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In vitro screening and in vivo colonization pilot model of *Lactobacillus plantarum* LP5 and *Campylobacter coli* DSPV 458 in mice

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Abstract

The objective of this work was to determine the antibacterial effect of *Lactobacillus plantarum* strains of pork origin against *Campylobacter coli* strains, and to conduct experimental colonization pilot models in mice for both microorganisms. Inhibition assays allowed evaluation and selection of *L. plantarum* LP5 as the strain with the highest antagonistic activity against *C. coli* and with the best potential to be used in in vivo study. Adult 6-week-old female *Balb/cCmedc* mice were lodged in two groups. The treated group was administered with 9.4 $\log_{10}$CFU/2 times/wk of *L. plantarum* LP5. *L. plantarum* LP5 was recovered from the feces and cecum of the inoculated mice. However, when bacteria stopped being administered, probiotic counts decreased. Experimental colonization with *C. coli* was carried out in five groups of mice. All animals were treated with antibiotics in their drinking water to weaken the indigenous microbiota and to allow colonization of *C. coli*. Four groups were administered once with different *C. coli* strains (DSPV458: 8.49 $\log_{10}$CFU; DSPV567: 8.09 $\log_{10}$CFU; DSPV570: 8.46 $\log_{10}$CFU; DSPV541: 8.86 $\log_{10}$CFU, respectively). After 8 h, mice inoculated with different *C. coli* strains were colonized because the pathogen was detected in their feces. *L. plantarum* LP5 tolerated the gastrointestinal conditions of murine model without generating adverse effects on the animals. *C. coli* DSPV458 colonized the mice without causing infection by lodging in their digestive tract, thus generating a reproducible colonization model. Both models combined could be used as protection murine models against pathogens to test alternative control tools to antibiotics.

Keywords Lactic acid bacteria · Food-borne pathogen · Pathogen challenge · Antibiotic alternative · Inhibition assays

Introduction

Thermotolerant *Campylobacter* (TC) species have taken great relevance because they are the main zoonotic agents that cause enteric food-borne diseases. Human campylobacteriosis is one of the zoonoses with the highest incidence in the last decade, being the most reported zoonosis in the European Union since 2005 (EFSA 2019). *Campylobacter coli*, most prevalent in pigs, together with *Campylobacter jejuni* are the main pathogenic species for humans within this genus. Transmission to humans occurs directly by contact of the person with animal stool, or indirectly by consumption of undercooked animal foods or by cross-contamination with ready-to-eat prepared foods (Rossler et al. 2017). Both thermotolerant species are capable of infecting and/or colonizing a wide variety of host species. The European Union estimates that a 50–80% of the TC strains that infect humans come from the poultry chain while a 15–20% come from the pork chain (AESAN 2012).

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In food-production animals, TC is part of the normal microbiota. In recent years, a high resistance to antimicrobials has been found (Signorini et al. 2018). The cause is possibly due to its overuse in the primary production of food. At the same time, the worrying appearance of multi-resistant strains at different production stages has been reported (Maćkiw et al. 2012; Zbrun et al. 2015). As an alternative to the use of antibiotics, new strategies are being studied to prevent or reduce the incidence of *C. coli* and *C. jejuni* in host animals. One strategy is the use of probiotic bacteria (Arena et al. 2016; Verso et al. 2017). This practice would reduce the incidence of antibiotic resistant strains since antibiotics would only be used for therapeutic purposes (Kemmett 2015).

Lactobacillus is a bacterial genus widely used in probiotic supplements for animals and humans. In recent years, interest in probiotics as nutritional supplements has increased significantly and the concept is being applied in different and varied matrices. The research to characterize a specific probiotic strain involves molecular typing and confirmation of its beneficial effects (Gosiewski and Brzychczy-Wloch 2015). *Lactobacillus plantarum* is a lactic acid bacterium that has a great capacity to adapt to environmental niches. The analysis of the complete *L. plantarum* WCFS1 sequence has shown that the WCFS1 strain has the coding capacity for absorption and use of different sugars, peptide uptake and amino acid formation linked to the potential of association to surfaces and substrates (Kleerebezem et al. 2003).

L. plantarum strains have the particularity of presenting antibacterial capacity against different pathogenic bacteria (Balasingham et al. 2017; Rajoka et al. 2018). This is a potential alternative, to be used as a probiotic, for the control of foodborne diseases related to pig meat consumption (Ruiz et al. 2017). There is a wide variety of methodologies to determine the antimicrobial effect in vitro of probiotic strains against pathogenic strains. Their study would allow to elucidate whether probiotic strains would have more chances of exerting beneficial effects in vivo. At the same time, it is desirable that in vitro and in vivo probiotic properties should be evaluated together. The interaction between host and microorganisms is key and, therefore, it must be considered during the probiotic selection process.

The objective of this work was to determine the antibacterial effect of *L. plantarum* strains against *C. coli* strains, and to conduct experimental colonization pilot models in mice for both microorganisms.

Methods

Bacterial strains

In this study, seven *L. plantarum* isolates (LP1, LP2, LP3, LP4, LP5, LP6 and LP7) obtained from a pig farm, a production room and pork retail (Ruiz et al. 2017), as well as a reference *L. plantarum* commercial strain (Lyofast BG112, Sacco), were used. The entrance to the farm, the production room and the retail were allowed within the framework of the Doctorate in Animal Science, accredited by CONEAU Resolution No. 235/08, which implies an agreement with the establishments. The work with animals on the farm was carried out in accordance with the Regulations established by the FCV Animal Welfare Committee (UNCPBA). The strains were previously identified by Sanger sequencing of the 16S ribosomal DNA, and their antibacterial activity was tested against pathogens involved in food outbreaks, Shiga toxin-producing *Escherichia coli*, *Salmonella* Typhimurium and *Staphylococcus aureus* (Ruiz et al. 2017).

L. plantarum LP5 was subjected to a rifampicin resistance process to be distinguished from the lactic population in stool samples. An individual colony was seeded in Man, Rogosa Sharpe agar (MRS, Biokar, France) with 1 µg/ml of rifampicin and incubated for 24–48 h in anaerobiosis at 37 °C. The procedure was repeated by gradually increasing the concentration of the antibiotic until a bacterial resistance to 100 µg/ml was achieved. Each plate was also supplemented with 20 µg/ml of vancomycin to increase medium selectivity for *Lactobacillus* spp.

The inhibitory effect of *L. plantarum* against different strains of *C. coli* was evaluated. To do this, reference strain NCTC 11,366 (Doyle 1948), kindly provided by Dr. Marta Cerdà-Cuéllar (Laboratory of Microbiology, CRESA, IRTA), and four strains isolated from animals (*C. coli* DSPV 458 from flies, *C. coli* DSPV 541 from poultry and *C. coli* DSPV 567 and *C. coli* DSPV 570 from pigs) in the Food Analysis Laboratory, ICIVET, UNL, were used. The latter wild type strains were selected based on the virulence and genetic profile previously studied (Rossler et al. 2017).

Genetic profile of L. plantarum isolates by PFGE

Seven isolates were analyzed by PFGE to compare the different genetic profiles and to differentiate the strains. *L. plantarum* was grown in MRS broth (Biokar, France) at 37 °C for 24 h in aerobiosis. Each cell suspension was adjusted to an OD600 of 2 ± 0.2 and centrifuged for 5 min at $13{,}000 \times g$. The pellet was suspended in 1 ml of 0.85% NaCl, and 150 µl of this suspension was added to 150 µl of 2% low melting agarose (Pulsed Field Certified Agarose, Bio-Rad®) with 1X TBE buffer and maintained at 55 °C.The mixture was immediately dispensed in duplicate in the wells of the sterile plugs mold, preventing the formation of bubbles and solidified at 4 °C for 5 min. Subsequently, the plugs were transferred to 2 ml of NET buffer (10 mM Tris/HCl, 1 M NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA), 1% p/v N-lauroylsarcosine, pH 7.6) with lysozyme (10 mg/ml, Sigma®) and incubated for 24 h at 37 °C (Lysis 1). Then, the plugs were

placed in 3 ml of lysis buffer (50 mM Tris, 50 mM EDTA pH 8.0, 1% N-lauroylsarcosine (Sigma®), pH 7.6) with proteinase K (0.5 mg/ml, Promega®, Madison, USA) and incubated for 24 h at 55 °C (Lysis 2). The four subsequent washes were performed every 15 min with 10 ml of sterile Tris–EDTA (TE) buffer (10 mM Tris; 1 mM EDTA, pH 8.0), tempered at 55 °C. The plugs were stored in 2 ml of sterile TE 1X buffer at 4 °C (Li et al. 2007; Doulgeraki et al. 2010). Enzymatic digestion with the *Sfi*I enzyme was performed for 5 h at 50 °C (Jordan & Dalmasso, 2015). *Salmonella Braenderup* H9812 was used as a reference marker (digested with *Xba*I, Fermentas®). Agarose plugs of *Salmonella* were performed according to the method described in the PulseNet protocol (www.pulsenetinternational.org/protocols/pfge). Digested DNA fragments were separated by electrophoresis in a 1% PFGE agarose gel in 0.5X TBE buffer (Tris–Borate-EDTA). Electrophoresis was performed in the CHEF-DR III (Bio-Rad®) device with 0.5X TBE buffer at 4.5 V/cm for 26 h at 14 °C (Jordan and Dalmasso 2015). Finally, the gels were stained with ethidium bromide (10 mg/ml) for 30 min and photo-documented. PFGE banding patterns were analyzed using BioNumerics version 6.6 (Applied Maths, Belgium). Matching and dendrogram of fingerprints were determined by the unweighted pair group method with averages (UPGMA) and performed using the Dice coefficient.

Inhibition assays

From each different *L. plantarum* genetic profile, one profile was selected for the inhibition assay. This was performed according to the methodology described by Santini et al. (2010) with modifications. The *C. coli* strains were reactivated in Muller Hinton agar (MH, Biokar, France) for 48 h at 37 °C in microaerophilia (H_2:CO_2:O_2 = 85:10:5). At the same time, *L. plantarum* strains were grown in MRS broth (Biokar, France) at 37 °C for 24 h in aerobiosis. The *L. plantarum* cultures were centrifuged at 6,000 × *g* for 15 min, and then washed and centrifuged again under the same conditions to obtain the cell-free extract (CFE). The CFE was lyophilized for 24 h and concentrated to 4X (Alpha 1.4 LD Plus, Martin Christ, Germany). A portion of the CFE obtained from each strain was neutralized to a pH of 6.3 with 10 N NaOH to obtain a neutralized cell-free extract (CFEn). From the *C. coli* culture, a bacterial suspension was made at an OD_{630} of 0.8 ± 0.2. This suspension (100 µl) was spread with a Digralsky spreader on the surface of plates with *Campylobacter* Blood-Free Selective Agar Base (CCDA, Oxoid, United Kingdom). After 3–5 min at room temperature, 40 µl of CFE and CFEn were inoculated in wells of 5 mm in diameter previously made on the agar. As a positive control, lactic acid (85%) diluted 1/32 in one of the wells was used. Plates were incubated for 48 h at 37 °C under microaerophilic conditions and analyzed by measuring the diameter of the zone of inhibition around each well with a caliper (0.05 mm sensitivity measurement). The results were reported as average values (mm) of the diameter of the zone of inhibition.

Experimental colonization of L. plantarum LP5 in mice

Six adult 6-week-old female mice (20 ± 1 g) were used (CMC-ICiVet-Litoral, CONICET-UNL). Mice from the *Balb/c* strain, identified with the international laboratory code *Balb/cCmedc*, issued by the Institute for Laboratory Animal Research (National Academies of Science, Engineering and Medicine, USA), were used in this study. This is a strain derived from animals of the Jackson Laboratory (USA), currently with an inbreeding higher than F15. The animals were kept in micro-ventilated grill systems, with automated environmental controls, under HEPA filtered air conditions and under veterinary supervision by specialized professionals. All the works were carried out at the Center for Comparative Medicine (CMC-ICiVet-Litoral, CONICET-UNL). All procedures were carried out according to the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and were approved by the Institutional Ethics and Security Committee of the *Facultad de Ciencias Veterinarias–Universidad Nacional del Litoral*, Santa Fe, Argentina (protocol number 518/19). The health status of the colony was periodically checked by Charles River Laboratories (Wilmington, MA, USA) through the Federation of European Laboratory Animal Science Associations (FELASA) Complete PRIA service. The mice were lodged in two groups of three animals each. Throughout the treatment (4 wk), the treated group (LAB-G) was administered with 0.1 ml of *L. plantarum* LP5 (9.4 $\log_{10}$CFU/2 times/wk) by gavage, and the control group (C–G) with physiological solution as placebo. Throughout the experiments, the mice were housed in cages and fed standard laboratory chow and tap water ad libitum. The animals were kept at 21 ± 2 °C, 55 ± 2% humidity and a 12 h light–dark cycle during the trial, with the light period starting at 8 AM. Any manipulation outside the cages was performed in a vertical laminar flow cabinet to prevent microbiological contamination. Inoculation was performed during the first three weeks of the study. Daily examination of the clinical signs of the animals was performed to evaluate possible harmful effects. Bacterial colonization was evaluated by microbiological feces sample analysis. Fecal samples were taken at weekly intervals to quantify populations of *L. plantarum* LP5 in $MRS_{rif\text{-}van}$ agar (supplemented with 100 µl/ml rifampicin and 20 µl/ml vancomycin), *Lactobacillus* spp. in MRS_{van} agar, β-glucuronide-positive *Escherichia coli* and β-glucuronide-negative bacteria in Tryptone Bilis X-glucuronide agar (TBX, Biokar, France), Enterobacteria in Violet Red Bilis Glucose agar (VRBG, Britania, Argentina), thermotolerant

Campylobacter in mCCDA agar (Oxoid, Germany) and yeast in a yeast glucose chloramphenicol modified medium (YGCm, Merck, Germany). At the end of the experiment, the animals were anesthetized subcutaneously in the interscapular region (Ketamine 5% (w/v) 95.8 mg/kg, xylazine 10% (w/v) 10.8 mg/kg and acepromazine 1% (w/v) 4.8 mg/kg) and intracardiac blood collection was performed. Then they were sacrificed by cervical dislocation. Translocation to internal organs and colonization in two intestinal regions were evaluated in the spleen and liver, and ileum and cecum, respectively. Samples were homogenized with 0.85% NaCl solution in Stomacher (Lab System, United Kingdom) and analyzed microbiologically with the same methodology as with feces.

Experimental colonization/infection of C. coli in mice

Fifteen adult 6-week-old female *Balb/cCmedc* mice (20 ± 1 g) were used (CMC-ICiVet-Litoral, CONICET-UNL). The animals were provided by the same center as the ones for the *L. plantarum* colonization test and maintained under the same biosecurity protocols, environmental and feeding conditions. The mice were lodged in five groups of three animals each. All mice were treated with antibiotics in their drinking water for 5 d: enrofloxacin (Floxagen-Vetanco, 60 mg/kg/d), florfenicol (Civet-Facyt, 120 mg/kg/d), trimetoprim sulfametoxazol (Civet-Facyt, 24 mg/kg/d and 120 mg/kg/d, respectively) and oxytetracycline (Proagro, 15 mg/kg/d). All animals were treated with antibiotics in their drinking water to weaken the indigenous microbiota and to allow colonization of *C. coli*. Antibiotic and drinking water replacement were performed daily. *C. coli* was administered two days after the antibiotics were withdrawn (day 7 of the experiment). All animals received 0.1 ml of $NaHCO_3$ (5% w/v) 15 min before being inoculated. Four groups: CAMPY1, CAMPY2, CAMPY3 and CAMPY4 were administered once with 0.1 ml of different *C. coli* strains (DSPV 458: 8.49 $\log_{10}$CFU; DSPV 567: 8.09 $\log_{10}$CFU; DSPV 570: 8.46 $\log_{10}$CFU; DSPV 541: 8.86 $\log_{10}$CFU, respectively), suspended in Brain Heart Infusion broth (BHI, Biokar, France). The control group (C–G) was administered with 0.1 ml of the same medium as placebo. Any manipulation outside the cages was performed in a vertical laminar flow cabinet (Allentown Phantom, USA) to prevent microbiological contamination. Daily examination of clinical signs of the animals was performed to evaluate possible harmful effects. Bacterial colonization/infection was evaluated by microbiological stool analysis. Fecal sampling was performed at 8, 24, 48, 72 and 96 h post-inoculation of the pathogen. Necropsies and treatment of fecal and intestinal samples to quantify bacterial populations were performed as described above for the colonization of *L. plantarum*.

Statistical analysis

A Generalized Lineal Model with Gamma distribution and logarithmic link function was applied to determine the differences between CFE and CFEn *versus C. coli*. Differences were considered significant for a value of $P \leq 0.01$. The experiment was analyzed with three replicates.

A Generalized Lineal Model of repeated measures with Gamma distribution and logarithmic link function was applied to determine the differences between the populations obtained from fecal samples throughout the *L. plantarum* LP5 experimental colonization. The same model but without repeated measures was used to analyze the differences between bacterial populations obtained from the cecum and ileum in both models. In addition, a Generalized Lineal Model with binomial distribution and logistics link function was applied to determine the differences between the populations obtained from fecal samples throughout the *C. coli* experimental colonization. Differences were considered significant for a value of $P \leq 0.01$ in all models.

Results

PFGE fingerprinting of L. plantarum

L. plantarum isolates were grouped into five perfectly defined profiles (Fig. 1). The strain profiles were different depending on the origin and the stage of production. Some of the strains with the same origin had different genetic profiles (PFGE profile 2 and 3 and PFGE profile 1 and 4, Fig. 1). However, strains isolated from different stages of the production chain showed the same profiles (PFGE profile 4). Likewise, all wild strains were different from the reference strain (Fig. 1).

L. plantarum inhibition assays

L. plantarum strains used for the inhibition assay were LP1, LP3, LP5 and LP7. The CFE pH of the four *L. plantarum* ranged between 4.0 and 4.5.

Each *L. plantarum* CFE generated halos of inhibition against all *C. coli* strains. The challenge was carried out between each one of the CFE and the *C. coli* strains. *C. coli* NCTC 11,366 showed the highest susceptibility. All *L. plantarum* CFEn also showed an inhibitory effect against all the *C. coli* studied. *C. coli* DSPV 541 and NCTC 11,366 were the most susceptible, followed by *C. coli* DSPV 458, *C. coli* DSPV 570 and *C. coli* DSPV 567 (Table 1; Fig. 2).

CFE of *L. plantarum* LP5 and *L. plantarum* LP3 had a greater inhibitory effect, followed by *L. plantarum* LP1 and *L. plantarum* LP7. CFEn of *L. plantarum* LP5, *L. plantarum* LP3 and *L. plantarum* LP1 had higher diameter inhibition

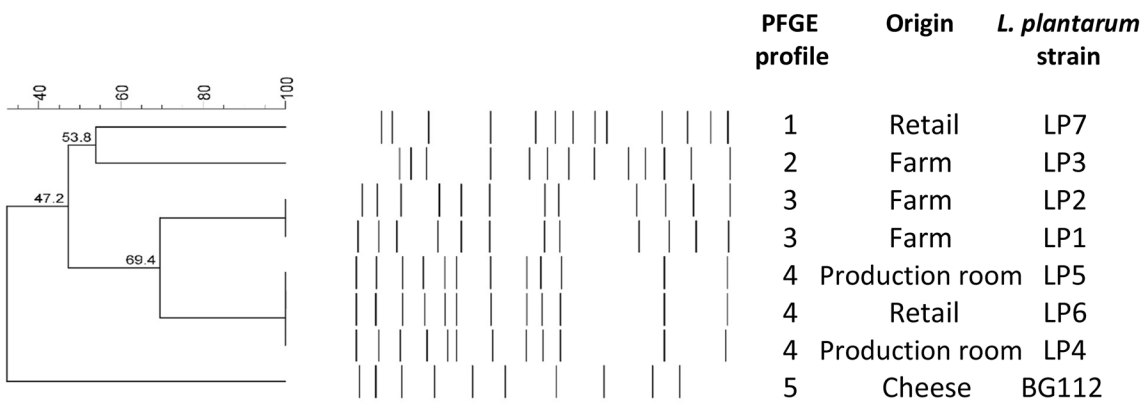

Fig. 1 PFGE dendrogram of *L. plantarum* using *Sfi*I restriction enzyme. Pulse field gel electrophoresis (PFGE)

Table 1 Inhibition halos generated by all *L. plantarum* CFE and CFEn against different strains of *C. coli* on CCDA agar

	Diameter of inhibition halo (mm)				
	C. coli NTCC 11,366	*C. coli* DSPV458	*C. coli* DSPV567	*C. coli* DSPV570	*C. coli* DSPV541
All LAB CFE	$21.3^{a} \pm 0.45$	$14.3^{b} \pm 0.30$	$14.2^{b} \pm 0.30$	$15.5^{c} \pm 0.32$	$18.1^{d} \pm 0.38$
All LAB CFEn	$13.3^{a} \pm 0.50$	$11.1^{b} \pm 0.41$	$10.0^{c} \pm 0.36$	$10.4^{c} \pm 0.38$	$14.2^{a} \pm 0.53$

Means followed by different letters within rows indicate significant differences according to GLM test ($P < 0.05$)

Lactic acid solution used as a positive control generated 11.6 mm halos

The challenge was carried out between each one of the CFE or CFEn and each one of the *C. coli* strains

CFE cell-free extract, *CFEn* naturalized cell-free extract

halos than *L. plantarum* LP7 (Table 2). The lactic acid used as a positive control generated a halo of smaller diameter in CFE tests. However, for CFEn strains LP1 and LP7, the halos shown were smaller than the 11.6 mm from lactic acid.

Mice colonization with L. plantarum LP5

Murine microbiota lacked LAB resistance to rifampicin at the start of the experiment. *L. plantarum* LP5 was recovered from the cecum and feces of the inoculated mice group. In the feces from LAB-G, *L. plantarum* LP5 reached maximum levels of 7.3 $\log_{10}$CFU/g in the second week and minimum levels of 3.4 $\log_{10}$CFU/g in the fourth week (Fig. 3). *L. plantarum* LP5 reached levels of 2.5 $\log_{10}$CFU/g of cecum content, while the strain supplied was not detected in the ileum. Rifampicin-resistant LAB in C–G was not found.

In feces, LAB counts remained between 9.2 $\log_{10}$CFU/g and 9.4 $\log_{10}$CFU/g and between 9.3 $\log_{10}$CFU/g and 10.2 $\log_{10}$CFU/g throughout the experiment in LAB-G and C–G, respectively.

The mean levels of LAB in all the experiment were 9.3 $\log_{10}$CFU/g and 9.7 $\log_{10}$CFU/g in LAB-G and C–G, respectively ($P > 0.05$, Fig. 3). Enterobacteria counts remained between 4.9 $\log_{10}$CFU/g and 6.4 $\log_{10}$CFU/g and between 5.3 $\log_{10}$CFU/g and 5.7 $\log_{10}$CFU/g in all the experiment

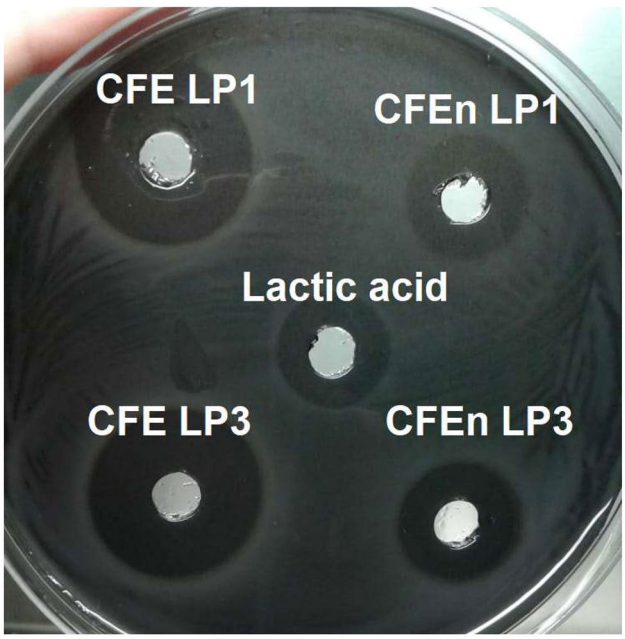

Fig. 2 Inhibition halo generated by CFE and CFEn from different strains of *L. plantarum* against different strains of *C. coli* in CCDA agar. Cell-free extract (CFE), neutralized cell free extract (CFEn)

Table 2 Inhibition halos generated by the CFE and CFEn of different *L. plantarum* strains against *C. coli* strains on CCDA agar

	Diameter of inhibition halo (mm)	
	All *C. coli* strains against CFE	All *C. coli* strains against CFEn
L. plantarum LP1	$17.3^{b} \pm 0.37$	$11.9^{a} \pm 0.38$
L. plantarum LP3	$18.8^{a} \pm 0.40$	$12.3^{a} \pm 0.41$
L. plantarum LP5	$19.2^{a} \pm 0.41$	$13.0^{a} \pm 0.43$
L. plantarum LP7	$16.5^{b} \pm 0.35$	$10.1^{b} \pm 0.33$

Means followed by different capital within column indicate significant differences according to GLM test ($P < 0.05$)

Lactic acid solution used as a positive control generated 11.6 mm halos

The challenge was carried out between each one of the CFE or CFEn and each one of the *C. coli* strains

CFE cell-free extract, *CFEn* naturalized cell-free extract

in LAB-G and C–G, respectively. In all the experiment, the mean levels of Enterobacteria in both groups were 5.5 $\log_{10}$CFU/g ($P > 0.05$, Fig. 4). *β*-glucuronide-negative bacteria counts remained between 1.9 $\log_{10}$CFU/g-5.8 $\log_{10}$CFU/g and between 5.8 $\log_{10}$CFU/g and 5.9 $\log_{10}$CFU/g in all the experiment in LAB-G and C–G, respectively. The mean levels of *β*-glucuronide-negative bacteria in all the experiment were 4.8 $\log_{10}$CFU/g and 5.9 $\log_{10}$CFU/g in LAB-G and C–G, respectively ($P > 0.05$, Fig. 4).

The bacterial populations studied in the cecum and ileum showed no differences between LAB-G and C–G ($P > 0.05$, data not shown). In the cecum, the mean levels of LAB were 9.5 $\log_{10}$CFU/g and 9.0 $\log_{10}$CFU/g in LAB-G and C–G, respectively; Enterobacteria were 5.9 $\log_{10}$CFU/g and 6.1 $\log_{10}$CFU/g in LAB-G and C–G, respectively; β-glucuronide-negative bacteria were 5.7 $\log_{10}$CFU/g and 6.3 $\log_{10}$CFU/g in LAB-G and C–G, respectively. In the ileum, the mean levels of LAB were 8.4 $\log_{10}$CFU/g and 8.5 $\log_{10}$CFU/g in LAB-G and C–G, respectively; Enterobacteria were 5.1 $\log_{10}$CFU/g and 4.8 $\log_{10}$CFU/g in LAB-G and C–G, respectively; β-glucuronide-negative bacteria were 4.3 $\log_{10}$CFU/g and 5.3 $\log_{10}$CFU/g in LAB-G and C–G, respectively.

Thermotolerant *Campylobacter*, β-glucuronide-positive *E. coli* and yeasts were not detected in any group in all the experiment in either fecal or intestinal contents. No microbial count in the spleen and liver was found in any experimental group. No characteristic clinical signs of disease were observed in any animal.

Mice colonization/infection with C. coli strains

Initially, LAB were found at levels of 9 $\log_{10}$CFU/g in fecal samples and were reduced after antibiotic treatment to 6.72 $\log_{10}$CFU/g in CAMPY1, 4.82 $\log_{10}$CFU/g in CAMPY2, 4.74 $\log_{10}$CFU/g in CAMPY3, 6.86 $\log_{10}$CFU/g in CAMPY4 and 5.95 $\log_{10}$CFU/g in the control group. Enterobacteria and β-glucuronide-negative bacteria counts were found in values of 5 $\log_{10}$CFU/g in fecal samples at the beginning of the experiment, but reached undetectable levels in the rest of the experiment.

All mice initially lacked thermotolerant *Campylobacter* in feces. In all groups of mice inoculated with the pathogen, colonization by *C. coli* was detected 8 h after inoculation and was maintained throughout the trial in at least

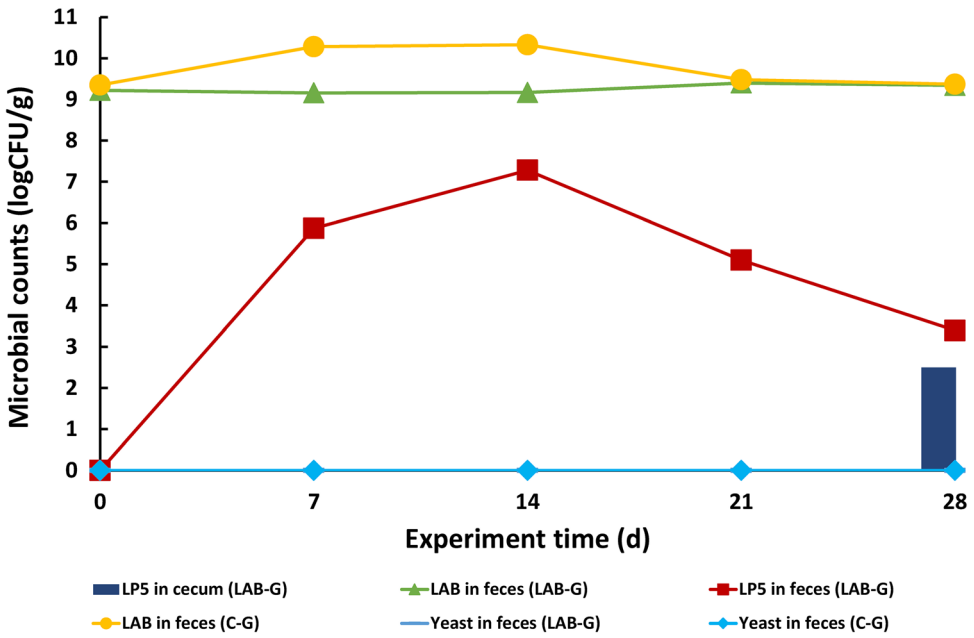

Fig. 3 Recovery of lactic acid bacteria, yeasts and *L. plantarum* LP5 in feces and in the intestinal tract of mice experimentally colonized with strain LP5. Counts of *L. plantarum* LP5 in the cecum of LAB-G mice (LP5 in cecum (LAB-G)), Counts of LAB in feces of LAB-G mice (LAB in feces (LAB-G)), Counts of *L. plantarum* LP5 in feces of LAB-G mice (LP5 in feces (LAB-G)), Counts of LAB in feces of C–G mice (LAB in feces (C–G)), Counts of Yeast in feces of LAB-G mice (Yeast in feces (LAB-G)), Counts of Yeast in feces of C–G mice (Yeast in feces (C–G))

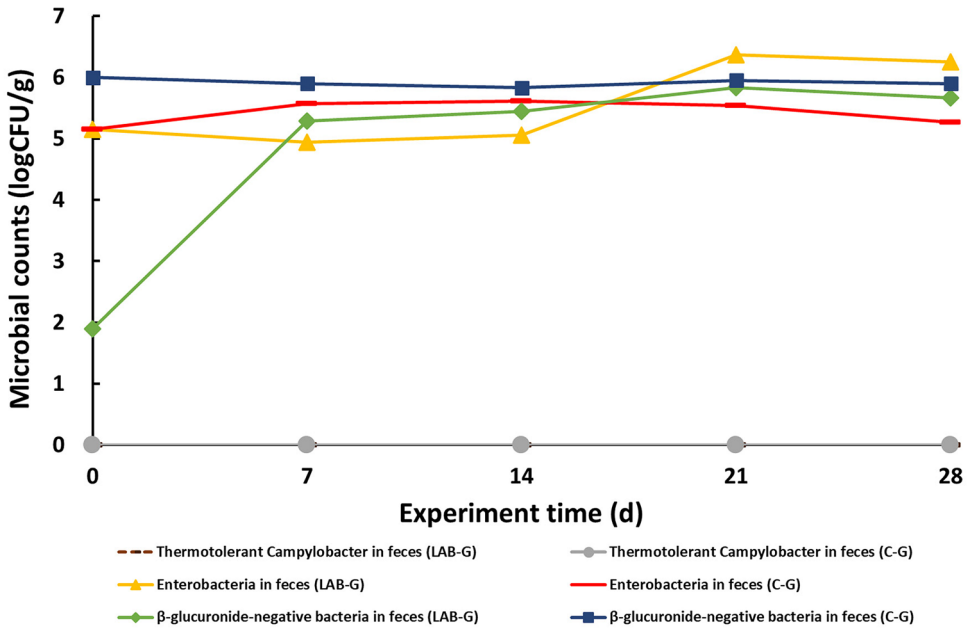

Fig. 4 Recovery of Enterobacteria, thermotolerant *Campylobacter* and *β*-glucuronide-negative bacteria in feces of mice colonized with *L. plantarum* LP5. Counts of thermotolerant *Campylobacter* in feces of LAB-G (Thermotolerant *Campylobacter* in feces (LAB-G)), Counts of thermotolerant *Campylobacter* in feces of C–G (Thermotolerant *Campylobacter* in feces (C–G)), Counts of Enterobacteria in feces of LAB-G (Enterobacteria in feces (LAB-G)), Counts of Enterobacteria in feces of C–G (Enterobacteria in feces (C–G)); Counts of *β*-glucuronide-negative bacteria in feces of LAB-G (β-glucuronide-negative bacteria in feces (LAB-G)), Counts of *β*-glucuronide-negative bacteria in feces of C–G (*β*-glucuronide-negative bacteria in feces (C–G))

one animal per group (1/3 colonized mice, Table 3). After administration of the pathogen, no colonization differences were found between the groups over time ($P=0.56$), nor were there differences in colonization among the different *C. coli* strains ($P=0.28$; Table 3). From 8 h, mice from all the inoculated groups showed significant differences ($P<0.05$) against the control group and against their previous state (not inoculated).

Finally, the four *C. coli* strains were detected in the cecum in one inoculated animal per group. Only in one group was *C. coli* detected in the ileum (Table 4). There were no detectable counts of any of the microbial populations studied in the spleen and liver, that is, LAB, Enterobacteria, *β*-glucuronide-negative bacteria, *β*-glucuronide-positive *E. coli*, yeasts, *L. plantarum* LP5 were not detected and neither were the *C. coli* inoculated

Table 3 *Campylobacter coli* intestinal colonization in mice detected by bacterial culture of feces

Experiment time (h)	Experimental groups				
	CAMPY1 *C. coli* DSPV 458	CAMPY2 *C. coli* DSPV 567	CAMPY3 *C. coli* DSPV 570	CAMPY4 *C. coli* DSPV 541	Control
	Proportion of colonized mice (#/total #)				
0	0/3	0/3	0/3	0/3	0/3
8	3/3	3/3	2/3	2/3	0/3
24	3/3	3/3	2/3	1/3	0/3
48	2/3	2/3	1/3	2/3	0/3
72	2/3	2/3	2/3	1/3	0/3
96	2/3	1/3	1/3	2/3	0/3

Experimental group inoculated with *C. coli* DSPV458 (CAMPY1 *C. coli* DSPV458), Experimental group inoculated with *C. coli* DSPV567 (CAMPY2 *C. coli* DSPV567), Experimental group inoculated with *C. coli* DSPV570 (CAMPY3 *C. coli* DSPV570), Experimental group inoculated with *C. coli* DSPV541 (CAMPY4 *C. coli* DSPV541)

strains (Table 4). The animals showed no clinical signs of disease throughout the experiment.

In the cecum, lactic acid bacteria (Fig. 5) and Enterobacteria counts showed no differences among the *C. coli* challenged groups and the control group. In the ileum, LAB counts were higher in the control, CAMPY3 and CAMPY2 groups than in the CAMPY4 and CAMPY1 groups ($P < 0.05$; Fig. 5). The Enterobacteria population was higher in the CAMPY3 and CAMPY1 groups than in the CAMPY2, CAMPY4 and control groups ($P < 0.05$). β-glucuronide-negative bacteria, yeasts and β-glucuronide-positive *E. coli* were not detected in the intestinal tract of any of the experimental groups.

Discussion

Genus and species identification of bacteria are one of the first steps for their characterization as probiotic bacteria. Bacterial identification is key to defining both taxonomic location and security level. This characterization is fundamental to elaborate functional foods. The beneficial properties described in both animals and humans have improved beneficial food demand by consumers (Castañeda Guillot 2018). In general, microorganisms can be identified by phenotypic and genotypic methods. LAB are usually identified

Table 4 Mice colonized in the intestinal tract and not colonized in internal organs (translocation assays) with different *C. coli* strains

Test	Tissue	Experimental groups				
		CAMPY1 *C. coli* DSPV 458	CAMPY2 *C. coli* DSPV 567	CAMPY3 *C. coli* DSPV 570	CAMPY4 *C. coli* DSPV 541	Control
		Proportion of colonized mice (#/total #)				
Colonization	Cecum	1/3	1/3	1/3	1/3	0/3
	Ileum	1/3	0/3	0/3	0/3	0/3
Translocation	Spleen	0/3	0/3	0/3	0/3	0/3
	Liver	0/3	0/3	0/3	0/3	0/3

Experimental group inoculated with *C. coli* DSPV458 (CAMPY1 *C. coli* DSPV458), Experimental group inoculated with *C. coli* DSPV567 (CAMPY2 *C. coli* DSPV567), Experimental group inoculated with *C. coli* DSPV570 (CAMPY3 *C. coli* DSPV570), Experimental group inoculated with *C. coli* DSPV541 (CAMPY4 *C. coli* DSPV541)

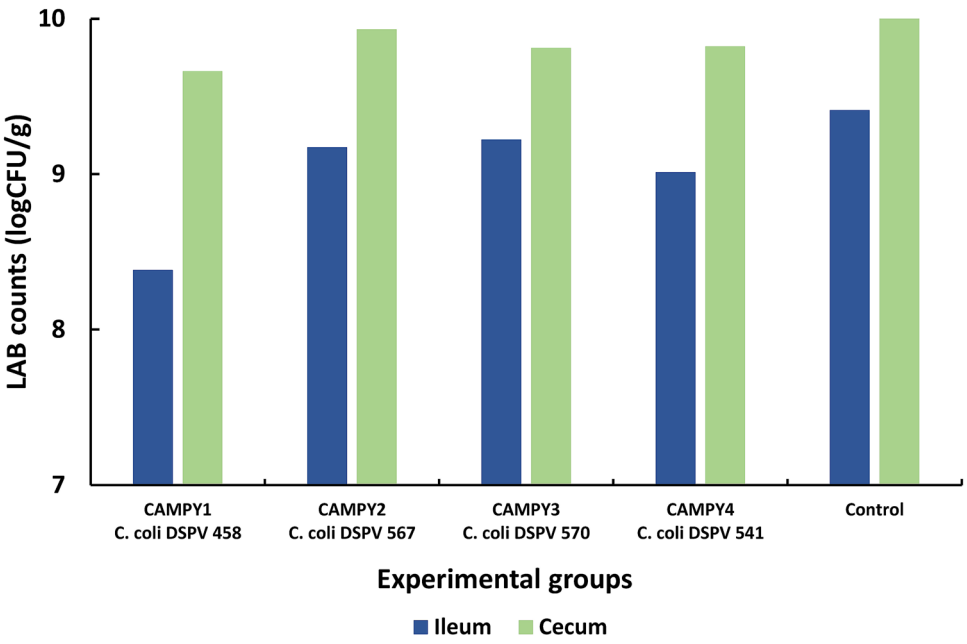

Fig. 5 Lactic acid bacteria counts in intestinal contents of mice challenged with different *C. coli* strains. Experimental group inoculated by *C. coli* DSPV458 (CAMPY1 *C. coli* DSPV458), Experimental group inoculated by *C. coli* DSPV567 (CAMPY2 *C. coli* DSPV567), Experimental group inoculated by *C. coli* DSPV570 (CAMPY3 *C. coli* DSPV570), Experimental group inoculated by *C. coli* DSPV541 (CAMPY4 *C. coli* DSPV541)

by biochemical determinations, such as Gram staining, catalase, mobility, and growth in a specific medium, among others (Avnİ Kırmacı 2016). These basic tests allow a first approach to the taxonomic groups. On the other hand, molecular epidemiology techniques based on fingerprinting, like PFGE, allow the differentiation of different subtypes within a bacterial species. In this study, a comparison of the genetic profiles using the PFGE technique allowed us to distinguish the *L. plantarum* subtypes isolated from different stages of the chain pork production. The selection of one representative strain from each profile allowed for the performance of the subsequent in vitro and in vivo assays. In vitro tests, such as probiotic inhibition assays against foodborne pathogens, are widely used in LAB detection to find possible strains for pathogen control by direct antagonism. The results of this study agree with numerous studies regarding the inhibitory capacity of the genus *Lactobacillus* spp., and specifically *L. plantarum*, against pathogenic bacteria (Vallejo et al. 2009; Roldán et al. 2011). All CFE of the *L. plantarum* strains had an inhibitory effect against all the *C. coli* strains studied. An important fact lies in the presence of some substance in addition to the acid with antibacterial activity since the wells-containing CFEn also produced an inhibitory effect. This differs with the study by Dec et al. (2018) in which, when neutralizing extracts, the inhibitory activity did not occur. In vitro inhibitory activity against food-borne pathogens is bacterial strain specific. Therefore, the results should not be extrapolated (Guarner and Malagelada 2003) and their use in murine models (Vinderola et al. 2017) provides more truthful in vivo information.

During the *L. plantarum* LP5 administration, mice showed a good recovery of the probiotic in feces and did not produce an imbalance between the intestinal microbiota components. The generation of the rifampicin-resistant LP5 mutant facilitated its enumeration and made it easy to differentiate it from the indigenous microbiota. However, when the bacteria stopped being administered, probiotic counts decreased. It has been shown that ingestion of probiotic strains does not cause lasting and measurable colonization and survival in the host. Invariably, microorganisms persist for days or weeks, but no longer (Tannock 1999; FAO 2006). The probiotic adhesion to the intestinal epithelium may contribute to its persistence on the mucosa surface (Collado et al. 2007). However, colonization of the intestine by orally administered probiotics seems to be only temporary. The interruption of probiotic administration causes them to leave the intestinal tract (Vinderola et al. 2017). Bacterial translocation is a good indicator of possible probiotic infectivity (Didari et al. 2014). *L. plantarum* LP5 and the rest of the populations evaluated showed no capacity to translocate to internal organs, or the host immune system eliminated them before they could be detected (Frizzo et al. 2010). That is, the strain administered has no invasive property and appears to be safe to be administered in the animals' diet.

The scarce scientific evidence of the inhibitory effect of *L. plantarum* against *C. coli* forces the development of models that allow the evaluation of in vivo control strategies of zoonotic pathogens that bear significance in public health. Murine models of *Campylobacter* infection present a high variability, lack of persistence and absence of consistent clinical or pathological findings (Chang and Miller 2006). All this has hindered the development of reproducible models that allow the study of the host-*Campylobacter* relationship (Chang and Miller 2006). Conventional laboratory mice can hardly be infected by thermotolerant *Campylobacter*. This is due to resistance to colonization caused by the composition of the mouse specific gut microbiota, which prevents pathogens from invading the murine host. A treatment with broad spectrum antibiotics weakens the indigenous microbiota and allows colonization by the pathogen. Treatment with an antibiotic´s combination in this study caused a murine microbiota imbalance. Enterobacteria and β-glucuronide-negative bacteria decreased to undetectable levels and lactic acid bacteria were greatly reduced. The colonization capacity of *C. coli* was favored by this antibiotic treatment. Field et al. (1984) have reported that adult mice could not be colonized with *C. jejuni* unless they had previously been treated with antibiotics. Other researchers have used this methodology to broaden the spectrum of the affected microbiota and to allow the colonization of *Campylobacter* (Lee et al. 1986; Giallourou et al. 2018). It has been shown that the depletion or alteration of the intestinal microbiota after an antibiotic treatment can overcome the resistance to colonization against *C. jejuni* produced by the specific microbiota of the murine host (Fauchere et al. 1985; Bereswill et al. 2011). Clinical, epidemiological and experimental studies suggest that the differences in the expression of *C. jejuni* pathogenicity are the result of a combination of bacterial strain properties and host factors (Mills et al. 2012). However, the understanding of the pathophysiology and the immune response to *C. coli* infection is severely restricted by the lack of an appropriate animal model. The absence of thermotolerant *Campylobacter* at the beginning of the experiment in all the mice allowed the evaluation of the evolution of colonization. Mice colonization was only verified through the animal's proportion with thermotolerant *Campylobacter* counts since the clinical signs of the infection and pathogen translocation were not found. Clinical disease or intestinal infection occasionally occurs in murine models (Chang and Miller 2006). All groups of inoculated mice were colonized; therefore, all the *C. coli* strains administered had that

colonizing capacity. The administered oral dose of *C. coli* strains in mice with an unbalanced intestinal microbiota was enough for an efficient establishment and reproducible colonization. The cecum represents a junction between a lower microbiota diversity within the small intestine and a higher microbiota diversity within the colon. That is the reason why the cecum functions as a bacterial reservoir both for the amount and diversity from the gut microbiota (Brown et al. 2018). In both in vivo studies, β-glucuronide-positive *E. coli* was not detected. This could be explained because from birth throughout the life of animals, some bacterial populations increase rapidly, reach extremely high levels, but then fall drastically within a few days, and can even disappear almost completely. *Escherichia coli*, enterococci and coliforms, when presenting a certain degree of infectivity, can establish themselves in the tissues, provoke a protective response and are eliminated or kept at a low population level under normal circumstances (Dubos et al. 1965). Furthermore, the sensitivity of the counting method used may have limited the detection of the low concentration of this microorganism. Despite the marked antibiotic effect generated, an important load of lactic acid bacteria was found in the small and large intestine at the end of the experiment. The less aggressive environment of the large intestine allowed the lodging of a greater microbial load, and *C. coli* was only found at that site. The *C. coli* DSPV 458 strain colonized the largest number of mice throughout the experiment; therefore, it could be used for future colonization models.

Conclusions

The inhibitory effect of *L. plantarum* LP5 demonstrated by the action of acid and other metabolites against different *C. coli* strains has rendered it a probiotic potential strain. *L. plantarum* LP5 tolerated the gastrointestinal conditions of the murine model without generating adverse effects on the animals. *C. coli* DSPV 458 colonized the mice without causing infection and was lodged in the digestive tract of the animals, thus generating a reproducible colonization model. Both models combined could be used as protection murine models against pathogens to test alternative control tools to antibiotics.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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