24 mL/L sodium lactate (60% *v*/*v*), 30 g/L casein peptone, 30 g/L yeast extract, 125 mM lithium chloride and 15 g/L agar, pH 6.8; the medium was sterilized at 121 °C for 20 min. Dilutions of each sample were seeded on the surface of agar plates and incubated for 10 days at 37 °C under anaerobic conditions provided by Anaerocult A (Merk KGaA, Germany) in an anaerobic jar (AnaeroGen system, Oxoid, UK).

Convex and punctual colonies with a creamy texture were selected and transferred to LAPTg agar medium [19] with the following composition: 15 g/L peptone, 10 g/L tryptone, 10 g/L yeast extract, 10 g/L glucose, 1 mL tween 80 and 15 g/L agar, pH 6.50; the medium was sterilized at 121 °C for 20 min. Plates were incubated at 37 °C, 7–10 days, under anaerobic conditions. Colonies were investigated by cell morphology and Gram-staining according to Bergey’s manual of Systematic Bacteriology [20]. Gram positive cultures of short or filamentous rods in arrangements that resemble V, Y or Chinese characters were selected. The isolated strains were stored at −20 °C in 10% (*w*/*v*) reconstituted non-fat milk (NFM) supplemented with 20% (*v*/*v*) glycerol. Prior to use, cultures were transferred three times to fresh LAPTg broth medium (2% *v*/*v* inoculum) after incubation for 24 h at 37 °C.

2.3. Other microorganisms

In some assays, three strains from the collection of CERELA (Centro de Referencia para Lactobacilos, CERELA-CONICET, Argentina), *Propionibacterium acidipropionici* CRL1198, *Propionibacterium jensenii* CRL928 and *Propionibacterium freudenreichii* subsp. *freudenreichii* CRL757, were used as reference strains. They were stored and activated in the same way as the newly isolated bacteria.

Salmonella enteritidis serovar Enteritidis (90/390) used in this study was provided by Instituto Nacional de Tecnología Agropecuaria de Balcarce (INTA-Balcarce), Argentina. The strain was stored at −20 °C in BHI broth supplemented with 10% (*v*/*v*) glycerol and activated by successive transferences in BHI broth with incubations at 37 °C prior to use.

2.4. Biochemical properties and short-chain fatty acids production

Biochemical properties were determined according to Bergey’s Manual of Systematic Bacteriology [20]. Glucose fermentation, esculin hydrolysis, gelatin liquefaction, indole production, nitrate reduction and catalase reaction were studied according to Holdeman et al. [21], Charfreitag et al. [22] and Gerhardt et al. [23]. The sugars fermentation profile was studied with the API 50 CH System Kit (BioMérieux, France) following the manufacturer’s instructions. The system was incubated at 37 °C and the results recorded after 2, 4 and 7 days.

Samples were taken from cultures in LAPTg medium incubated at 37 °C for 72 h and centrifuged at 10,000 ×*g* for 10 min at 4 °C. Aliquots of one mL were deproteinized with H_2SO_4 (final concentration 0.1 M); tubes were maintained at 4 °C for 15 min and centrifuged at 10,000 ×*g* for 10 min. Twenty μL of supernatant were injected into an HPLC system (Knauer, Germany) equipped with a Smartlinepump 100, a refractive index detector (Knauer, K-2301), a smart line auto sampler AS 3800 plus and a BIO-RAD Aminex HPX-87H (300 × 7.8 mm) column. The different components were eluted by H_2SO_4 5 mM at a 0.6 mL/min flow rate. Samples quantification was carried out with acetic, propionic, butyric and lactic acids as standard solutions.

2.5. Genus-specific PCR and 16s rDNA sequencing

DNA extraction was carried out according to Pospiech and Neumann [24]. For *Propionibacterium* genus-specific PCR assays, primers PB1 (5′-AGTGGCGAAGGCGGTTCTCTGGA-3′) and PB2 (5′-TGGGGTCGAGTTGCAGACCCCAAT-3′), corresponding to nucleotide positions 720 to 742 and 1305 to 1328 of the 16S rDNA of *E. coli*, were used [25]. Reaction mixture (20 μL) consisted of 1.5 mmol/L $MgCl_2$, 2 μL of 10× reaction buffer, a 100 μmol/L concentration of each dNTP, a 0.5 μmol/L concentration of each primer, 1 μL bacterial DNA, and 1 U of recombinant *Taq* DNA polymerase (Invitrogen, USA). The PCR was performed in a MyCycler device (Bio-Rad Laboratories, Hercules, CA) according to Rossi et al. [25]. The amplification products were separated by electrophoresis at 80 V on 1.5% (*w*/*v*) agarose gel stained with SYBR® Safe DNA stain (Invitrogen, USA) in 0.5× TAE buffer (40 mmol/L Tris/acetate, 1 mmol/L EDTA, pH 8.0). Strains of classical propionibacteria from the culture collection of CERELA were used as controls.

The 16S rRNA gene of each isolated strain was also amplified by using a universal primers pair and the PCR products were then sequenced. For this purpose, amplification of 16S rRNA gene was carried out as follows: 5 min of denaturation at 94 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 2 min and extension at 72 °C for 2 min, with a final extension step at 72 °C for 7 min. Primers used were 27F (5′-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CACGGATCCTACGGGTACCTTGTTACGACTT-3′), corresponding to nucleotide positions 8 to 36 and 1478 to 1508 of the 16S rDNA of *E. coli*, respectively [26]. Reaction mixture (50 μL) consisted of 1.5 mmol/L $MgCl_2$, 5 μL of 10× reaction buffer, 100 μmol/L of each dNTP, 0.5 μmol/L of each primer, 4 μL bacterial DNA, and 1.5 U of recombinant *Taq* DNA polymerase (Invitrogen, USA). PCR reaction was performed in a MyCycler device (Bio-Rad Laboratories, Hercules, USA). The amplification products were separated by electrophoresis at 80 V on 0.8% (*w*/*v*) agarose stained with SYBR® Safe DNA Gel Stain (Invitrogen, USA) in 1× TAE buffer (40 mmol/L Tris/acetate, 2 mmol/L EDTA, pH 8.0). PCR products were purified using NucleoSpin Extract II (MACHEREY-NAGEL, France) and sequenced at INTA – Castelar (Argentine) using a 3130xl Genetic Analyzer (Applied Biosystems, USA).

2.6. Sequence alignments and analyses

The fragments of sequences were edited with Chromas Pro software (Version 1.5 Technelysium Pty. Ltd. 2003–2009), and assembled with DNAman software (Version 2.6, Lynnon-Biosoft, Canada). They were compared to other 16S rRNA gene sequences of the GenBank/EMBL/DDBJ database using Basic Local Alignment Search Tool (BLAST http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine their approximate phylogenetic affiliations. A phylogenetic tree was constructed using 16S rRNA gene available sequences of propionibacteria and related microorganisms from database with Molecular Evolutionary Genetics Analysis version 5.0 (MEGA5) [27], by applying the neighbor-joining method and the Maximum Composite Likelihood method.

2.7. Resistance to in vitro gastrointestinal digestion

The resistance to gastric and intestinal digestions was sequentially assessed with a protocol adapted from Zarate et al. [28]. Temperature, time and pH of each treatment were adjusted to the corporal temperature, retention time of solid markers and mean pH values in different segments of the avian digestive tract [29].

Cultures of each strain in the exponential phase of growth were adjusted to 1×10^8 CFU/mL and washed twice with sterile PBS. A volume of 0.5 mL of a solution of 2 mg/mL mucin in PBS, pH 7.0, was mixed with 1.75 mL of each cell suspension and incubated at 41 ± 0.5 °C for 5 min to stabilize the mixtures. Then, 2.25 mL of simile gastric juice (125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$, 3 g/L pepsin) were added; the pH was adjusted to 3.0–3.5 with HCl and then, the mixtures were incubated at 41 ± 0.5 °C for 1 h (simulating the retention in proventriculus plus gizzard). Three mL of simile intestinal juice (0.75% *w*/*v* bile salts, 2 mg/mL pancreatin) were added; the pH was adjusted to 7.5–8.0 with NaOH and the mixtures were incubated at 41 ± 0.5 °C during 2 h (simulating the retention in the small intestine). Then, the digested mixtures were centrifuged (10,000 ×*g*, 10 min, 4 °C) and the pellets suspended in 1.75 mL of PBS. Finally, 100 μL of each suspension was stained with 2 μL of 1 mg/mL propidium iodide and 2 μL of 100 ng/μL 4′, 6-diamidino-2-phenylindole (DAPI) solutions. The number of live (blue) and dead (red) cells was determined by direct visual counting. For this purpose, cells from 10 microscopic fields were counted on a conventional fluorescence microscope (Carl Zeiss Axio Scope A1, Gottingen, Germany) fitted with the appropriated filters and the average number of live and dead cells per field was calculated. The total number of cells per milliliter of suspension was determined by using a factor of 1.525×10^{-6} mL per field that represents the volume of the liquid between the slide and cover slip in the field of view under the experimental conditions used [30].

2.8. Ex vivo adherence assay

Portions of ileum were taken from clinically healthy poultry intestine and treated as described previously [31] with modifications. Ileum portions were cut into sections of 5 cm long, open along the mesenteric border and washed with PBS/FBS (Fetal Bovine Serum) 10% pH 7.2 at least 3 times at 4 °C. Clean tissues were placed in RPMI 1640 medium supplemented with penicillin 100 UI/mL and streptomycin 100 μg/mL (GIBCO) for 30 min at 37 °C and then in fresh medium to eliminate antibiotics. Prior to the adhesion assay, the tissues were washed three times with sterile PBS. The ability of adhesion to intestinal tissue fragments was studied in the presence and absence of mucus. When was necessary, the mucus layer was aseptically removed from the underlying tissue by gentle scraping of the surface with a slide, in a laminar flow cabin to preserve the tissue sterility.

For the adhesion assays, tissue fragments (ileum area 200–400 mm^2) were inoculated (1×10^8 CFU/mL) with one strain each in a final volume of 2 mL of RPMI medium. They were then incubated at 41 ± 0.5 °C for 1 h in a humid chamber gassed with a mixture of 5% CO_2 and 95% O_2 (Nuaire Co., MN, USA). The tissues were washed three times to eliminate non-adhered bacteria and then homogenized with RPMI medium. Viable counts were determined after plating serial dilutions in LAPTg agar and incubating at 37 °C for 5 days under anaerobic conditions. The adhesion results were expressed as log CFU of adhered bacteria per mm^2 of tissue. Tissue samples incubated without the strains were used as control of the effectiveness of the antibiotics treatment. Adhesion results were

admissible when no colony or counts lower than 1 log CFU/mm^2 was obtained in the LAPTg agar plates of the control tissues.

2.9. Growth and SCFA production in cecal media

Hens fed an antibiotic-free diet were obtained from a commercial farm. In each experiment, two hens were randomly chosen and sacrificed by cervical dislocation. The ceca of each bird were collected aseptically, tied from open sides, placed into a sterile plastic bag on ice and immediately transported to the laboratory. The cecal contents were squeezed into a pre-weight sterile bottle under laminar flow and then mixed at 10% (*w*/*v*) thoroughly with sterile saline solution to obtain a uniform and pooled sample. The pH of the resulting slurry was 7.66 ± 0.07. It was centrifuged at 10,000 ×*g* for 20 min; the supernatant was sequentially sterilized by filtration with 8 and 0.22 μm pore membranes (Millipore) and conserved at −20 °C until use. This sterile solution was named cecal water medium (CW).

Cultures of 24 h in LAPTg broth medium of each strain were harvested (10,000 ×*g* for 20 min) and the pellets suspended in sterile PBS pH 7.4. Strains suspensions were inoculated in the sterile cecal water (CW) to obtain an initial absorbance of 0.15 at 560 nm and incubated at 41 ± 0.5 °C for 10 h in an Anaerobic Jar (Oxoid, Cambridge, UK) with anaerobic atmosphere of 95% N_2 and 5% CO_2 generated by the evacuation/replacement technique. At the end of incubation, counts of propionibacteria were performed by plating serial dilutions in LAPTg agar and incubating at 37 °C for 5 days under anaerobic conditions. Culture samples were centrifuged at 10,000 ×*g* for 10 min at 4 °C and short chain fatty acids concentrations were determined by HPLC as above described (Section 2.3).

Cecal homogenates (CH) at 10% (w/v) in sterile saline solution were obtained as above described and inoculated with a suspension of the strain *P. acidipropionici* LET 105 at a level of 1 × 10^6 CFU/mL. Homogenates were incubated at 41 ± 0.5 °C for 10 h in an Anaerobic Jar and at the end of incubation, were centrifuged at 10,000 ×*g* for 10 min at 4 °C. Short chain fatty acids concentrations were determined by HPLC as above described (Section 2.3).

2.10. Effect of SCFA on Salmonella Enteritidis in cecal water

A mixture of pure acetic, propionic and butyric acids was added to sterile cecal water (CW) to reach concentrations of each SCFA equal to the average concentrations measured when cecal homogenates (CH) were incubated with *P. acidipropionici* LET 105 (Subsection 2.9). The pH of supplemented and non-supplemented CW was adjusted to 7.5 or 5.5 with sterile solutions of NaOH or HCl. The media were inoculated with 100 μL of 12 h cultures of *Salmonella* Enteritidis (SE) in brain hearth infusion broth (BHI; Fluka, Sigma–Aldrich Argentina). Media were incubated at 41 ± 0.5 °C for 10 h in an Anaerobic Jar with anaerobic atmosphere and at the end of incubation, counts of *Salmonella* were performed by plating serial dilutions in BHI agar; plates were incubated at 37 °C for 24–48 h in aerobic conditions. BHI broth pH 7.5, without SCFA supplementation, was used as control of *Salmonella* growth.

2.11. Competition for adhesion sites on the mucosa

For adhesion assays, tissue samples of the ileum (area 200–400 mm^2) were prepared as above described (Subsection 2.8) and inoculated with 1 × 10^8 CFU/mL of *P. acidipropionici* LET 105, 1 × 10^6 CFU/mL of *Salmonella* Enteritidis or a mixture of both in a final volume of 2 mL in RPMI medium. They were then incubated at 41 ± 0.5 °C for 1 h in a humid chamber gassed with a mixture of 5% CO_2 and 95% O_2 (Nuaire Co., MN, USA). The tissues were washed three times to eliminate non-adhered bacteria and then homogenized with RPMI medium. Viable counts were determined after plating serial dilutions in SSA and modified Lactate agar, incubated at 37 °C for 5 days under aerobic and anaerobic conditions, respectively. The adhesion results were expressed as log CFU of adhered bacteria per mm^2 of tissue. The sterility of the tissue used was controlled as described in Subsection 2.8.

3. Statistical analysis

Results were expressed as mean ± SD and were compared by one-way analysis of variance (ANOVA) in multiple groups and by Student's unpaired *t*-test between two groups. $P < 0.05$ was considered statistically significant.

4. Results

4.1. Isolation and biochemical properties

A total of 24 hens from intensive production system and 10 backyard hens were used in the study. Samples of ileum, ceca and large intestine, as well as food and cloacal swabs were serially diluted and plated in modified Lactate agar. After 7–10 days of incubation, colonies of ivory color and creamy appearance of convex and discoid shapes and diameter of 2–4 mm were transferred to LAPTg agar, which contains glucose instead of lactate, to promote the fast growth of microorganisms; colonies were then evaluated for the typical morphology of propionibacteria and Gram staining. No colony with the typical features of the genus *Propionibacterium* was observed in samples from commercial food. In contrast, 26% of the animals subjected to the study showed colonies with these characteristics in samples of large intestine or cloacal swabs in a range of 4–5 log of CFU/g of content. No typical colony was isolated from the ileum or ceca in the wide range of dilutions examined, which is in agreement with previous reports [13].

Nine isolates with morphology and Gram reaction typical of the genus *Propionibacterium*, each one from a different hen, were selected. Four isolates were obtained from conventional poultry production systems and five from backyard hens. They were designated as LET (Laboratorio de Ecofisiología Tecnológica) followed by the numbers 101–109.

In order to classify them into the two known groups of this genus, gelatin liquefaction, esculin hydrolysis and nitrate reduction [8,20] were considered main features (Table 1). Catalase reaction was positive or weakly positive depending on the strain. Gelatin liquefaction, a character absent in the classical group of propionibacteria and variable in *P. propionicum* and *Propionibacterium granulosum* [8], was negative for the strains LET 102, 103, 105, 107 and 109. The nitrate reduction and esculin hydrolysis were positive for them indicating that they are not strains of *P. granulosum* (nitrate negative) or *P. propionicum* (esculin hydrolysis negative) species. Among the classical propionibacteria (gelatine liquefaction negative, esculin positive), the nitrate reduction is a character assigned to *P. acidipropionici* and some *P. freudenreichii* species, which are differentiated by their carbohydrates fermentation profile. On the other hand, all the strains that produced gelatine liquefaction, LET 101, 104, 106 and 108, were grouped as cutaneous propionibacteria. They also hydrolyzed esculin, which is a character present in *Propionibacterium avidum* and absent in the other species of this group. These four strains also showed negative result for nitrate reduction and indole production, as expected for the *P. avidum* species.

Fermentation profiles were studied with the API 50 CH kit. All the isolates utilized glycerol, erythritol, D-arabinose, ribose, galactose, D-glucose, D-fructose, D-mannose, mannitol, esculin, maltose, sucrose, trehalose, melezitose, starch, D-turanose and D-arabitol

Table 1
Biochemical test differentiating isolates of *Propionibacterium*.

Biochemical test[b]	Isolates[a]								
	1	2	3	4	5	6	7	8	9
Catalase activity	+	w	w	+	w	+	w	+	w
Gelatin liquefaction	+	−	−	+	−	+	−	+	−
Esculin hydrolysis	+	+	+	+	+	+	+	+	+
Nitrate reduction	−	+	+	−	+	−	+	−	+
Indole production	−	−	−	−	−	−	−	−	−

Acids from[c]	Isolates[a]									Acids from[c]	Isolates[a]								
	1	2	3	4	5	6	7	8	9		1	2	3	4	5	6	7	8	9
Adonitol	+	+	+	+	+	−	+	+	+	Lactose	−	−	+	−	+	−	+	−	+
L-Arabinose	−	+	+	−	+	−	+	−	+	Maltose	+	+	+	+	+	+	+*	+	+*
L-Arabitol	−	+	+	−	+	−	+	−	+	Mannitol	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	−	+	−	+	−	+	Mannose	+	+	+	+	+	+	+	+	+
Erytritol	+	+	+	+	+	+	+	+	+	Melibiose	−	+	+	−	+	−	−	−	−
D-Galactose	+	+	+	+	+	+	+	+	+	D-Raffinose	−	+	+	−	+	−	+*	−	+*
Gentibiose	+	−	−	+*	−	−	−	−	−	Rhamnose	−	+	+	−	+	−	+	−	+
Gluconate	+*	+*	+*	−	−	−	−	−	+*	Salicin	+	+	+*	+*	−	−	−	−	−
α-Me-D-Gluc	+	−	−	+*	−	−	−	−	−	Sorbitol	−	+	+	−	+	−	+	−	+
N-Ac-D-Gluc	+	−	−	+	−	+	−	+	−	Sucrose	+	+	+	+	+	+	+*	+	+*
Inositol	−	+	+	−	+	−	+	−	+	Trehalose	+	+	+	+	+*	+	+*	+	+*

[a] Isolates 1–9 were named after identification as follow: 1, *P. avidum* LET 101; 2, *P. acidipropionici* LET 102; 3, *P. acidipropionici* LET 103; 4, *P. avidum* LET 104; 5, *P. acidipropionici* LET 105; 6, *P. avidum* LET 106; 7, *P. acidipropionici* LET 107; 8, *P. avidum* LET 108; 9, *P. acidipropionici* LET 109.
[b] Symbols: +, present character; −, absent character; w, weak reaction, *positive after 5–7 days of incubation.
[c] Main substrates used to identify the isolates.

(some results are shown in Table 1). In contrast, D and L-xylose, β-methyl-D-xiloside, sorbose, amygdalin, α-Methyl-D-mannoside, inulin, glycogen, D-lyxose, D-tagatose, D and L-fucose and 2 and 5-keto-gluconate were not used. Other carbohydrates were fermented only by some isolates, establishing two groups with different profiles. The strains LET 102, LET 103, LET 105, LET 107 and LET 109 presented a long profile of fermentation, agreeing with *P. acidipropionici* [8,20]. On the other hand, strains LET 101, LET 104, LET 106 and LET 108 showed positive results in sorbitol, maltose and sucrose fermentations, which are differential carbohydrates in the cutaneous group. On the bases of these results, the strains LET 102, 103, 105, 107 and 109 were grouped as dairy propionibacteria and preliminary identified as *P. acidipropionici*, while LET 101, 104, 106 and 108 were included in the cutaneous group and preliminary identified as *P. avidum*.

Propionic and acetic acids were fermentation products of the bacteria isolated; their concentrations and the ratio between them are shown in Table 2. Neither lactic acid nor butyric acids were detected. The best producers of propionic acid were LET 101, 103, 105, 107 and 109 with values around 55–70 mM in cultures of 72 h of incubation; they also showed the highest concentrations of acetic acid. The ratios of propionic acid:acetic acid were higher than 2:1 for six out of the nine strains. This is in agreement with characteristics of the genus *Propionibacterium* although this ratio may vary widely [32].

Table 2
Short-chain fatty acids production in culture medium.[a]

Strains	Acetic acid[b] (mM)	Propionic acid[b] (mM)	Propionic/acetic
LET 101	27.16 ± 3.04	68.59 ± 9.21	2.53
LET 102	17.79 ± 2.07	43.79 ± 6.05	2.46
LET 103	20.37 ± 2.98	53.33 ± 7.80	2.62
LET 104	16.46 ± 2.45	29.87 ± 4.60	1.81
LET 105	19.39 ± 2.85	52.93 ± 7.85	2.73
LET 106	16.67 ± 2.18	24.13 ± 3.79	1.45
LET 107	26.07 ± 3.25	67.43 ± 9.98	2.59
LET 108	22.14 ± 3.02	36.37 ± 4.45	1.64
LET 109	25.88 ± 2.98	61.43 ± 9.41	2.37

[a] Cultures of each strain in exponential growth phase were inoculated (2% *v*/*v*) in LAPTg broth and incubated for 72 h in anaerobic conditions. Acetic and propionic acids were quantified by HPLC in the culture supernatants.
[b] Data are mean ± standard deviation.

4.2. Genus-specific PCR, 16S rDNA sequencing and phylogenetic analysis

Identification by biochemical tests of isolated bacteria was confirmed by genus-specific PCR and amplification of 16S rRNA gene and sequence analysis. In order to clearly identify *Propionibacterium* at genus level, primer pair PB1–PB2 was used and a unique PCR product with expected size (610 bp) was obtained in all samples.

PCR amplification of the 16S rRNA gene corresponding to *E. coli* rDNA sequence between positions 27 and 1492 allowed obtaining sequences of 1300–1500 nucleotide bases for all strains. Alignments were performed using the BLAST program. The query sequences of strains LET 102, LET 103, LET 105, LET 107 and LET 109, showed the highest BLAST scores (2590–2518; *E*-value 0.0) and 99% of maximum identity with *P. acidipropionici*. The query sequences of the remaining strains, LET 101, LET 104, LET 106 and LET 108 (BLAST scores: 2400–2300; *E*-value 0.0), showed 98% identity with uncultured bacteria (clone FB03B09), *Propionibacterium* sp.H456, *P. avidum* DSM 4901 (ATCC25577) and *P. propionicum* DSM 43307.

A phylogenetic tree was constructed to show the relationship between 16S rRNA gene sequences of the strains isolated and related type strains by using MEGA software version 5.0 (Fig. 1). For the phylogenetic tree, the 16S rDNA of *Lactobacillus johnsonii* ATCC33200 was used as outgroup. A close relationship between the strains LET 102, LET 103, LET 105, LET 107, LET 109 and the reference strains *P. acidipropionici* NCFB 570, NCFB 563, DH42 and CRL1198 was observed. Remarkably, the strains LET 101, LET 104, LET 106 and LET 108 were in a new cluster, but nearest to cutaneous propionibacteria. These strains were finally identified as *P. avidum* based on their biochemical behavior. The 16S rDNA sequences of the nine strains isolated were deposited in the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/Submission/index.htmL). The accession numbers are given in Table 3.

4.3. Tolerance to the gastrointestinal tract conditions

Strains of propionibacteria showed different degree of resistance to the gastrointestinal digestion. *P. acidipropionici* strains showed to be highly resistant as counts were diminished only by 0.59–0.11 log bacteria/mL at the end of the experiment. *P. acidipropionici* LET 105 and LET 107 exhibited higher tolerance than other strains of the same species in this assay. In contrast, *P. avidum* strains showed a high reduction of counts with values of 2.43–2.90 log bacteria/mL lower than the initial values (Fig. 2).

4.4. Ex vivo adherence

All *Propionibacterium* strains from poultry origin were able to adhere to the raw intestinal tissue with mean adhesion values in a range of 4.70–5.40 log CFU/mm^2 of tissue (Fig. 3). The lower and higher values corresponded to the strains *P. avidum* LET 108 and LET 106 respectively. When the mucus layer was removed from the tissue some strains significantly reduced their adherence. Four *P. acidipropionici* and one *P. avidum* strains did not show statistically significant differences between the adhesion to tissues with and without the mucus layer. The results suggested that these strains

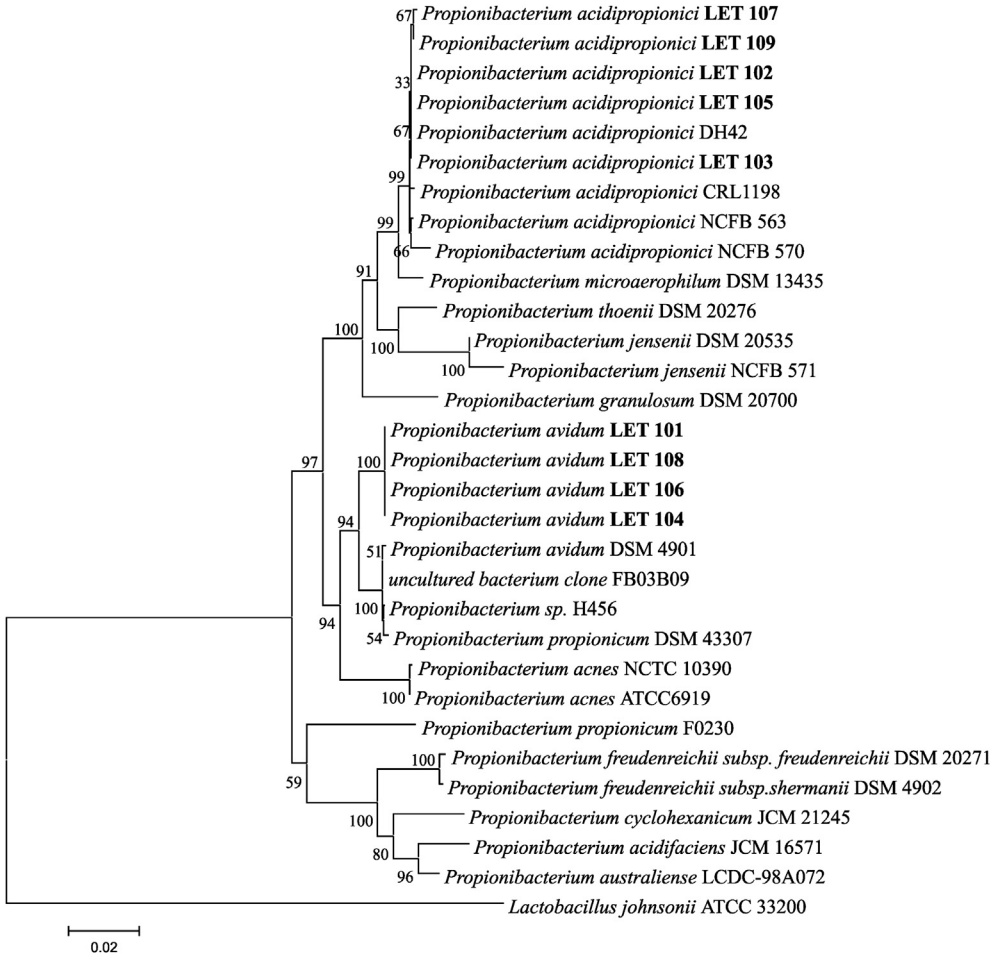

Fig. 1. Phylogenetic tree based on the 16S rDNA of members of the genus *Propionibacterium* showing the relative positions of strains isolated from the gastrointestinal tract of hens in this investigation as inferred by the Neighbor-Joining method. Published sequences in database of the 16S rDNA of *Propionibacterium* strains from different collections were used for the construction of the tree. Bootstrap values for a total of 1000 replicates are shown at the nodes of the tree.

have higher ability to bind to remnants of the inner thin layer of mucus firmly adhered to the epithelium or to the exposed epithelial cell surface after the mucus removal.

4.5. Growth and SCFA production in cecal water and homogenates

Counts of viable bacteria was performed in order to determine the ability of the strains to grow in a natural medium containing products derived from the metabolic activity of the gut microbiota and the residues of non-digestible dietary carbohydrates (Fig. 4).

Table 3
Source and identity of strains of *Propionibacterium* isolated in this study.

Strains	Source[a]	Sequence size	Species identified	Accession number
LET 101	Cloaca	1385 bp	*P. avidum*	FN824481
LET 102	Large intestine	1405 bp	*P. acidipropionici*	FN824482
LET 103	Large intestine	1411 bp	*P. acidipropionici*	FN824483
LET 104	Cloaca	1359 bp	*P. avidum*	FN824484
LET 105	Large intestine	1409 bp	*P. acidipropionici*	FN824485
LET 106	Cloaca	1388 bp	*P. avidum*	FN824486
LET 107	Cloaca	1401 bp	*P. acidipropionici*	FN824487
LET 108	Cloaca	1382 bp	*P. avidum*	FN824488
LET 109	Large intestine	1411 bp	*P. acidipropionici*	FN824489

[a] Strains: LET 101, 104, 106, 107 and 108 were isolated from backyards hens and LET 102, 103, 105 and 109 from conventional production systems.

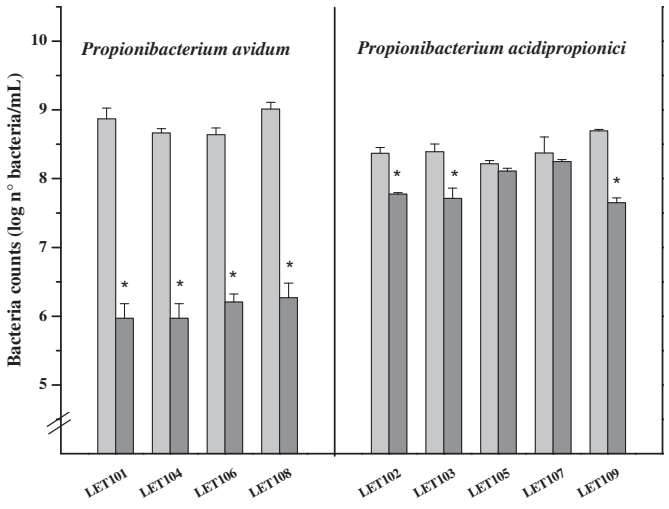

Fig. 2. Viability of *Propionibacterium* strains before (□) and after (■) *in vitro* gastrointestinal digestion adapted to the avian digestive tract conditions. Cultures in the exponential phase of growth were used. Suspensions of strains in sterile PBS were subjected to digestion with artificial gastric and intestinal fluids at 41 ± 0.5 °C during 1 h and 2 h, respectively. Live and dead cells were assessed by fluorescence microscopy. Results of counts of live bacteria are expressed as means ± standard deviation. Significant differences in counts before and after digestion for each strain are indicated with asterisks ($P \leq 0.05$).

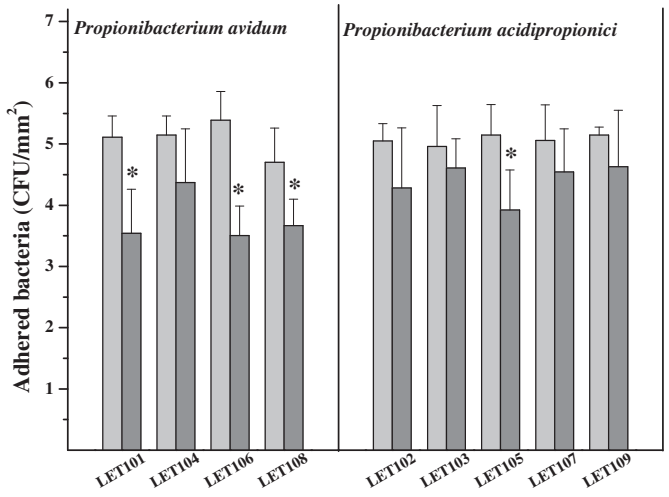

Fig. 3. Adhesion of *Propionibacterium* strains to intestinal tissue with (■) and without mucus layer (■). Clean ileum portions of healthy poultry were treated with antibiotics prior to incubation with strains suspensions of 1×10^8 CFU/mL for 1 h at 41 ± 0.5 °C in a humid chamber gassed with 5% CO_2 and 95% O_2. The assay was carried out also with tissues without the mucus layer. After incubation, tissues were washed and homogenized. Adhered bacteria were counted by plating dilutions of the homogenates in LAPTg agar medium. Tissue fragments incubated without propionibacteria were used to control the efficiency of the antibiotic treatment. Results are expressed as means ± standard deviation of the log CFU of adhered bacteria per mm^2 of tissue. Significant differences in the adhesion of each strain to tissues with and without the mucus layer are indicated with asterisks ($P \leq 0.05$).

Initial counts of propionibacteria strains in the cecal water were in the range of 7.9–8.6 log CFU/mL and, after 10 h of incubation, counts showed differences that depended on the studied strain. *P. acidipropionici* LET 102 and LET 103 were not able to grow in the cecal water without the additional supply of carbohydrates but moderate increment of 0.6–0.8 log CFU/mL was observed for the strains LET 105, LET 107 and LET 109 of this species. Scarce development was registered for the strain *P. avidum* LET 101 but a remarkable growth was observed for *P. avidum* strains LET 104, LET 106 and LET 108 which increased their counts in 2.0, 2.3 and 1.7 log CFU/mL, respectively.

The fermentation products were detected in low concentrations in this medium (Fig. 5). In this trial, average values of acetic, propionic, butyric and total SCFA in cecal water control were 58.0 ± 10.5, 18.8 ± 1.7, 10.0 ± 3.10 and 86.8 ± 15.1 μmol/g of cecal content, respectively. These acids concentrations represent the metabolic activity of the cecal microbiota before the cecal water was prepared and sterilized. Cultures of propionibacteria after 10 h of incubation contained more SCFA than the control, suggesting that all strains of propionibacteria were viable and functionally active in cecal water. The highest acetic acid concentrations were observed for the strains *P. acidipropionici* LET 103, LET 107 and LET 102 with 74.96 ± 2.37, 72.61 ± 3.84 and 72.54 ± 9.52 μmol/g of cecal content respectively. The main propionic acids producers were *P. avidum* LET 104, LET 101 and LET 108 and *P. acidipropionici* LET 103. Concentrations of propionic acid in cecal water were in the range of 36.71 ± 4.27 (*P. avidum* LET 104) and 32.84 ± 3.99 μmol/g of cecal content (*P. acidipropionici* LET 103). Molar ratios propionic acid:acetic acid in this medium were different from ratios found in LAPTg medium incubated during 72 h. Values of the ratios propionic acid : acetic acid produced, after subtracting the acids concentrations of the water cecal control medium, were 3.3, 2.9, 2.3 and 1.8 for *P. avidum* LET 106, LET 101, LET 108 and LET 104 respectively. In contrast, lower ratios were found for *P. acidipropionici* strains with values of 0.9 for LET 109 and LET 105, 0.8 for LET 103 and LET 107, and 0.6 for LET 102. As expected, no difference was observed in the butyric acid concentration among cultures of propionibacteria and the control sample of sterile cecal water.

The strain *P. acidipropionici* LET 105 was selected for further studies. Cecal homogenates supplemented with 1×10^6 CFU/mL of this strain showed higher production of SCFA after 10 h of anaerobic incubation relative to the control without supplementation (Table 4), but molar ratio of acetic, propionic and butyric acids was similar. Average concentrations of 203.0, 99.4 and 33.8 μmol/g of cecal content were obtained for acetic, propionic and butyric acids respectively in control homogenates, while average values of 261.9, 134.3 and 47.1 μmol/g were detected in homogenates inoculated with propionibacteria.

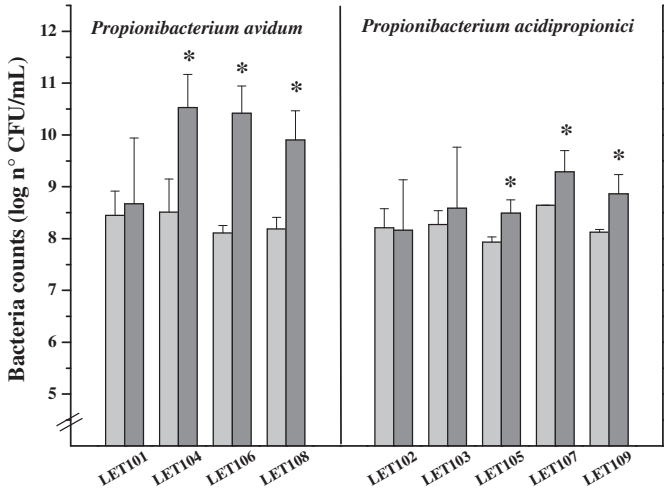

Fig. 4. Growth of *Propionibacterium* strains in cecal water. The cecal content of healthy animals was suspended in saline solution (10% w/v), homogenized, centrifuged and filtered (8 and 0.22 μm pore membranes). Aliquots of the sterile cecal water (CW) obtained were inoculated with bacterial suspensions and incubated at 41 ± 0.5 °C for 10 h in anaerobic conditions. Initial (■) and final (■) viable counts were determined by plating dilutions in LAPTg agar medium. Results are expressed as means ± standard deviation of the log CFU/g of cecal content. Significant differences in counts after 10 h with counts before incubation are indicated with asterisks ($P \leq 0.05$).

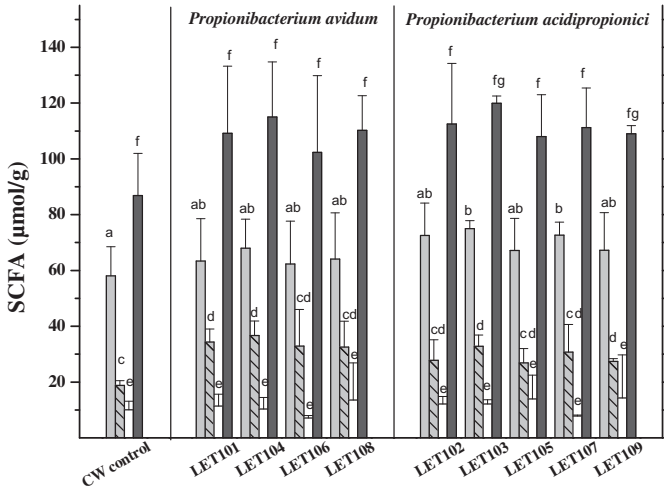

Fig. 5. Production of short chain fatty acids by propionibacteria in an *in vitro* model of cecal water. Sterile cecal water (CW) was inoculated with cultures of propionibacteria in the exponential phase of growth and incubated at 41 ± 0.5 °C for 10 h in anaerobic conditions. Acetic (■), propionic (▨) and butyric acid (□) and total SCFA (■) were quantified. Results are expressed as means ± standard deviation. The same lowercase letter on columns indicates values not significantly different ($P \leq 0.05$).

Table 4
Short-chain fatty acids production in cecal homogenates inoculated with *P. acidipropionici* LET 105.[d]

SCFA	Basal values[e] (μmol/g)	Control homogenate[f] (μmol/g)	+ LET 105 homogenate[f] (μmol/g)
Acetic acid	74.3 ± 9.2[a]	203.0 ± 20.2[b]	261.9 ± 29.7[c]
Propionic acid	27.2 ± 4.9[a]	99.4 ± 15.5[b]	134.3 ± 16.4[c]
Butyric acid	9.4 ± 0.8[a]	33.8 ± 8.1[b]	47.1 ± 7.5[b]

[d] Data are the mean value ± standard deviation of three independent assays.
[e] Basal values of SCFA in cecal homogenates before incubation (*t*0).
[f] SCFA in cecal homogenates (CH) incubated 10 h in anaerobic conditions (*t*10) without additions (Control homogenate) and supplemented with 10^6 CFU of *P. acidipropionici* LET 105 per gram of cecal content (+LET 105). Different superscript characters indicate significant differences in the values obtained ($P < 0.05$).

4.6. Inhibition of Salmonella by SCFA and competition for adhesion sites on the mucosa

A study to estimate the ability of propionibacteria to inhibit *Salmonella* only trough SCFA produced in the cecal environment was performed. The influence of SCFA on the growth of *Salmonella* was assessed in sterile cecal water (58.0 ± 10.5, 18.8 ± 1.7 and 10.0 ± 3.1 μmol/g of acetic, propionic and butyric acids) and cecal water supplemented with concentrations of SCFA equal to mean values observed in cecal homogenates (CH) supplemented with propionibacteria. *Salmonella* Enteritidis grew in lower extension in the cecal water medium than in BHI broth due to the shortage of nutrients of the former. Growth of *S.* Enteritidis was 0.9 log CFU/mL lower in cecal water supplemented with SCFA at pH 7.5 than in the same medium without supplementation. At pH 5.5, the inhibitory effect was evident in both cecal water media (Fig. 6A).

In another trial, the ability of *P. acidipropionici* LET 105 to interfere the adhesion of *Salmonella* Enteritidis to the intestinal epithelium was assessed. As it is shown in Fig. 6B, *P. acidipropionici* LET 105 was able to compete with *Salmonella* for adhesion sites on the mucosa. Counts of *Salmonella* in SSA were lower in the presence of the strain LET 105 than in tissues without exposition to propionibacteria. Counts of propionibacteria in tissues incubated with both type of bacteria were almost the same than in tissues only incubated with the strain LET 105.

5. Discussion

Up to now, 12 species have been recognized in the genus *Propionibacterium* (http://www.bacterio.cict.fr/) and grouped as "cutaneous" and "dairy or classical" propionibacteria mainly on the basis of their natural habitats. The main species of the cutaneous group are *P. acnes*, *P. avidum*, *P. propionicum*, *Propionibacterium lymphophilum* and *P. granulosum*. The classical group of *Propionibacterium* includes *P. freudenreichii*, *P. acidipropionici*, *P. jensenii* and *Propionibacterium thoenii* as the most commonly isolated species. The genus *Propionibacterium* has been previously detected by culture-dependent methods in poultry intestine [11–14] and in Competitive exclusion (CE) or Nurmi type cultures (NTCs) [15,16], but the species identification by molecular methods was not in agreement with these previous results [16,17]. Propionibacteria are in low amount in the intestinal content of humans and in contents and tissues of different animals where they were found in a range of 3–7 log CFU/g [9,33–36]. Dairy propionibacteria used as human probiotics or DFM in animals, were also recovered in counts lower than 7 log CFU/g [9,36–38]. With respect to the frequency of detection of this genus in poultry, Salanitro et al. [12] reported the presence of *P. acnes* in 1 out of 6 studied birds (nearly 17%).

Intestinal bacteria have special growth requirements that are not fully known at present and make very difficult the use of isolation methods adapted to species from a different ecosystem. Furthermore, the selective agents used in culture media may not be as effective as desired when they are used for bacteria from a very complex microbiota. Hence, the isolation of a specific genus is less probable if the population is in low numbers in the ecosystem. In the present work, a complex medium containing L-lactate as energy source with the addition of lithium chloride was used for isolation from the intestine of hens. The lithium cation in high concentration is toxic for some microorganisms but it is well tolerated by Actinobacteria like bifidobacteria [39] and propionibacteria [9], and helps to reduce the number of Gram positive and negative unwanted bacteria. Colonies with typical morphology of propionibacteria in this medium were in a level of 4–5 log CFU/g of intestinal content in only 26% of the total hens studied. This count represents less than 1% of the total microbiota in the cecum, which is at a level higher than 10 log CFU/g of content [13,40]. This may explain the lack of

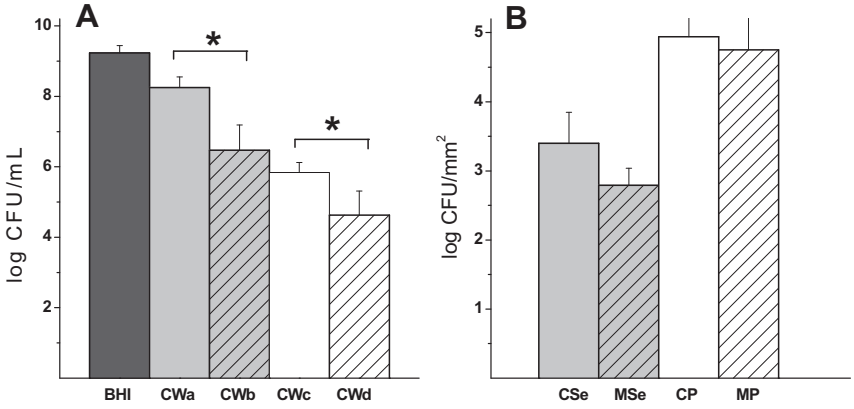

Fig. 6. Antagonic activity of SCFA at different pH values against *Salmonella* Enteritidis (A) and competition of the pathogen with *P. acidipropionici* LET 105 for adhesion sites on the mucosa (B). A: Sterile cecal water (CW) was supplemented with a mixture of SCFA in concentrations equal to values found in cecal homogenates (CH) after incubation with *P. acidipropionici* LET 105. The CW was adjusted to pH 7.5 and 5.5 and inoculated with *Salmonella* Enteritidis. Columns: Control of the growth of *Salmonella* Enteritidis in BHI broth (BHI); growth in cecal water al pH 7.5 (CWa), in cecal water supplemented with the mixture of SCFA at pH 7.5 (CWb), in cecal water at pH 5.5 (CWc) and in cecal water supplemented with the mixture of SCFA at pH 5.5 (CWd). B: Ileum fragments were treated with antibiotics, washed and incubated with *Salmonella* Enteritidis, *P. acidipropionici* LET 105 or a mixture of both microorganisms at 41 ± 0.5 °C during 1 h in a humid chamber gassed with 5% CO_2 and 95% O_2. Counts of *Salmonella* and propionibacteria were performed in BHI agar and modified Lactate agar, respectively. Columns: CSe, counts of *Salmonella* Enteritidis after interacting alone with the tissue; MSe, counts of *S.* Enteritidis after adhesion of a mixture of the pathogen with *P. acidipropionici* LET 105; CP, counts of *P. acidipropionici* LET 105 after interacting alone with the tissue; MP, counts of *P. acidipropionici* LET 105 after adhesion of a mixture of this strain with *S.* Enteritidis.

detection of the genus *Propionibacterium* in the PCR-DGGE analysis of some NTCs [41].

Nine colonies of presumptive propionibacteria, each belonging to a different hen, were confirmed both by biochemical methods and genus-specific PCR. The 16S rRNA gene of each isolated *Propionibacterium* strain was further amplified, sequenced and compared with known sequences to determine their phylogenetic affiliations. In previous reports of the presence of propionibacteria in poultry, *P. acnes* [11,12] and *P. propionicum* [16] were identified by biochemical methods while some strains, which were not identified, were named *Propionibacterium* sp. The species of the classical group of propionibacteria have not been previously identified as autochthonous bacteria in poultry. Different assays were used in our work to distinguish between cutaneous and dairy or classical strains. Five out of 9 strains were recognized as belonging to the classical group while the other 4 were placed in the cutaneous group mainly taking into account their ability to hydrolyze gelatin. Fermentations profiles evaluated by the API 50 CH identified the classical group strains as *P. acidipropionici* and those of the cutaneous group as *P. avidum*. The 16S rRNA gene sequences confirmed the biochemical identification of propionibacteria of the classical or dairy group; in contrast, sequences retrieved from strains presumptively identified as *P. avidum* exhibited 98% of sequence similarity with the genes of strains of this and other cutaneous species and were included in a new cluster (Fig. 1). Nevertheless, considering the metabolic profile of the strains LET 101, LET 104, LET 106, and LET 108, they were assigned to the *P. avidum* species. The availability of other 16S rDNA sequences from database of cutaneous propionibacteria, from the same or a different niche would be necessary to compare these strains.

The early colonization of the gastrointestinal tract by bacteria derived from the intestine of adult specimens is a natural phenomenon in free-range poultry that promote the development of the gut mucosa and the immune system, and protect newly hatched chicks of pathogens infections. Probiotics administration in the intensive production systems offers an alternative to ensure the establishment of beneficial bacteria in the intestine of chicken which are housed without contact with healthy adult poultry. Moreover, probiotics may exert protection against intestinal pathogens in adult animals when they are subjected to stressing conditions of housing or during displacements. The main bacteria used as poultry probiotics are lactic acid producers. Bearing in mind the recognized beneficial effects of other organic acids like SCFA in the intestinal ecosystem, indigenous propionibacteria may be of interest for probiotic development.

In the present study a natural medium of cecal water was assayed to evaluate the viability and growth of the strains in limiting nutrients conditions and in presence of metabolites produced by the resident microbiota. SCFA were produced in lower amount in the cecal water medium than in LAPTg broth, and the molar ratios of propionic acid:acetic acid were also different. Higher ratios were observed for *P. avidum* than for *P. acidipropionici* independently of the total amount of acids produced. Moreover, with the exception of *P.* avidum LET 101, strains with better growth in the cecal medium evidenced higher molar ratios. These metabolic differences seem to indicate that *P. avidum* is better adapted to the scarce nutrients of the cecal water, probably due to their proteolytic activity. However, the results of SCFA production suggested that all strains were viable and functionally active and they would be capable to produce more amount of SCFA if additional carbohydrates enter the intestine with the diet.

The strain *P. acidipropionici* LET 105 was selected for further studies due to the QPS (qualified presumption of safety) statement of this species [42], the high tolerance to stressful conditions of the gastrointestinal tract and the location of the isolation place, the large intestine, which was the niche of almost all the strains of this species isolated in our investigation (Table 2). This strain adhered to the raw intestinal tissue as well as the other strains studied. The lower adhesion to tissue deprived to mucus suggested that LET 105 strain is adapted to the growth on the external mucus layer exposed to the luminal content where the main fermentation of dietary components take place.

Cecal homogenates have been previously used to study SCFA production by the mouse microbiota when propionibacteria and/or complex carbohydrates were added [30]. This *in vitro* model allows assessing the activity of the bacterial community by measuring the concentration of products that come from the fermentation of mucus and dissolved substrates or particles entering with feed. In this cecal model, SCFA concentrations measured are result of the production and cross-feeding of the intestinal populations. According to the higher amount of available substrates and the presence of the microbiota, SCFA concentrations were higher in cecal homogenates (CH) than in cecal water (CW) after 10 h of anaerobic incubation. Moreover, values obtained in samples of CH inoculated with *P. acidipropionici* LET 105 were 30% higher than in control samples without inoculation (Table 4). Acids production with antimicrobial activity is involved in the exclusion of exogenous pathogens like *Salmonella* species. They are damaged by dissipation of the proton motive force across the cell membrane and acidification of the cytoplasm caused by diffusion of non-dissociated molecules of SCFA [6]. van Der Wielen et al. [43] observed concentrations of 70, 8 and 24 μmol/g of cecal content for acetic, propionic and butyric acids, respectively in broilers from day 15 onwards while Van Immerseel et al. [44] reported concentrations of these acids of 33.17, 12.03 and 5.77 μmol/g of cecal content, respectively in 18 days old chicks. In our study, cecal homogenates of hens had the same acetic acid concentration than the reported by van Der Wielen et al. [43], but inverted amount of propionic and butyric acids that could be due to differences in animals or diets. Inhibition of the growth of *Salmonella* Enteritidis was reported in Luria-Bertoni medium (LB) supplemented with concentrations of 25–100 mM acetic, propionic or butyric acids at pH 6, while very small differences in the growth were observed with the same acids concentrations at neutral pH [44]. The concentrations observed in the cecal content of chicken were not enough to inhibit the growth of *Salmonella* in LB medium or to avoid further invasion to tissues. In our study, basal concentrations of SCFA of the CW medium produced a slight reduction in counts of *Salmonella* relative to the control of BHI medium, possibly due to the different availability of nutrients. The CW medium after addition of SCFA to reach concentrations similar to the produced by the incorporation of propionibacteria to the intestinal microbiota, revealed the antimicrobial effect against *Salmonella* at both pH values assayed. These results suggested that propionibacteria may contribute to the control of *Salmonella* in the intestinal environment by the SCFA production.

The adhesion property to epithelial cells and mucus is of great interest in probiotic strains selection because the adhesion to tissue avoids the rapid removal of the bacteria by the intestinal transit. This property has been recently reviewed in dairy propionibacteria by Cousin et al. [45] as a mechanism to reduce binding of pathogens to the intestinal mucosa. In the present study, it was observed that propionibacteria from poultry have also the ability to adhere *ex vivo* to the intestinal epithelia and through this interaction block adhesion sites for *Salmonella*. The ability to produce SCFA and the blockage of the adhesion of the pathogen are valuable contributions of propionibacteria to reinforce the protective effects of the microbial community in the intestine of poultry. The high resistance of some strains, i.e. *P. acidipropionici* LET 105, to the stressful conditions of the gastrointestinal tract suggests that they may be used in

poultry by oral administration. However, antibiotic resistances and safe doses must be assessed before recommend these propionibacteria as probiotics for poultry.

6. Conclusions

The present study resulted in the identification of nine *Propionibacterium* strains isolated from hens' intestine and highlighted the presence of *P. acidipropionici* as an autochthonous bacterium in the gastrointestinal tract of poultry. To the best of our knowledge, the presence of *P. avidum* in this ecological niche has not been previously reported; moreover, there are not enough entries for this species from any source in the available database. Therefore, nucleotide sequences of the four strains isolated were included in a public access database with the aim to contribute to the growing knowledge of the genus *Propionibacterium*. Comparative studies on the functional properties of the isolated strains revealed the potential of some propionibacteria for probiotic production for poultry. The high resistances of *P. acidipropionici* strains to the stressful conditions of the gastrointestinal tract, their ability to adhere to epithelial cells and mucus and compete with *Salmonella* Enteritidis for adhesion sites, besides of remain metabolically active and improving SCFA production in the intestinal environment, suggests that these strains may be evaluated as probiotics. They could be orally administered to provide protection against *Salmonella* spp. and other enteric pathogens in poultry. Further studies to assess the efficiency and safety of different doses must be carried out in an *in vivo* infection model.

Acknowledgments

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References

[1] Schneitz C. Competitive exclusion in poultry – 30 years of research. Food Control 2005;16:657–67.
[2] Callaway TR, Dowd SE, Wolcott RD, Sun Y, McReynolds JL, Edrington TS, et al. Evaluation of the bacterial diversity in cecal contents of laying hens fed various molting diets by using bacterial tag-encoded FLX amplicon pyrosequencing. Poult Sci 2009;88:298–302.
[3] Feighner SD, Dashkevicz MP. Subtherapeutic levels of antibiotics in poultry feeds and their effects on weight gain, feed efficiency, and bacterial cholyltaurine hydrolase activity. Appl Environ Microbiol 1987;53:331–6.
[4] Kabir SML. The role of probiotics in the poultry industry. Int J Mol Sci 2009;10:3531–46.
[5] Tellez G, Pixley C, Wolfenden RE, Layton SL, Hargis BM. Probiotics/direct fed microbials for Salmonella control in poultry. Food Res Int 2012;45:628–33.
[6] Van Immerseel F, Fievez V, de Buck J, Pasmans F, Martel A, Haesebrouck F, et al. Microencapsulated short-chain fatty acids in feed modify colonization and invasion early after infection with Salmonella enteritidis in young chickens. Poult Sci 2004;83:69–74.
[7] Huyghebaert G, Ducatelle R, Van Immerseel F. An update on alternatives to antimicrobial growth promoters for broilers. Vet J 2011;187:182–8.
[8] Stackebrandt E, Cummins CS, Johnson JL. Family Propionibacteriacea: the genus Propionibacterium. In: Dworkin M, Falkow S, Rosemberg E, Schleifer KH, Stackebrandt E, editors. The prokaryotes: a handbook on the biology of bacteria. Singapore: Springer; 2006. p. 400–18.
[9] Jan G, Leverrier P, Proudy I, Roland N. Survival and beneficial effects of propionibacteria in the human gut: in vivo and in vitro investigations. Lait 2002;82:131–44.
[10] Zárate G, Babot J, Argañaraz-Martínez E, Lorenzo-Pisarello MJ, Perez Chaia A. Dairy propionibacteria: technological importance and probiotic potential for application on human and animal nutrition. In: Filip R, editor. Multidisciplinary approaches on food science and nutrition for the XXI century. India: Editorial Research Signpost; 2010. p. 175–213.
[11] Barnes EM. The intestinal microflora of poultry and game birds during life and after storage. Address of the president of the Society for Applied Bacteriology delivered at a meeting of the society on 10 January 1979. J Appl Bacteriol 1979;46:407–19.
[12] Salanitro JP, Fairchilds IG, Zgornicki YD. Isolation, culture characteristics, and identification of anaerobic bacteria from the chicken cecum. Appl Microbiol 1974;27:678–87.
[13] Salanitro JP, Blake IG, Muirehead PA, Maglio M, Goodman JR. Bacteria isolated from the duodenum, ileum, and cecum of young chicks. Appl Environ Microbiol 1978;35:782–90.
[14] Apajalahti JH, Sarkilahti LK, Maki BR, Heikkinen JP, Nurminen PH, Holben WE. Effective recovery of bacterial DNA and percent-guanine-plus-cytosine-based analysis of community structure in the gastrointestinal tract of broiler chickens. Appl Environ Microbiol 1998;64:4084–8.
[15] Corrier DE, Nisbet DJ, Scanlan CM, Hollister AG, Caldwell DJ, Thomas LA, et al. Treatment of commercial broiler chickens with a characterized culture of cecal bacteria to reduce salmonellae colonization. Poult Sci 1995;74:1093–101.
[16] Waters SM, Murphy RA, Power RF. Characterisation of prototype Nurmi cultures using culture-based microbiological techniques and PCR-DGGE. Int J Food Microbiol 2006;110:268–77.
[17] Wagner RD, Paine DD, Cerniglia CE. Phenotypic and genotypic characterization of competitive exclusion products for use in poultry. J Appl Microbiol 2003;94:1098–107.
[18] Malik AC, Reinbold GW, Vedamuthu ER. An evaluation of the taxonomy of Propionibacterium. Can J Microbiol 1968;14:1185–91.
[19] Raibaud P, Caulet M, Galpin JV, Mocquot G. Studies on the bacterial flora of the alimentary tract of pigs II. Streptococci: selective enumeration and differentiation of the dominant group. J Appl Bacteriol 1961;24:285–91.
[20] Cummins CS, Johnson JL. Genus *Propionibacterium*. In: Sneath PH, Mair ME, Sharpe ME, Holt JG, editors. Bergey's manual of systematic bacteriology. Baltimore: The Williams & Wilkins; 1986. p. 1346–53.
[21] Holdeman LV, Cato EP, Moore WEC. Anaerobe laboratory manual. Blacksburg, VA: Virginia Polytechnic Institute and State University; 1977.
[22] Charfreitag O, Collins MD, Stackebrandt E. Reclassification of Arachnia propionica as Propionibacterium propionicus comb. nov. Int J Syst Bacteriol 1988;38:354–7.
[23] Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, et al. Manual of methods for general bacteriology. Washington, DC: American Society for Microbiology; 1981.
[24] Pospiech A, Neumann B. A versatile quick-prep of genomic DNA from gram-positive bacteria. Trends Genet 1995;11:217–8.
[25] Rossi F, Torriani S, Dellaglio F. Genus- and species-specific PCR-based detection of dairy propionibacteria in environmental samples by using primers targeted to the genes encoding 16S rRNA. Appl Environ Microbiol 1999;65:4241–4.
[26] Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt MGE, editor. Nucleic acid techniques in bacterial systematics. New York, NY: John Wiley and Sons; 1991. p. 115–75.
[27] Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731–9.
[28] Zárate G, Chaia AP, Gonzalez S, Oliver G. Viability and beta-galactosidase activity of dairy propionibacteria subjected to digestion by artificial gastric and intestinal fluids. J Food Prot 2000;63:1214–21.
[29] Denbow DM. Gastrointestinal anatomy and physiology. In: Whittow GC, editor. Sturkie's avian physiology. New York: Academic Press; 2000. p. 299–325.
[30] Lorenzo-Pisarello MJ, Gultemirian ML, Nieto-Penalver C, Perez Chaia A. Propionibacterium acidipropionici CRL1198 influences the production of acids and the growth of bacterial genera stimulated by inulin in a murine model of cecal slurries. Anaerobe 2010;16:345–54.
[31] Borruel N, Carol M, Casellas F, Antolín M, de Lara F, Espín E, et al. Increased mucosal tumour necrosis factor a production in Crohn's disease can be downregulated ex vivo by probiotic bacteria. Gut 2002;51:659–64.
[32] Deborde C, Boyaval P. Interactions between pyruvate and lactate metabolism in Propionibacterium freudenreichii subsp. shermanii: in vivo (13)C nuclear magnetic resonance studies. Appl Environ Microbiol 2000;66:2012–20.
[33] Collado MC, Sanz Y. Quantification of mucosa-adhered microbiota of lambs and calves by the use of culture methods and fluorescent in situ hybridization coupled with flow cytometry techniques. Vet Microbiol 2007;121:299–306.
[34] Davidson CA. The isolation, characterization and utilization of propionibacterium as a direct-fed microbial for beef cattle. Stillwater: Oklahoma State University; 1998.
[35] Oshio S, Tahata I, Minato H. Effects of diets differing in ratios of roughage to concentrate on microflora in the rumen of heifers. J Gen Appl Microbiol 1987;33:99–100.
[36] Perez Chaia A, Nader de Macias ME, Oliver G. Propionibacteria in the gut: effect on some metabolic activities of the host. Lait 1995;75:435–45.
[37] Adams MC, Luo J, Rayward D, King S, Gibson R, Moghaddam GH. Selection of a novel direct-fed microbial to enhance weight gain in intensively reared calves. Anim Feed Sci Technol 2008;145:41–52.
[38] Luo J, King S, Adams MC. Effect of probiotic Propionibacterium jensenii 702 supplementation on layer chicken performance. Benef Microbes 2010;1:53–60.
[39] Lapierre L, Undeland P, Cox LJ. Lithium chloride-sodium propionate agar for the enumeration of bifidobacteria in fermented dairy products. J Dairy Sci 1992;75:1192–6.

[40] Gong J, Forster RJ, Yu H, Chambers JR, Wheatcroft R, Sabour PM, et al. Molecular analysis of bacterial populations in the ileum of broiler chickens and comparison with bacteria in the cecum. FEMS Microbiol Ecol 2002;41:171–9.
[41] Muyzer G, de Waal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 1993;59:695–700.
[42] EFSA Panel on Biological Hazards (BIOHAZ); Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2011 update). EFSA J 2011;9(12):2497. http://dx.doi.org/10.2903/j.efsa.2011.2497.
[43] van Der Wielen PW, Biesterveld S, Notermans S, Hofstra H, Urlings BA, van Knapen F. Role of volatile fatty acids in development of the cecal microflora in broiler chickens during growth. Appl Environ Microbiol 2000;66:2536–40.
[44] Van Immerseel F, De Buck J, Pasmans F, Velge P, Bottreau E, Fievez V, et al. Invasion of Salmonella enteritidis in avian intestinal epithelial cells in vitro is influenced by short-chain fatty acids. Int J Food Microbiol 2003;85:237–48.
[45] Cousin F, Deutsch SM, Perez Chaia A, Foligne B, Jan G. Interactions between probiotic dairy propionibacteria and the intestinal epithelium. Curr Immunol Rev 2012;8:216–26.