Research article

Characterization of *Lactobacillus fermentum* UCO-979C, a probiotic strain with a potent anti-*Helicobacter pylori* activity

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ABSTRACT

Background: Helicobacter pylori is considered as the main risk factor in the development of gastric cancer. In the present study, we performed a detailed characterization of the probiotic properties and the anti-*H. pylori* activity of a previously isolated lactobacillus strain — *Lactobacillus fermentum* UCO-979C — obtained from human gut.
Results: The strain tolerated pH 3.0; grew in the presence of 2% bile salts; produced lactic acid and hydrogen peroxide; aggregated in saline solution; showed high hydrophobicity; showed high adherence to glass; Caco-2 and gastric adenocarcinoma human cells (AGS) cells; showed an efficient colonization in Mongolian Gerbils; and potently inhibited the growth and urease activity of *H. pylori* strains. *L. fermentum* UCO-979C significantly inhibited *H. pylori*-induced IL-8 production in AGS cells and reduced the viability of *H. pylori*. With regard to innocuousness, the strain UCO-979C was susceptible to several antibiotics and did not produce histamine or beta-haemolysis in blood agar containing red blood cells from various origins.
Conclusion: The results demonstrated that *L. fermentum* UCO-979C is a very good candidate as a probiotic for the protection of humans against *H. pylori* infections.

1. Introduction

Probiotics are defined as live microorganisms that can confer beneficial properties for health when consumed by individuals [1,2,3]. To be considered as probiotics, microorganisms must meet several requirements, including functional and safety aspects [4,5]. Probiotic bacteria must be non-pathogenic and should produce antibacterial substances to successfully compete with pathogens [6,7]. They should also be tolerant to gastric acid and bile salts, and should be able to colonize and persist in the gastrointestinal epithelium [8,9,10]. In addition, potential probiotic bacteria should meet safety aspects, including the absence of transmissible antibiotic-resistance genes [11] and the capacity to synthesize haemolysin or other toxic compounds such as nocive biogenic amines (histamine, tyramine, putrescine, cadaverine phenylethylamine or tryptamine) [12]. However, if biogenic amines are produced, they must be present at low levels [4, 13,14].

Human probiotic bacteria that exert beneficial effects on the gastrointestinal tract include members of the genus *Lactobacillus* [2,10, 15]. These gram-positive bacteria are members of the normal human microbiota [1] and are considered GRAS (generally recognized as safe) microorganisms [16]. *Lactobacillus* strains have been successfully used in prophylactic or therapeutic treatments of several diseases including gastric disorders, inflammatory syndrome, bowel syndrome, gastric cancer, or *Helicobacter pylori* infection [16,17,18]. Because *H. pylori* colonizes in the gastric tract and is a causative agent of gastric diseases, it is not surprising that several studies were carried out on the effects of probiotics on *H. pylori*. Numerous *in vitro* studies demonstrating bacterial inhibition were followed by preclinical and clinical studies. These studies showed promising results of the use of probiotics against *H. pylori* when administered alone or administered together with the eradication treatment [18,19].

Considering that the Chilean population shows a high prevalence of *H. pylori*-infected individuals [20,21] and of gastric cancer [22], our laboratory has focused on the search of new and effective prophylaxis

strategies against *H. pylori* infection. Presently, no vaccine is available for the prophylaxis against *H. pylori*, and eradication therapy shows increased failure mainly due to clarithromycin resistance of the clinical isolates [23,24,25]. Thus, two fields of research arise as promissory against *H. pylori* infection: the detection of natural compounds with anti-*H. pylori* activity [26,27] and the isolation of probiotic bacteria with anti-*Helicobacter* activities, such as *Lactobacillus* spp. [18,24].

The present study aimed to establish a detailed characterization of the probiotic properties and the anti-*H. pylori* activity of a previously isolated lactobacillus strain — *Lactobacillus fermentum* UCO-979C — obtained from human gut.

2. Material and methods

2.1. Isolation and identification of L. fermentum UCO-979C

Lactobacillus UCO-979C was isolated from a human gastric biopsy in the year 2007 [17]. The strain was identified as *L. fermentum* (95.0%) through an API 50 CHL V5.2 kit. The final identity of the strain as *L. fermentum* was confirmed by the complete genome sequencing [28].

2.2. Control strains

Lactobacillus rhamnosus GG (ATCC 53103), *Lactobacillus johnsonii* La1 and *Lactobacillus casei* Shirota reference strains were used as control for the assay. The Shirota strain was isolated from a yogurt sample (Yakult) obtained from a local supermarket at Montevideo, Uruguay. Strains GG and La1 were kindly provided by Dr. Martin Gotteland (University of Chile, Santiago, Chile).

Unless otherwise stated, all strains of *Lactobacillus* spp. were grown in MRS (Man-Rogosa-Sharpe, Difco, France) agar or broth at 37°C under microaerophilic conditions (10% CO_2) for 48 h.

2.3. Physiological and functional properties of L. fermentum UCO-979C

2.3.1. Acid and bile tolerance

Acid tolerance was investigated as described by Kaushik et al. [29] with minor modifications. Briefly, 100 µL of a 24-h liquid culture of *L. fermentum* UCO-979C or controls (*L. rhamnosus* GG or *L. casei* Shirota) in MRS broth was inoculated in 10 mL of MRS adjusted to pH 2 or 3. Cultures were incubated at 37°C under 10% CO_2 atmosphere, and aliquots were obtained at 0, 1, 2, 3, and 24 h of incubation to determine viable bacterial counts by the micro-drop assay [30]. For studying bile tolerance, a similar assay was performed. MRS broth was supplemented with 1.5 or 2.0% Oxgall bile salt (Neogen, USA) [29]. Strains maintaining their viable count or growth ability in the presence of bile salts were considered as tolerant.

2.3.2. Hydrophobicity

An organic solvent partitioning assay (MATH: Microbial Adhesion To Hydrocarbons [31]) was used to re-evaluate hydrophobicity as described previously [17]. Briefly, UCO-979C strain or controls (*L. rhamnosus* GG and *L. johnsonii* La1) were grown in MRS broth for 24 h, collected by centrifugation at 5000*g* for 5 min, and suspended in phosphate buffered saline (PBS) supplemented with 3 M urea and 0.8 mM $MgSO_4$ on glass tubes at OD_{400nm} 0.8–1.0 and OD_{600nm} 0.4–0.6. The bacterial suspension (1.2 mL) was mixed with 0.3 mL of xylene, thoroughly shaken in a Vortex for 2 min and allowed to stand for further 10 min. The aqueous phase was separated and the OD at 400 nm and 600 nm were measured. The hydrophobic percentage (% H) was calculated using Ocaña et al. criteria [32] for classifying the strains as high hydrophobicity (% H: 71–100), medium hydrophobicity (% H: 36–70) or of low hydrophobicity (% H: 0–35).

2.3.3. Saline aggregation test

This test was performed according to Ocaña et al. [32] without modifications. Twenty microliters of a phosphate buffered saline (PBS) cell suspension (1×10^9 CFU mL^{-1}) of *L. fermentum* UCO-979C strain or controls (*L. rhamnosus* GG or *L. johnsonii* La1) was mixed with 20 µL of 2 M ammonium sulphate on the surface of a glass slide, allowed to stand for 2 min before sealing the sample with a coverslip. Samples were analysed using a light microscope to determine the saline aggregation status of the strains and classified as non-aggregative, medium aggregative and highly aggregative [32].

2.3.4. Glass adherence

L. fermentum UCO-979C strain or controls (*L. rhamnosus* GG or *L. johnsonii* La1) were grown as described below. Cells were collected by centrifugation and suspended in 20 mL MRS broth at a cellular density equivalent to McFarland No.2 and incubated for 1 h. Sterile glass slides were introduced into the culture flask and incubated for 4 h, stained with crystal violet (0.1% w/v) for 5 min, washed and dried for 10 min at room temperature. Samples were observed under a light microscope (100× objective lens). The strains were classified as non-adherent (less than 20 bacterial cells per field), mildly adherent (20–50 bacterial cells per field), and strongly adherent (over 50 bacterial cells per field).

2.4. Innocuousness assays for L. fermentum UCO-979C

2.4.1. Antibiotic susceptibility test

Susceptibility of *L. fermentum* UCO-979C and *L. rhamnosus* GG to the antibiotics amikacin, ampicillin, amoxicillin, cefotaxime, cefuroxime, ciprofloxacin, clarithromycin, chloramphenicol, erythromycin, streptomycin, gentamicin, kanamycin, neomycin, levofloxacine, benzylpenicillin, rifampicin, sulphamethoxazole/trimethoprim, tetracycline and vancomycin were determined by an agar diffusion test as described previously [33]. The criteria of Tang et al. [34] and Georgieva et al. [35] were used for classifying the strains as susceptible or resistant to each antibiotic.

2.4.2. Determination of biogenic amines

Sample preparation was performed in 1 mL glass reaction vial. For this purpose, 100 µL of culture supernatant filtrate of *Lactobacillus* UCO-979C strain or *L. casei* Shirota was dansylated by adding 400 µL of carbonate–bicarbonate buffer (pH 10), 300 µL of acetone and 200 µL of Dns-Cl solution. The mixture was vortexed for 30 s and then incubated at 47°C for 60 min. This solution was filtered through a 13-mm PVDF syringe mounted filter (0.45 µm) before HPLC injection. Chromatography was performed using a Waters HPLC system (Milford, USA) consisting of a 600 controller binary pump, a 717 plus autosampler, a 2475 multi-λ fluorescence detector, a 5CH column oven and a VWR international L-7614 online degasser (West Chester, Pennsylvania). Separation was carried out on a Waters C_{18} column YMC-Pack ODS-A (150 mm × 4.6 mm, 5 µm) set at 45°C using methanol (A) and water (B) as mobile phase. The flow rate was of 1.2 mL min^{-1} achieving a complete separation of all biogenic amines under study in 45 min. Detection was performed by fluorescence using 330 nm and 520 nm as excitation and emission wavelengths, respectively.

2.4.3. Haemolysis assay

The strain UCO-979C was assayed for detecting haemolytic activity on blood agar using Columbia agar base supplemented with 5% blood from sheep, pig, and goat. The strain was incubated as described above, and the results were expressed as positive for β-haemolysis when a clear zone surrounding the colonies was observed, γ-haemolysis-producing strain if no haemolysis was observed surrounding the colonies or α-haemolysis-producing strain

when a green colour was observed surrounding the colonies [8]. Thus, α- and γ-haemolytic strains are considered non-haemolytic ones.

2.5. H. pylori inhibition by L. fermentum UCO-979C

The method of Sgouras et al. [33] with minor modifications was used to evaluate the anti-*H. pylori* 43504 activity [17]; two new reference strains (*H. pylori* J99 and G27) were also used. Agar plates containing 20 mL of Columbia agar (Oxoid, UK) supplemented with 5% defibrinated horse blood were inoculated with *H. pylori* (ATCC43504, J99 or G27 strain) by using a swab. Wells of 6 mm diameter were made and 50 μL of the *L. fermentum* UCO-979C inoculum at a cellular density of McFarland No. 2 was deposited in each well. Plates were incubated at 37°C for 72 h under microaerophilic conditions (Campygen, Oxoid, UK). To evaluate inhibition, the criteria of Gaudana et al. [36] were followed, with the scorings as follows: no inhibition (diameter of 1 mm or less), mild inhibition (diameter of more than 1 mm and up to 2 mm), strong inhibition (diameter of more than 2 mm and up to 5 mm), and very strong inhibition (diameters more than 5 mm). The final values of the inhibition diameters were obtained by subtracting the well size (6 mm) to the inhibition zone measured. All experiments were performed in triplicate. MRS broth was used as negative control and *L. casei* Shirota, *L. rhamnosus* LGG and *L. johnsonii* La1 were used as positive controls.

2.5.1. Death curves

The kinetics of *H. pylori* ATCC43504 inhibition by *L. fermentum* UCO-979C was determined as follows: *H. pylori* 43504 was cultivated on Columbia agar supplemented with 10% whole horse blood and incubated for 72 h at 37°C under 10% CO_2. The colonies obtained were suspended in 3 mL Brain–Heart Infusion (BHI) broth at McFarland No. 2. A 1.8-mL suspension was mixed with 200 μL of a cell-free supernatant of *L. fermentum* UCO-979C obtained by growing the strain for 24 h in MRS followed by filtration through a 0.22-μm diameter pore filter. Viable cell counts of *H. pylori* were determined at 0, 3, and 24 h (first experiment) and at 0, 0.5, 1, 1.5, 2, 4, 5, and 6 h (second experiment) after the challenge by removing 20 μL aliquots in triplicate at each sampling time and plating it in Columbia Agar supplemented with 10% horse blood. A killing curve with amoxicillin was used as control for bacterial death. Both sterile MRS broth and BHI broth were also used as control for measuring endogenous anti-*H. pylori* activity [37].

2.5.2. Urease inhibition assay

Urease inhibition assay was performed as described by Sgouras et al. [33] with minor modifications. A 1-mL suspension of McFarland No. 2 inoculum of *H. pylori* ATCC43504 grown in BHI broth was mixed with 100 μL of a cell-free supernatant obtained after filtering (using a 0.22-μm membrane) a 48-h culture of *Lactobacillus* UCO-979C strain. Amoxicillin (20 μg mL^{-1}) was used as positive control and Brain–Heart Infusion (BHI, Oxoid, UK) broth as negative control. Each suspension was incubated at 37°C under 10% CO_2 atmosphere and samples of 150 μL were obtained after 0, 3 and 24 h of incubation. A 100-μL suspension was mixed with 900 μL of 20% urea in 10 mM phosphate buffer, pH 6.5 and 0.012% phenol red. The mixture was incubated for additional 1 h and the absorbance at 550 nm was recorded to establish the percentage of urease inhibition.

2.5.3. Adherence and protection assays with AGS cells

AGS cell, an adherent human gastric adenocarcinoma epithelial cell line, was used to further evaluate *L. fermentum* UCO-979C strain. The adherence of *H. pylori* to AGS cells was performed using the phenol red method according to Pastene et al. [27]. Briefly, AGS cells were cultured in T-75 flasks containing Roswell Park Memorial Institute medium (RPMI) 1640 medium supplemented with 10% foetal calf serum (FCS) and 1% antibiotics solution (10 U/mL penicillin, 10,000 μg/mL streptomycin and 25 μg/mL amphotericin B) for 4 d until more than 90% confluence was reached. AGS cells were trypsinized (0.25% trypsin and 2.21 mM ethylenediaminetetraacetic acid (EDTA) solution), counted using trypan blue and a Neubauer chamber [28] and adjusted to a concentration of 5×10^3 CFU mL^{-1}.

The potential protection provided by *L. fermentum* UCO-979C to AGS cells infected by *H. pylori* was assessed by adhesion assays. AGS cells were cultured in a 96-well microplates and treated with the UCO-979C strain at different concentrations: 10^5, 10^6, 10^7, 10^8 or 10^9 CFU mL^{-1}. After 4 h, AGS cells were infected with *H. pylori* ATCC 43504 (McFarland No. 2). AGS cells infected only with strain ATCC 43504 were used as control. After 4 h of incubation, the supernatants were removed and conserved for cytokine analysis (-20°C) and, phenol red indicator was added (0.003% phenol red, 2% urea, pH 6.8). Finally, microplates were read using a spectrophotometer at 570 nm wavelength.

2.5.4. Regulation of IL-8 production

To determine the possible anti-inflammatory effect of *L. fermentum* UCO-979C on AGS cells during *H. pylori* ATCC 43504 infection, the levels of IL-8 were measured using an enzyme-linked immunosorbent assay (ELISA). The cellular supernatants obtained in the experiments as described in Section 2.5.3 were kept at -20°C to be used for IL-8 production analysis. After supernatants were thawed, IL-8 was quantified using the DuoSet kit (R&D Systems) following the manufacturer's instructions.

2.6. Analysis of antimicrobial compounds synthetized by L. fermentum UCO-979C

2.6.1. L-lactic acid determination

L-lactic acid stock solution was prepared in ultra-pure water at a concentration of 1 mg/mL and chromatography was performed on HPTLC plates from Merck, coated with 0.2 mm of silica gel 60 F$_{254}$. A calibration curve with L-lactic acid between 3 and 15 μg/mL was used.

The samples of *L. fermentum* UCO-979C, *L. rhamnosus* and *L. casei* Shirota were centrifuged at 15000g for 5 min, and the supernatant filtrated through a 0.45-μm pore diameter filter mounted in a syringe prior to be used in the assays. Samples (2–3 μL) and 3–15 μL standard of L-lactic acid were applied to the column by using an Automatic TLC Sampler (ATS4) from CAMAG (Muttenz, Switzerland).

Chromatography was performed using a mixture of diisopropyl ether:formic acid:water (80:15:5 v/v) as mobile phase. The plates were then dried under a stream of warm air. Lactic acid was detected after a post-chromatographic derivatization with an ethanolic solution of bromophenol blue (0.5 mg mL^{-1}) and 0.1 aqueous NaOH is carefully added until the colour of the solution changes to blue. The plates were then heated on a TLC plate heater (CAMAG) for 2 min at 100°C. Scanning was performed using a TLC Scanner 3 (CAMAG) in visible absorption mode at 430 nm. All instruments were controlled by WinCats software 1.4.2 Planar Chromatography Manager (CAMAG).

2.6.2. Hydrogen peroxide (H$_2$O$_2$) production

The semiquantitative method described by Felten et al. [38] was used for determining H_2O_2 production. Briefly, the strains of lactobacilli were inoculated on MRS agar supplemented with 3, 3′, 5, 5′-tetramethylbenzidine and H_2O_2, incubated during 48 h at 37°C under 10% CO_2 and the colour developed in colonies was recorded. A strain of *Enterococcus faecium* was used as a positive control.

The criterion applied for the interpretation of the results was as follows: negative synthesis (-), white colonies; low synthesis (+), light blue colonies; moderated synthesis (++), dark light blue colonies; and strong synthesis (+++), the colonies show a blue colour [38].

Table 1
Lactobacillus strains tolerance to pH 3.0.

Strain	Viable counts (CFU/mL) at pH 3.0					
	Before exposure	0 h	1 h	2 h	3 h	24 h
L. fermentum UCO-979C	1.5×10^8	8.0×10^6	8.3×10^6	5.4×10^{6a}	4.4×10^6	4.5×10^6
L. casei Shirota	1.0×10^8	7.2×10^7	6.2×10^7	7.1×10^{7a}	9.9×10^7	1.0×10^8
L. rhamnosus GG	1.9×10^6	2.4×10^6	2.7×10^6	2.9×10^{6b}	2.6×10^6	2.1×10^6

Different superscripts (a and b) indicate significant differences ($P < 0.05$). For details, see Material and methods.

2.7. Colonization assays in Mongolian gerbils

Six female Mongolian gerbils (*Meriones unguiculatus*) (24- to 28-weeks-old) were divided in two groups of three animals each: one used as the challenge group and the second was used as the control group. The gerbils were obtained from the closed colony kept at the Laboratory of Pharmacognosy (Universidad of Concepción, Chile). All experiments were carried out in compliance with the Guide for Care and Use of Laboratory Animals and approved by the Ethical Committee of Animal Care at the University of Concepción, Chile and in accordance with the U.K. Animals and associated guidelines, EU Directive.

During the days of study, the animals were kept in controlled environmental conditions with light dark cycles of 12 h, and isolated in plastic cages to minimize contact with environmental particles. All animals were fed with balanced conventional diet and sterilized water *ad libitum*. The animals were fasted 20 h before receiving inoculation of probiotic bacteria *L. fermentum* UCO-979C. Labelling of *L. fermentum* UCO-979C was performed by suspending bacterial pellets in PBS buffer to a concentration of McFarland No. 4. Then, 10 μL of fluorescein isothiocyanate (FITC) was added, and the suspension was incubated at 37°C in dark for 1 h. Bacterial suspensions were centrifuged twice at 5000 rpm for 5 min in PBS. The animals were anaesthetized by isoflurane inhalation (Baxter Forane 100%). Once animals were anaesthetized, 1 mL of FITC-labelled *L. fermentum* UCO-979C (McFarland No. 4) was inoculated orally into their stomach. The challenge group was inoculated with 1 mL of the strain UCO-979C (1.2×10^9 CFU mL^{-1}) labelled with FITC. The control group was inoculated with only 1 mL of PBS. The animals were euthanized after 10 h post-inoculation, and their stomachs were excised and observed under fluorescence using the UVP i-Box Scientia Small Animal Imaging System (California, USA).

2.7.1. L. fermentum UCO-979C bacterial counts

For bacterial counts, another group of six gerbils was used. The animals were managed and challenge as mentioned above, but in this case, the strain was not labelled with FITC. Pyloric antrum and corpus samples were aseptically obtained 14 d after the inoculation; the samples were homogenized with PBS, and 20 μL of macerated organs was collected and used for microdroplet technique on MRS agar. The samples were incubated at 37°C for 24 h under microaerophilic conditions (10% CO_2) and bacterial counts were determined.

Table 2
Lactobacillus strains tolerance to 2.0% bile salts.

Strain	Viable counts (CFU/mL)				
	Before exposure	0 h	1 h	2 h	24 h
L. fermentum UCO-979C	2.9×10^{6a}	6.4×10^{6a}	4.2×10^{6a}	4.2×10^{6a}	2.6×10^{7b}
L. casei Shirota	4.1×10^6	4.9×10^6	6.1×10^6	6.3×10^6	3.8×10^6
L. rhamnosus GG	3.1×10^6	5.9×10^6	3.9×10^6	7.1×10^6	3.0×10^7

Different superscripts (a and b) indicate significant differences ($P < 0.05$). For details, see Material and methods.

2.8. Statistical analysis

Experiments were performed in triplicate, and the results were expressed as mean ± standard deviation (SD). After verification of the normal distribution of data, 2-way ANOVA was used. Tukey's *post hoc* (for pair wise comparisons of the means) was used to test for differences between the groups. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Physiological and functional properties of L. fermentum UCO-979C for its potential use as probiotic

Several physiological properties of *L. fermentum* UCO-979C related to its probiotic potential were evaluated, including tolerance to acid and bile, hydrophobicity, saline aggregation and *in vitro* adherence. Bile and pH tolerance tests showed that *L. fermentum* UCO-979C had a similar behaviour compared to the control strains *L. casei* Shirota and *L. rhamnosus* GG. All the strains were susceptible to pH 2 since the acidic medium inhibited bacteria prior to 1 h of incubation (data not shown). In contrast, all the strains remain viable after 24 h of incubation at pH 3 (Table 1). A sudden decrease of two logarithms in the count of viable *L. fermentum* UCO-979C was consistently detected at the beginning of the exposure to pH 3. No statically significant differences ($P < 0.05$) were observed among the viable counts of the UCO-979C strain during the 24 h of exposure to pH 3. In addition, bile tolerance test indicated that all the strains were resistant to 2.0% of bile salts (Table 2).

L. fermentum UCO-979C showed both high hydrophobicity and positive saline aggregation (Table 3), indicating that this strain could have an increased capacity for cellular adherence. In fact, *L. fermentum* UCO-979C was highly adherent in the glass assay and to Caco-2 cells in culture, similar to *L. rhamnosus* GG and *L. johnsonii* La1.

3.2. Safety assessment of L. fermentum UCO-979C for its potential use as probiotic

To evaluate the safety of *L. fermentum* UCO-979C, antibiotic resistance, haemolytic activity and production of biogenic amines were studied. The probiotic strain was susceptible to all the 19 antibiotics assayed with the exception of benzylpenicillin (Table 4), suggesting that this strain does not harbour resistance plasmids. On the other hand, the commercial probiotic strain *L. rhamnosus* GG used as control showed resistance to vancomycin, kanamycin, streptomycin and sulphamethoxazole/trimethoprim (Table 4).

Table 3
Physiological properties of *Lactobacillus* strains.

Assay	*L. fermentum* UCO-979C	*L. johnsonii* La1	*L. rhamnosus* GG
Hydrophobicity	Strong	Strong	Moderate
Saline aggregation test	Positive	Negative	Negative
Glass adherence	++	++	++

Table 4
Antibiotic susceptibility profile of *Lactobacillus* strains.

Antibiotic	(µg)	Strains			
		L. fermentum UCO-979C		*L. rhamnosus* GG	
		C1	C2	C1	C2
Amikacin	30	S	S	–	MS
Amoxicillin	10	–	SS	–	MS
Ampicillin	10	–	SS	–	S
Cefotaxime	30	–	SS	–	SS
Cefuroxime	30	–	S	–	S
Ciprofloxacin	5	S	–	S	SS
Clarithromycin	5	–	S	–	SS
Chloramphenicol	30	S	SS	S	S
Erythromycin	15	–	S	S	SS
Streptomycin	10	S	S	R	MS
Gentamicin	10	S	S	S	S
Kanamycin	30	–	S	–	R
Levofloxacin	5	–	S	–	SS
Neomycin	30	–	S	–	S
Penicillin G	10	R	MS	S	S
Rifampicin	5	–	S	–	SS
Sulfatrimethoprim	25	S	S	R	R
Tetracycline	30	S	SS	S	SS
Vancomycin	30	–	MS	R	R

C1: criterion 1, Tang et al. [34]; C2: criterion 2, Georgieva et al. [35]; MS: moderately susceptible; S: susceptible; SS: strongly susceptible; R: resistant.

Our strain UCO-979C showed α-haemolysis in blood agar assays, which is indicative of a non-β-haemolytic strain according to the criteria of Peres et al. [8].

As shown in Table 5, the total content of biogenic amines was 5.43 and 8.59 mg/L for *L. casei* Shirota and *L. fermentum* UCO-979C, respectively. The Bonferroni statistical test revealed no significant differences ($P > 0.05$) between Shirota and UCO-979C strains. Interestingly, histamine, which is the most important biogenic amine, was not detected in any of the strains.

3.3. Anti-H. pylori activity of L. fermentum UCO-979C

The UCO-979C strain showed the highest antibacterial activity against the three *H. pylori* strains used as indicators. Moreover, the antibacterial activity of *L. fermentum* UCO-979C was higher than the activity of the reference *L. rhamnosus* GG, *L. casei* Shirota and *L. johnsonii* La1 strains (Table 6). Inhibition kinetics studies further showed that *L. fermentum* UCO-979C exhibited an antibacterial activity against *H. pylori* ATCC 43504 that was similar to that shown by amoxicillin used as positive control (Fig. 1a). Thus, in accordance with the bacterial death criteria of Pearson et al. [37], the effect of UCO-979C strain at 24 h can be considered as lethal for *H. pylori*. On the other hand, after repeating this assay at shorter time intervals and by using the same Pearson criteria [37], the inhibition of *H. pylori* was still observed after 4 h of treatment with *L. fermentum* UCO-979C (Fig. 1b).

Table 5
Biogenic amine content in MRS broth.

Biogenic amines (mg L^{-1})	Strains	
	L. fermentum UCO-979C	*L. casei* Shirota
2-Phenylethylamine	2.70 ± 0.16	1.07 ± 0.05
Putrescine	2.34 ± 0.29	1.56 ± 0.19
Cadaverine	1.44 ± 0.07	1.74 ± 0.11
Histamine	ND	ND
Tyramine	0.44 ± 0.04	0.53 ± 0.12
Spermidine	TR	TR
Spermine	1.68 ± 0.49	0.53 ± 0.01
Total	8.59	5.43

ND: not detected, TR: traces (not quantifiable).

Table 6
Antimicrobial activity spectrum exhibited by various lactobacilli on *Helicobacter pylori* strains.

Lactobacillus strains	*Helicobacter pylori* strains					
	ATCC43504		J99		G27	
L. rhamnosus GG	2.3 ± 0.05[a]	++	2 ± 0.15	+	3 ± 0.12	++
L. casei Shirota	3.8 ± 0.05	++	3.5 ± 0.05	++	4 ± 0.1	++
L. johnsonii La1	2.7 ± 0.05	++	2.5 ± 0.15	++	3 ± 0.05	++
L. fermentum UCO-979C	5.1 ± 0.05	+++	5 ± 0.15	++	6 ± 0.1	+++

The inhibition zones 1 mm, 2 mm, 2–5 mm and more than 5 mm were classified as strains of no (+/-), mild (+), strong (++) and very strong (+++) inhibition, respectively (Gaudana et al. criterion [36]).
[a] Results are expressed as mean ± standard deviation of three independent biological replicates.

Urease activity of *H. pylori* ATCC43504 showed a slight decrease in the presence of amoxicillin (Fig. 2). This inhibition effect was more intense when the cell-free supernatant obtained from a culture of *L. fermentum* UCO-979C was used, showing that a diffusible molecule should be present in this supernatant.

The ability of *L. fermentum* UCO-979C to inhibit *H. pylori* adhesion was evaluated in AGS cells. The results demonstrated that the UCO-979C strain could decrease *H. pylori* adhesion to AGS cells in a dose-dependent manner (Fig. 3). The lowest *H. pylori* adherence to AGS cells occurred when *L. fermentum* UCO-979C was used at a concentration of 10^8 or 10^9 CFU mL^{-1} ($P < 0.05$) (Fig. 3).

The capacity of *L. fermentum* UCO-979C to modulate IL-8 production in AGS cell was also evaluated. Treatment of gastric cells with the UCO-979C did not induce significant changes in the production of IL-8 (Fig. 4). Challenge of AGS cells with *H. pylori* ATCC 43504 significantly increased the levels of IL-8 in agreement with the capacity of the pathogen to mount a robust inflammatory response in gastric cells. However, when AGS cells were colonized with *L. fermentum* UCO-979C previous to *H. pylori* challenge, a significant decrease in IL-8 production was observed (Fig. 4).

3.4. Analysis of the production of antimicrobial compounds by L. fermentum UCO-979C

In vitro analysis showed that the lactic acid content in the growth medium of *L. fermentum* UCO-979C and *L. casei* Shirota was 3.149 mg/mL and 2.764 mg/mL, respectively, with no statistical differences among the strains studied. Similarly, both strains produced +++ H_2O_2, which according to the criteria of Felten et al. [38] is high, and no significant differences were observed between the two strains.

3.5. Colonization assays in Mongolian gerbils

Fluorescence studies demonstrated that *L. fermentum* UCO-979C strain adheres to the gastric mucosa of Mongolian gerbils (Fig. 5). This adhesive capacity of the UCO-979C strain was confirmed by the determination of bacterial counts. Animals were initially inoculated with 1.2×10^9 CFU mL^{-1} of the UCO-979C strain and after 14 d, we detected a count of 2.5×10^7 and 2.8×10^7 CFU mL^{-1} in the pyloric antrum and corpus of the gerbil stomachs, respectively.

4. Discussion

Among the pathogenic bacteria that can colonize and affect the human stomach, *H. pylori* is the most important pathogen. *H. pylori* selectively colonizes stomach epithelium, and in some individuals, early infection and persistence of bacteria cause chronic gastric inflammation and tissue damage, changes that could progress to severe diseases such as peptic ulcer or gastric adenocarcinoma [38]. In
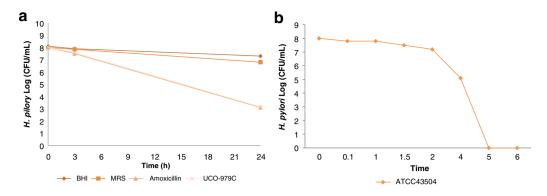
Fig. 1. Inhibition kinetics of *Lactobacillus fermentum* UCO-979C strain against *Helicobacter pylori* ATCC 43504. (a) Samples taken at 3 different times from 0 to 24 h. (b) Samples taken at 6 different times from 0 to 6 h.

addition to pathogenic bacteria, the combination of traditional analytical tools and culture-independent molecular methods has shown wide diversity of the bacterial microbial ecosystem of the stomach. This fact shows that although the extremely acidic environment of the stomach could theoretically prevent bacterial colonization, it does not mean that some bacteria have not adapted to these extreme conditions, such as acidophilus bacteria that require or can survive at low pH [39,40]. This fact implies that the microbiota of the gastric mucosa can be an interesting source of potential probiotic strains with the ability to prevent or reduce the colonization of pathogens such as *H. pylori*.

Interestingly, several studies were carried out on the effects of beneficial probiotic bacteria on *H. pylori*. Numerous *in vitro* studies demonstrating bacterial inhibition were followed by preclinical and clinical studies that indicated only partial efficacy of probiotics against *H. pylori* when administered alone, but increase in efficacy and/or reduction of side effects when probiotics were administered together with the eradication treatment [19]. Moreover, few studies were performed with probiotics strains isolated from human stomach. In the present study, we aimed to characterize a potential probiotic property of *L. fermentum* strain isolated previously from a human stomach.

To be considered as a potential probiotic for human use, bacterial strains must comply with a series of characteristics grouped into two main categories: functional properties and innocuousness or safety. Several studies have been performed to determine whether the candidate probiotic strain is not toxic and whether it possesses the claimed properties [4,3,10]. Therefore, we studied several characteristics of *L. fermentum* UCO-979C to determine its safety and functional capacities.
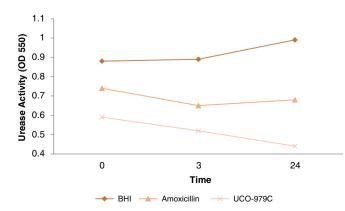
The present study demonstrated that the UCO-979C strain was resistant to one antibiotic: penicillin G; this would be an undesirable property [23,41]. Nevertheless, penicillin resistance has been observed in diverse *Lactobacillus* strains, and it is usually codified in the chromosome, suggesting a low probability of transferring it to other bacterial cells [42]. Additionally, the capacity of the UCO-979C strain to produce biogenic amines was also evaluated. The increase in the levels of biogenic amines in serum, especially histamine, is deleterious for human health. These amines can cause several detrimental effects including hypotension, hypertension, nausea, diarrhoea, headache, and respiratory or skin problems [43,44,45]. It must be emphasized that *L. fermentum* UCO-979C did not produce detectable levels of histamine. These results clearly indicate that *L. fermentum* UCO-979C could be used as a safe probiotic microorganism.

Tolerance to acid and bile salts are desirable properties in bacterial species to be considered as human probiotics because of the stress conditions found in the gastrointestinal tract of humans. In this regard, probiotic microorganisms thought to colonize the stomach region should be tolerant to pH 3 [6,40] or, at least, tolerant to pH 4, which is the pH of the gastric mucus layer. New species belonging to the genus *Lactobacillus* sp. isolated from human stomach mucosa have been described (*Lactobacillus gastricus*, *Lactobacillus antri*, *Lactobacillus kalixensis* and *Lactobacillus ultunensis*) that show high resistance to acidic conditions allowing them to persist in this rigorous environment [46,47]. As shown here, *L. fermentum* UCO-979C strain survived at pH 3 for 24 h and it had a similar behaviour to that observed for other probiotics strain such as *L. rhamnosus* GG with respect to its tolerance to pH and bile salts. Moreover, the *in vivo* experiments in Mongolian gerbils clearly demonstrated the capacity of the UCO-979 strain to colonize and survive in the gastric mucosa.

Fig. 2. Inhibitory activity of *Lactobacillus fermentum* UCO-979C strain over the activity of *Helicobacter pylori* urease. Samples taken at times 0, 3 and 24 h.
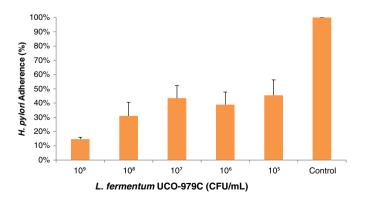

Fig. 3. Percentage of *Helicobacter pylori* (ATCC 43504) adherence on AGS cells previously colonized by *Lactobacillus fermentum* UCO-979C (preventive treatment) strain at different bacterial concentrations.
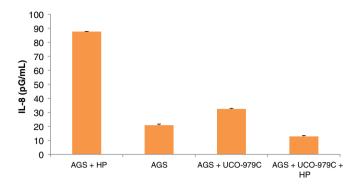
Fig. 4. Determination of IL-8 levels produced by AGS cells infected with *H. pylori* ATCC 43504 or/and colonized with *Lactobacillus fermentum* UCO-979C and non-infected (control) by using an ELISA assay.

We demonstrated that *L. fermentum* UCO-979C has several characteristics making it an excellent candidate as a probiotic to prevent infection by *H. pylori*. The UCO-979C strain inhibited bacterial pathogen growth, reduced *H. pylori* urease activity, decreased pathogen adhesion to gastric cells and beneficially regulated the inflammatory response. There are several reports describing probiotic strains with anti-*H. pylori* activity through the production of antimicrobial compounds such as bacteriocins, autolysins and organic acids [7,48]. In this regard, inhibition of *H. pylori* by the production of lactic acid has been reported in *Lactobacillus salivarius*, *Lactobacillus acidophilus*, *L. rhamnosus* and *L. casei* strain Shirota [49], in both *in vitro* and animal studies. Lactic

acid, in addition to its antimicrobial effect by the decrease in pH, could inhibit the urease enzyme of *H. pylori*.

L. fermentum UCO-979C could reduce colonization of *H. pylori* and decrease the production of the inflammatory chemokine IL-8 in human gastric epithelial cells. Human immune response has an important role in the development of serious diseases after *H. pylori* infection since increased pro-inflammatory cytokine expression was found in the gastric mucosa of infected patients [50]. Regulation of *H. pylori*-induced inflammation was reported to play important roles in the prevention of chronic gastric damage and cancer. *H. pylori* infection can activate NF-$_k$B in gastric epithelium cells and subsequently up-regulate IL-8 gene transcription that greatly contributes to disease evolution. On the other hand, some probiotics, including lactobacilli, have previously been shown to decrease inflammatory markers in *H. pylori* infection models both *in vitro* and *in vivo* [51,52]. Here, we showed that *L. fermentum* UCO-979C could induce a significant reduction in IL-8 production in *H. pylori*-infected cells, indicating its potential as a beneficial immunomodulator. More detailed studies about *L. fermentum* UCO-979C immunomodulatory properties would allow us to include this strain in the group of bacteria known as immunobiotics [53,54].

In conclusion, the results presented here indicate that *L. fermentum* UCO-979C is a good candidate for use as human probiotic for gastric protection against *H. pylori*, since this strain can tolerate the stress conditions of this habitat in addition to its capacity to produce antimicrobial compounds and to beneficially modulate inflammatory response. In this regard, it should be noted that antimicrobial and immunomodulatory activities are independent properties. In fact, it was reported that some probiotic bacteria have a positive effect on
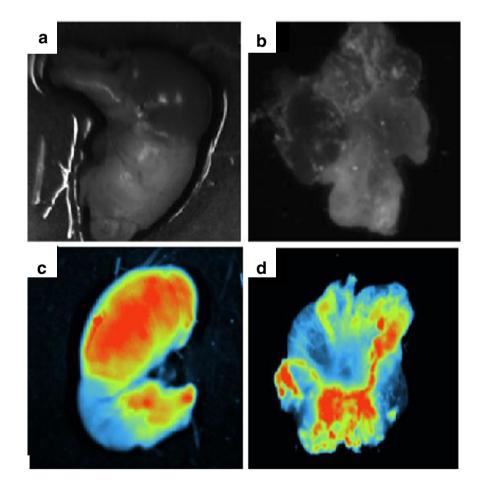

Fig. 5. Adherence of *Lactobacillus fermentum* UCO-979C strain to the stomachs of Mongolian gerbils detected by fluorescence. (a) and (b): control stomach; (c) and (d): fluorescence in the stomach caused by the adherence of the UCO-979C strain.

H. pylori-associated inflammation without clearing the infection [55,56]. *L. fermentum* UCO-979C that harbours both these properties has a high probability of functioning as an effective anti-*Helicobacter* probiotic.

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Conflict of interest

The authors declare that they have no conflict of interest in the present publication.

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