Curr Microbiol (2012) 64:371–378
DOI 10.1007/s00284-012-0080-0

Antagonistic Activity of *Lactobacillus acidophilus* ATCC 4356 on the Growth and Adhesion/Invasion Characteristics of Human *Campylobacter jejuni*

Raffaella Campana · Sara Federici · Eleonora Ciandrini · Wally Baffone

Received: 19 October 2011 / Accepted: 22 December 2011 / Published online: 22 January 2012

Abstract The aim of this research was to determine the potential probiotic activity of *Lactobacillus acidophilus* ATCC 4356 against several human *Campylobacter jejuni* isolates. The ability to inhibit the pathogen's growth was evaluated by co-culture experiments as well as by antimicrobial assays with cell-free culture supernatant (CFCS), while interference with adhesion/invasion to intestinal Caco-2 cells was studied by exclusion, competition, and displacement tests. In the co-culture experiments *L. acidophilus* ATCC 4356 strain reduced the growth of *C. jejuni* with variable percentages of inhibition related to the contact time. The CFCS showed inhibitory activity against *C. jejuni* strains, stability to low pH, and thermal treatment and sensitivity to proteinase K and trypsin. *L. acidophilus* ATCC 4356 was able to reduce the adhesion and invasion to Caco-2 cells by most of the human *C. jejuni* strains. Displacement and exclusion mechanisms seem to be the preferred modalities, which caused a significant reduction of adhesion/invasion of pathogens to intestinal cells. The observed inhibitory properties of *L. acidophilus* ATCC 4356 on growth ability and on cells adhesion/invasion of *C. jejuni* may offer potential use of this strain for the management of *Campylobacter* infections.

Introduction

Campylobacter jejuni is well identified as a leading cause of bacterial-induced enterocolitis in humans in most countries across the globe [3, 31]. Typically, acute diarrhea develops ranging from watery stools to dysentery [37]. Some serious post-infectious sequelae, such as reactive arthritis, irritable bowel syndrome [16, 25], and the paralytic neuropathy Guillain–Barrè syndrome [36, 38], also have been associated with antecedent *C. jejuni* infections. Normally, *Campylobacter* infections are self-limiting and treatment with antibiotics is needed only in individuals with invasive or very severe disease. In these cases, therapy may be complicated by the fact that antimicrobial resistance in *Campylobacter* isolates from human infections has become increasingly common [4, 23, 33]. Thus, there is a need for alternative strategies that would complement currently employed methods aimed at reducing *C. jejuni*-induced disease burden in humans.

Lactobacilli are recently gaining increased attention due to their probiotic properties. In fact, when lactobacilli are administered in adequate quantities [12, 34], they should be an attractive alternative strategy to interrupt *C. jejuni* infection cycle and/or treatment of active *Campylobacter*-related disease, both in poultry and humans. The roles of probiotic bacteria include aid in lactose digestion, resistance to enteric pathogens, anticolon cancer effect, small bowel bacterial overgrowth, allergy and immune stimulation [18]. The proposed mechanisms of action of probiotics comprise competitive exclusion, alteration of the intestinal microbial communities, enhancement of host barrier defenses, and modification of host signaling [28, 30]. Many criteria have been suggested for the selection of probiotic strains, such as safety, tolerance to gastrointestinal conditions, ability to adhere to intestinal mucosa, and competition with pathogens [5, 8, 22]. In particular, the adhesion to the intestinal epithelium would allow the colonisation by probiotic microorganisms of the human intestinal tract [29] and this property has been related to immune system modulation [10, 15].

R. Campana · S. Federici · E. Ciandrini · W. Baffone (✉)
Department of Biomolecular Science, Division of Toxicology, Hygienic and Environmental Science, University of Urbino "Carlo Bo", via S. Chiara 27, Urbino, Italy
e-mail: wally.baffone@uniurb.it

The objective of our research was to the examine the antimicrobial effect of *L. acidophilus* ATCC 4356, a human isolate employed as dietary adjuncts in various cultured dairy products, on the growth of several human *C. jejuni* isolates and its interference with their adhesion/invasion to an intestinal cell line.

Materials and Methods

Bacterial Strains and Culture Conditions

Lactobacillus acidophilus ATCC 4356, a reference strain, which has shown competitive properties against some intestinal pathogens [7], was routinely grown on de Man–Rogosa–Sharpe (MRS) agar (Oxoid; Milan, Italy) at 37°C under microaerophilic condition (5% O_2; 10% CO_2; 85% N_2) and kept at −80°C in nutrient broth n° 2 (Oxoid) containing 15% glycerol. *L. acidophilus* ATCC 4356 was previously examined for adhesion to Caco-2 cells, survival at pH 2.5, and resistance to 0.3% bile salts conditions; the strain demonstrated remarkable ability to adhere to intestinal cells, as well as to survive to the intestinal artificial conditions (data not shown).

Nine *C. jejuni* strains (Hom 107, ISS 9, ISS 3, Hom 13, 241, ISS 1, Hom 88, Hom 14, Hom 7), previously isolated in the Pesaro-Urbino area (Central Italy) from human clinical samples, were used in this study. The strains were identified on the basis of their biochemical characteristics and biomolecular profiles [4]. The adhesion properties of all the *C. jejuni* strains were first determined on Caco-2 cell monolayers (data not shown). *C. jejuni* ATCC 33291 was also included in the study. All the strains were grown on Columbia agar base (Oxoid) supplemented with 5% of Laked Horse Blood (Oxoid) and Preston *Campylobacter* Selective Supplement (Oxoid) for 48 h at 42°C under microaerophilic conditions.

Antagonistic Activity in Co-culture

Co-culture inhibitory effect of *L. acidophilus* ATCC 4356 on each human *C. jejuni* isolate was performed as described by Chaveerach et al. [6] with several modifications. Mueller-Hinton broth (MH, Oxoid) was prepared and distributed (34 ml) into three bottles (each 100 ml) successively sterilized at 121°C for 15 min. These bottles were kept at 4°C until their use in co-culture experiments; briefly, 500 µl (10^6 cfu ml^{-1}) of each overnight culture of *C. jejuni* in MH broth (Oxoid) was aseptically transferred into Bottle 1; then 500 µl (10^6 cfu ml^{-1}) of the overnight culture of *L. acidophilus* ATCC 4356 grown in MRS broth (Oxoid) was transferred to the same Bottle 1. The controls of each strains of *C. jejuni* (500 µl) and *L. acidophilus* ATCC 4356 (500 µl) were separately transferred into Bottle 2 (MH broth) and Bottle 3 (MRS broth), respectively. The inoculated bottles were then incubated at 37°C. One-milliliter aliquots were taken aseptically at 0, 6, 12, 24, 30, 36, and 48 h, serially diluted and spread onto Columbia agar base (Oxoid) for *Campylobacter* cultivability and on MRS agar (Oxoid) plates for *Lactobacillus* cultivability. All the plates were incubated at 37°C under microaerophilic conditions for 24–48 h; at the end of incubation, the colonies were counted and expressed as colony forming units per milliliter (cfu ml^{-1}).The data reported represent mean values of three independent experiments; each experiment was performed in duplicate.

Agar Well Diffusion Method (AWDM)

AWDM was carried out according to Santini et al. [26] with several modifications. Previously, cells of *L. acidophilus* ATCC 4356 from an overnight culture were pelleted at 17,000×*g* for 15 min at 4°C and the supernatants (cell-free culture supernatant, CFCS), adjusted to pH 6.5 and 5.5 with 10 N NaOH, were collected and filtered (0.22 µm pore size) to remove any remaining bacteria. The aliquots of CFCSs were then kept at −20°C until use.

Four to six colonies were drawn from each plate of *C. jejuni* isolates to prepare bacterial suspensions in 30 ml of Brucella broth (Difco Laboratories, Detroit, MI, USA) and incubated by gentle shaking (150 rpm) at 42°C for 24 h under microaerophilic conditions. At this point, 500 µl of a *Campylobacter* culture at the concentration of 10^7 cfu ml^{-1} was added to 20 ml of Nutrient agar (Oxoid) maintained at 50°C, poured into petri dishes, and allowed to solidify for 20 min. Wells of 5 mm in diameter were made on the agar with sterile stainless steel cylinders and 50 µl of the CFCS were finally dropped into the holes. After 48 h incubation under microaerophilic conditions at 42°C, the diameter of the zone of inhibition around each hole was measured and the antimicrobial activity was expressed as the mean of inhibition diameters produced by CFCS.

To characterize the nature of any possible inhibitory effect generated by *L. acidophilus* ATCC 4356 and secreted to CFCSs, the AWDM was repeated by adding to different aliquots of CFCS the enzymes trypsin and proteinase K (2 mg ml^{-1}) (Sigma, Milan, Italy). To evaluate the thermal stability of the antimicrobial compounds, aliquots of CFCS were incubated at 80°C for 15, 20, and 60 min, and at 100°C for 10 min and then used in the AWDM as described above. The data reported represent mean values of three independent experiments; each experiment was performed in duplicate.

Cell Culture

Caco-2 (human colon adenocarcinoma) cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 15% fetal bovine serum (FBS, Pbi, Milan, Italy), 1% non-essential amino-acids (Sigma), and 1% antibiotics solution (5,000 U of streptomycin–penicillin; Sigma) at 37°C in a 5% CO_2 humidified atmosphere.

For the experimental assays Caco-2 cells, cultured for 14 days at 37°C in an atmosphere of 5% CO_2 to develop the characteristics of mature enterocytes, were seeded at 2×10^4 cells well^{-1} in 6-well plastic plates (IWAKI brand, Science Product Dept., Ashai glass Co., LTD, Japan) and grown until semi-confluence. Before the assays, the cell monolayers were washed twice with phosphate-buffered saline (PBS) pH 7.2.

Interference Studies

Interference studies were carried out based on the methods of Forestier et al. [13] with several modifications, in order to differentiate exclusion, competition, or displacement of *C. jejuni* strains from Caco-2 cell monolayers by *L. acidophilus* ATCC 4356. In brief, the bacteria were harvested by centrifugation (1,145×*g* for 15 min) from 37°C overnight incubation in MRS broth (Oxoid) for *L. acidophilus* ATCC 4356 and 42°C overnight incubation in MHB (Oxoid) for *C. jejuni* strains under microaerophilic conditions. After centrifugation, the pellets were resuspended in DMEM (Sigma) containing 1% FBS (Pbi) and adjusted spectrophotometrically to approximately 10^8 cfu ml^{-1}. These bacterial suspensions were finally utilized for the adhesion inhibition assays.

For the exclusion test, Caco-2 cell monolayers were incubated with 1 ml of *L. acidophilus* ATCC 4356 suspension (10^8 cfu ml^{-1}) for 1 h at 37°C in 5% CO_2. Afterward, non-adhering bacteria were removed by three washings with 1 ml of PBS, and 1 ml of *C. jejuni* suspensions (10^8 cfu ml^{-1}) was added to wells and incubated for another 4 h at 37°C in 5% CO_2. Then, unbound bacteria were removed by three washings with 1 ml of PBS and Caco-2 cells were lysed by the addition of 1 ml of a 0.05% Triton-X 100 solution. The adherent bacteria, representing the total bacteria associated with intestinal cells (both extracellular and intracellular pathogens), were enumerated by plating serial dilutions of the lysates on Columbia agar base (Oxoid) with 5% of Laked Horse Blood (Oxoid) and on MRS agar (Oxoid). Resultant colonies (cfu ml^{-1}) after 24–48 h of 42°C incubation under microaerophilic conditions were finally counted.

For the competition test, a 1-ml suspension of 50% *L. acidophilus* ATCC 4356 and 50% *C. jejuni* cells (10^8 cfu ml^{-1}, respectively) was added to Caco-2 cell monolayers and incubated for 4 h at 37°C in 5% CO_2. The infected cells were then washed with PBS and the experimental scheme described above for bacterial enumeration was carried out.

For the displacement test, *C. jejuni* suspensions (10^8 cfu ml^{-1}) were added to Caco-2 cell monolayers and incubated for 4 h at 37°C in 5% CO_2. Afterward, the unbound pathogens were removed by PBS washings and 1 ml of *L. acidophilus* ATCC 4356 suspension (10^8 cfu ml^{-1}) was added and incubated for 1 h at 37°C in 5% CO_2. Then, the wells were washed again and the bound bacteria were released and counted as described above.

Invasion inhibition assay was performed as described by Wine et al. [35] with slight modifications. In brief, the above described interference tests (exclusion, competition and displacement) were carried out adding in each well 1 ml of antibiotic-free culture media containing gentamicin (150 μg ml^{-1}) and incubating for another 2 h at 37°C. After this period, the cells were washed by PBS and lysed by addition of a 0.05% Triton-X 100 solution. The internalized bacteria were enumerated by plating serial dilutions of the lysates on Columbia agar base (Oxoid) with 5% of Laked Horse Blood (Oxoid) and on MRS agar (Oxoid); resultant colonies (cfu ml^{-1}) after 24–48 h of 42°C incubation under microaerophilic conditions were finally counted.

The data reported represent mean values of three independent experiments; each experiment of adhesion and invasion were performed in duplicate. For each interference assay a set of Caco-2 cells, infected only with *C. jejuni* strains, was included as control. The adhesion or invasion inhibition was calculated as the difference between the percentage of pathogen adhesion/invasion in absence and presence of the probiotic strain.

Statistical Analysis

Statistical analysis was performed using Prism version 5.0 (GraphPad Software, Inc., La Jolla, USA). The assumptions for parametric tests were checked prior to carry out the analyses. Since results of the interference assays did not follow the Gaussian distribution, Kruskal–Wallis non-parametric test and Dunn's multiple comparison test were utilized. In all the cases, significance was noted at $P < 0.05$.

Results

Inhibitory Activity in Co-Culture

In the co-culture experiments, *L. acidophilus* ATCC 4356 was able to inhibit the growth of *C. jejuni* isolates with

Table 1 Percentage growth inhibition of ten human *C. jejuni* strains in co-culture experiments with *L. acidophilus* ATCC 4356

C. jejuni strains	Percentage of growth inhibition						
	3 h	6 h	9 h	24 h	30 h	36 h	48 h
Hom 107	20.86	22.85	15.45	8.37	8.36	7.92	7.81
ISS 9	17.91	17.36	13.74	26.94	12.11	12.09	12.08
ATCC 33291	10.87	13.32	19.34	16.84	22.45	19.99	20.07
ISS 3	0.56	16.05	25.10	10.60	3.35	2.26	0.49
Hom 13	3.50	27.31	11.30	2.70	1.50	0.19	0.11
241	3.29	15.74	9.71	12.73	2.79	1.32	0.71
ISS 1	1.54	3.52	9.65	10.59	0.00	0.00	0.00
Hom 88	0.00	0.00	9.12	6.36	0.00	0.00	0.00
Hom 14	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Hom 7	0.00	0.00	0.00	0.00	0.00	0.00	0.00

The data reported represent mean values of three independent experiments; each experiment was performed in duplicate

The growth of pathogens in the absence of probiotic strain was assigned as 100%

The percentage of growth inhibition was expressed by the formula: [(cfu ml^{-1} in the control) − (cfu ml^{-1} in co-culture)/cfu ml^{-1} in the control] × 100

percentages of inhibition variable and related to the contact time between probiotic and pathogen strains. The percentages of growth inhibition, defined as reduction in percentage of recovered cfu ml^{-1} after co-culture incubation, are reported in Table 1. As shown, in most cases, the growth reduction of *C. jejuni* strains was obtained by 6, 9, and 24 h of co-culture, with the highest values of growth inhibition of 27.31, 25.10, and 26.94% for *C. jejuni* Hom 13, *C. jejuni* ISS 3, and *C. jejuni* ISS 9, respectively. *C. jejuni* ATCC 33291 strain exhibited a remarkable growth inhibition at all times of incubation, with an increased rate from 3 to 48 h of co-culture.

Antimicrobial Activity

The CFCS of *L. acidophilus* ATCC 4356 at pH 6.5 showed inhibitory activity against eight of the human *C. jejuni* strains (Table 2). The nature of the antimicrobial compound was determined by testing the effects of common proteolytic molecules (trypsin and proteinase K) on the inhibitory activity of CFCS against the pathogen strains, as well as the pH influence and the thermal stability. The exposure of CFCS adjusted to pH 5.5 demonstrated that the inhibitory compounds were stable to pH acid, in fact no diminution in inhibition diameters was observed. Moreover, CFCS was thermo-stable showing no relevant differences in the inhibitory activity of CFCS after treatment at 80°C for 15, 20, and 60 min and at 100°C for 20 min. On the other hand, the treatments by trypsin and proteinase K diminished the antimicrobial activity of CFCS at the enzymatic concentrations assayed. These findings let us consider that the inhibitory action can be due to a proteinaceous molecule.

Adhesion and Invasion Inhibition

Since the ability of *C. jejuni* strains to invade epithelial cells is cell type dependent [35], we determined the capacity of *L. acidophilus* ATCC 4356 to inhibit *C. jejuni* adhesion and invasion in human colon cells using exclusion, competition, and displacement tests. The results, reported in Table 3, indicated that *L. acidophilus* ATCC 4356 was able to reduce the adhesion and invasion of most human *C. jejuni* strains to Caco-2 cells. As shown, the total association of *C. jejuni* strains with Caco-2 cells decreased in the interference tests with *L. acidophilus* ATCC 4356, and this effect was more evident in the displacement test, where a decrease of adherent bacteria between 10.88 and 52.66% ($P < 0.05$) was observed.

In regard to the invasion inhibition ability of *L. acidophilus* ATCC 4356 toward *C. jejuni* human strains, the measure of invasiveness, representing the internalized bacteria, was obtained after gentamicin killing of extracellular *C. jejuni*. The displacement test showed the most pronounced effect, with a detectable decrease of cfu ml^{-1} values between 11.25 and 52.34% ($P < 0.05$), less evident with the other two interference tests (Table 3).

Discussion

The initial screening of strains using in vitro methods remains a useful preliminary step in the detection of probiotic candidates, despite the difficulties encountered to characterize reliable probiotic strains in this way.

Lactobacillus acidophilus ATCC 4356, a strain of human origin, as many species of the genus *Lactobacillus*,

Table 2 Antimicrobial activity of *L. acidophilus* ATCC 4356 cell-free supernatants (CFCSs) toward *C. jejuni* human strains performed by agar well diffusion method

C. jejuni strains	Antimicrobial activity of *L. acidophilus* 453 CFCS*							
	pH		Enzymes		Heat treatments			
	6.5	5.5	Trypsin	Proteinase K	80°C for 15 min	80°C for 20 min	80°C for 60 min	100°C for 20 min
Hom 107	++	++	±	±	++	+	+	+
ISS 9	++	++	+	+	++	++	++	++
ATCC 33291	++	++	+	+	+	+	+	+
ISS 3	++	++	+	+	++	++	++	++
Hom 13	+	+	±	±	+	+	+	+
241	++	+	+	+	+	+	+	+
ISS 1	+	+	±	±	+	+	+	+
Hom 88	++	++	+	+	++	++	++	++
Hom 14	−	ND	ND	ND	ND	ND	ND	ND
Hom 7	−	ND	ND	ND	ND	ND	ND	ND

Antimicrobial activity was detected as zone of inhibition with widths of (−), no inhibition; (±), <8 mm; (+), 8–10 mm; (++) >10 mm

ND not determined because no inhibition zone was observed by CFCS at pH 6.5

* The CFCSs were utilized at pH 6.5 and 5.5, with the addition of enzymes (trypsin and proteinase K) and after heat treatments

possesses an S-layer, which functions include it being a protective sheath against hostile environmental agents and having an important role in the establishment of *L. acidophilus* in the gastrointestinal tract [2, 9, 20]. Moreover, in previous studies, a new enzymatic functionality for the surface layer (S-layer) of *L. acidophilus* ATCC 4356 was described to control bacterial growth in Gram-negative bacteria [7]. As with other lactobacilli, this strain can produce H_2O_2, which can inhibits or kills other microbes and pathogens, particularly those that lack or have low levels of H_2O_2-scavenging enzymes [11]. In our study, *L. acidophilus* ATCC 4356 demonstrated some probiotic characteristics, such as survival under low pH conditions and tolerance to bile acids under in vitro conditions.

To better understand the probiotic profile of *L. acidophilus* ATCC 4356, its effect on the growth of several human *C. jejuni* isolates and interference with adhesion/invasion characteristics of these pathogens were determined.

Several mechanisms for pathogen inhibition have been suggested; for example, some metabolic products from lactobacilli, such as lactic acid and/or bacteriocins, may inhibit the growth of pathogenic bacteria [28], while some other products may enhance the systematic immune response, activating macrophages activity. Since the antimicrobial activity of lactic acid strains is known to be multifactorial [28], the possibility that mechanisms underlying *L. acidophilus* activity against *Campylobacter* strains involve both lactic acid-producing strains and secreted non-lactic acid molecules acting synergistically cannot be excluded. In this report, the ability of the *L. acidophilus* ATCC 4356 strain to inhibit the in vitro growth of human *C. jejuni* strains was evaluated in co-culture experiments and by AWDM with CFCS. These experiments showed the antagonistic activity of *L. acidophilus* ATCC 4356 toward *C. jejuni* strains. In the co-culture studies, a decrease of the inhibitory effect on growth for all the pathogens, but *C. jejuni* ATCC 33291, over time was observed. Since in the literature few data are reported on co-culture experiments using *Lactobacillus* spp. and *C. jejuni,* our results could be compared only with those of Chaveerach et al. [6]. These authors investigated the inhibitory effect of *Lactobacillus* P93 and *Enterococcus* P67 on a mixture of ten *C. jejuni* strains and *Lactobacillus* P93 and *Lactobacillus* P104 just on one strain (*C. jejuni* C2150); in opposition to us, they pointed out a steady growth inhibition, with a dramatic decline below the detection limit after 48 h incubation. On the other hand, in contrast to other authors [6], we observed that the inhibitory effect of *Lactobacillus* ATCC 4356 on the growth of the pathogens by AWDM was not enhanced at low pH level. The bactericidal effect against *Campylobacter* probably results from the production of organic acids, as already evidenced by Chaveerach et al. [6]. In our case, the CFCSs inhibitory activity may not depend on medium acidification, since in our experiments a pH 6.5 CFCS was used; the observed effect might be due to the production of a proteinaceous molecule, as confirmed by the loss of CFCSs activity after protease treatment. Moreover, because of catalase activity of our *C. jejuni* strains, the inhibitory activity of *Lactobacillus* ATCC 4356 is not mainly caused by hydrogen peroxide, but by a combination of organic acids and probably antimicrobial peptide

Table 3 Effect of *L. acidophilus* ATCC 4356 (LA) on adhesion and invasion abilities of *C. jejuni* human strains performed with three different interference tests

	C. jejuni strains (log cfu ml^{-1})									
	Hom 107	ISS 9	ATCC 33291	ISS 3	Hom 13	241	ISS 1	Hom 88	Hom 14	Hom 7
Interference test										
Exclusion										
Control (*C. jejuni* alone)	6.90 ± 4.78	6.78 ± 5.32	6.44 ± 5.41	6.34 ± 5.30	6.78 ± 6.28	6.46 ± 5.41	6.48 ± 5.12	5.60 ± 4.00	6.40 ± 5.70	6.71 ± 4.88
Adhesion[a] of *C. jejuni* + LA	6.01 ± 5.66	6.48 ± 5.00	6.21 ± 5.51	5.65 ± 4.58*	4.70 ± 3.97*	5.21 ± 4.84	6.42 ± 5.31	5.18 ± 4.79	5.23 ± 4.84	6.37 ± 4.30
Invasion[b] of *C. jejuni* + LA	5.90 ± 5.04	6.41 ± 5.72	6.01 ± 4.76	5.60 ± 5.43	4.14 ± 3.56	4.27 ± 3.87	6.43 ± 5.96	4.74 ± 4.12	5.27 ± 4.18	5.01 ± 3.76
Competition										
Control (*C. jejuni* alone)	6.90 ± 4.78	6.78 ± 5.32	6.44 ± 5.41	6.34 ± 5.30	6.78 ± 6.28	6.46 ± 5.41	6.48 ± 5.12	5.60 ± 4.00	6.40 ± 5.70	6.71 ± 4.88
Adhesion[a] of *C. jejuni* + LA	6.85 ± 4.66	6.70 ± 5.70	5.26 ± 3.85	5.88 ± 4.00	6.03 ± 6.00	6.18 ± 5.40	6.32 ± 5.06	4.48 ± 3.41*	5.21 ± 4.47	6.61 ± 6.04
Invasion[b] of *C. jejuni* + LA	6.85 ± 6.08	6.42 ± 5.85	5.07 ± 4.46	6.25 ± 6.00	6.52 ± 6.40	6.27 ± 5.62	6.24 ± 6.09	4.15 ± 3.41*	4.21 ± 3.86	6.31 ± 5.18
Displacement										
Control (*C. jejuni* alone)	6.90 ± 4.78	6.78 ± 5.32	6.44 ± 5.41	6.34 ± 5.30	6.78 ± 6.28	6.46 ± 5.41	6.48 ± 5.12	5.60 ± 4.00	6.40 ± 5.70	6.71 ± 4.88
Adhesion[a] of *C. jejuni* + LA	5.90 ± 4.18*	4.88 ± 3.30*	3.15 ± 2.56*	5.65 ± 4.55*	4.92 ± 3.70*	3.18 ± 2.60*	4.39 ± 3.06*	5.09 ± 3.46	3.03 ± 2.91*	4.26 ± 3.12*
Invasion[b] of *C. jejuni* + LA	5.82 ± 5.34*	4.62 ± 3.88*	3.10 ± 2.06*	5.57 ± 5.74*	4.19 ± 3.91*	3.16 ± 2.75*	4.05 ± 2.75*	4.97 ± 3.72	3.05 ± 1.77*	4.19 ± 3.69*

The results are presented as log cfu ml^{-1} (mean ± SD) of three independent experiments; each experiment was performed in duplicate

* Value statistically significant ($P < 0.05$) compared to the corresponding control value

[a] Total number of *C. jejuni* (log cfu ml^{-1}) associated with intestinal cells

[b] Number of *C. jejuni* (log cfu ml^{-1}) representing the bacteria inside intestinal cells recovered after gentamicin killing

production as reported for *Lactobacillus* P93 by Chaveerach et al. [6].

Epithelial cell adhesion and invasion by *C. jejuni* play important roles in the pathogenesis of the disease and are associated with other well-defined disease traits, including induction of cell death [17] and disruption of mucosal barrier function [35]. As shown for other intestinal pathogens, interfering with the ability of bacteria to adhere and invade epithelial cells can prevent intestinal injury and improve clinical outcomes [27]. Adhesion and colonisation of probiotic bacteria in the gastrointestinal tract of the host is believed to be one of the essential features required for the delivery of their health benefits [19]. It is known that good adhesion of a probiotic microorganism to the intestinal cells is related to many beneficial effects. In fact, adhesion is a prerequisite for colonisation [1], stimulation of the immune system [10], and for antagonistic activity against enteropathogens [5]. Adherent strains are, however, preferred, since their establishment in the intestines is fundamental to the expression of probiotic effects. In our experience, *L. acidophilus* ATCC 4356 showed marked adhesion to intestinal cells, probably related to its ability to produce the S-layer, and for this reason was utilized for the interference studies with human pathogens.

The proposed mechanisms by which *Lactobacillus* affects adhesion to cells are displacement, exclusion, and competition. Our investigations, carried out on Caco-2 cells, a cell line widely used as an in vitro model for intestinal epithelium [35], showed that *L. acidophilus* ATCC 4356 strain interfered with the adhesion/invasion of all *C. jejuni* strains; the displacement and the exclusion seemed to be the preferred modalities, which caused a significant reduction of adhesion and invasion of the pathogen to the intestinal cells. A wide bibliography shows that the displacement activity exerted by probiotic bacteria toward enteropathogens is related to mechanisms other than mere competition for common adhesion sites. Lievin et al. [24] demonstrated that *Bifidobacterium* strains isolated from infants was able to produce antibacterial lipophilic factor(s) effective in inhibiting *Salmonella enterica* serovar *Typhimurium* invasion of Caco-2 cells and in killing intracellular enteropathogenic bacteria. Moreover, Fujiwara et al. [14] reported a proteinaceous factor which inhibited in vitro adhesion of an enterotoxigenic *E. coli* strain to gangliotetraosylceramide molecules [32]. Probably, inhibition could be related to specific receptors and adhesins that probiotics and pathogens are competing for, as shown for other microorganisms [21]. Although the in vitro model used does not completely mimic the in vivo setting, it provides a valuable opportunity to study the host epithelial cells interaction between an enteric pathogen and a probiotic strain.

This study adds to the understanding of the mechanisms developed by *L. acidophilus* ATCC 4356 against pathogenic microorganisms by showing evidence of the implication of antimicrobial peptides. The observed inhibitory properties on growth ability and on epithelial cells adhesion/invasion of human *C. jejuni* strains, may turn the attention on the possible use of *L. acidophilus* ATCC 4356 as new potential antimicrobial agent for the management of *Campylobacter* infections. However, deep studies are necessary to better understand the mechanism involved in the competition between *L. acidophilus* ATCC 4356 and *C. jejuni* in order to obtain more information on the human benefit due to probiotics.

References

1. Alander M, Satokari R, Korpela R, Saxelin M, Vilpponen-Salmela T, Mattila-Sandholm T, vonWright A (1999) Persistence of colonization of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG, after oral consumption. Appl Environ Microbiol 65:351–354
2. Avall-Jaaskelainen S, Palva A (2005) *Lactobacillus* surface layers and their applications. FEMS Microbiol Rev 29:511–529
3. Boyanova L, Gergova G, Spassova Z, Koumanova R, Yaneva P, Mitov I, Derejian S, Krastev Z (2004) *Campylobacter* infection in 682 Bulgarian patients with acute enterocolitis, inflammatory bowel disease, and other chronic intestinal diseases. Diagn Microbiol Infect Dis 49:71–74
4. Campana R, Patrone V, Federici S, Fulvi S, Baffone W (2010) Antibiotic resistance of *Campylobacter* spp. isolated from chickens and humans in central Italy. J Food Saf 30:924–940
5. Candela M, Perna F, Carnevali P, Vitali B, Ciati R, Gionchetti P, Rizzello F, Campieri M, Brigidi P (2008) Interaction of probiotic *Lactobacillus* and *Bifidobacterium* strains with human intestinal epithelial cells: adhesion properties, competition against enteropathogens and modulation of IL-8 production. Int J Food Microbiol 125:286–292
6. Chaveerach P, Lipman LJA, van Knapen F (2004) Antagonistic activities of several bacteria on in vitro growth of 10 strains of *Campylobacter jejuni/coli*. Int J Food Microbiol 90:43–50
7. Chen X, Xu J, Shuai J, Chen J, Zhang Z, Fang W (2007) The S-layer proteins of *Lactobacillus crispatus* strain ZJ001 is responsible for competitive exclusion against *Escherichia coli* O157:H7 and *Salmonella typhimurium*. Int J Food Microbiol 115:307–315
8. Collado MC, Gueimonde M, Hernandez M, Sanz Y, Salminen S (2005) Adhesion of selected *Bifidobacterium* strains to human intestinal mucus and its role in enteropathogen exclusion. J Food Protect 68:2672–2678
9. Delgado S, Florez AB, Mayo B (2005) Antibiotic susceptibility of *Lactobacillus* and *Bifidobacterium* species from the human gastrointestinal tract. Curr Microbiol 50:202–207
10. Dong H, Rowland I, Tuohy KM, Thomas LV, Yaqoob P (2010) Selective effects of *Lactobacillus casei* Shirota on T cell activation, natural killer cell activity and cytokine production. Clin Exp Immunol 161:378–388
11. Eschenbach DA, Davick PR, Williams BL, Klebanoff SJ, Young-Smith K, Critchlow CM, Holmes KK (1989) Prevalence of hydrogen peroxide-producing *Lactobacillus* species in normal women and women with bacterial vaginosis. J Clin Microbiol 27:251–256
12. Food and Health Agricultural Organization (FHAO) of the United Nations and Word Health Organization (2002) Guidelines for the

evaluation of probiotics in food. Working Group Rep. 2002. Food and Health Agricultural Organization of the United Nations and Word Health Organization, Washington
13. Forestier C, De Champs C, Vatoux C, Joly B (2001) Probiotic activities of *Lactobacillus casei rhamnosus*: in vitro adherence to intestinal cells and antimicrobial properties. Res Microbiol 152:167–173
14. Fujiwara S, Hashiba H, Hirota T, Forstner JF (1999) Purification and characterization of a novel protein produced by *Bifidobacterium longum* SBT2928 that inhibits the binding of enterotoxigenic *Escherichia coli* Pb176 (CFA/II) to gangliotetraosylceramide. J Appl Microbiol 86:615–621
15. Galdeano CM, de Leblanc AdeM, Carmuega E, Weill R, Perdigón G (2009) Mechanisms involved in the immunostimulation by probiotic fermented milk. J Dairy Res 76:446–454
16. Garcia Rodriguez LA, Ruigomez A, Panes J (2006) Acute gastroenteritis is followed by an increased risk of inflammatory bowel disease. Gastroenterology 130:1588–1594
17. Kalischuk LD, Inglis GD, Buret AG (2007) Strain-dependent induction of epithelial cell oncosis by *Campylobacter jejuni* is correlated with invasion ability and is independent of cytolethal distending toxin. Microbiology 153:2952–2963
18. Klein M, Sanders ME, Duong T, Young HA (2010) Probiotics: from bench to market. Ann NY Acad Sci 1212(Suppl 1):E1–14
19. Kos B, Suskovic J, Vukovic S, Simpraga M, Frece J, Matosic S (2003) Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. J Appl Microbiol 94:981–987
20. Jakava-Viljanen M, Palva A (2007) Isolation of surface (S) layer protein carrying *Lactobacillus* species from porcine intestine and faeces and characterization of their adhesion properties to different host tissues. Vet Microbiol 124:264–273
21. Lee YK, Puong KY (2002) Competition for adhesion between probiotics and human gastrointestinal pathogens in presence of carbohydrate. Brit J Nutr 88:101–108
22. Lee YK, Puong KY, Ouwehand AC, Salminen S (2003) Displacement of bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. J Med Microbiol 52:925–930
23. Lehtopolku M, Nakari UM, Kotilainen P, Huovinen P, Siitonen A, Hakanen AJ (2010) Antimicrobial susceptibilities of multidrug-resistant *Campylobacter jejuni* and *C. coli* strains: in vitro activities of 20 antimicrobial agents. Antimicrob Agents Chemother 50:1232–1236
24. Lievin V, Peiffer I, Hudault S, Rochat F, Brassart D, Neeser JR, Servin AL (2000) *Bifidobacterium* strains from resident infant human gastrointestinal microflora exert antimicrobial activity. Gut 47:646–652
25. Marshall JK, Thabane M, Garg AX, Clark WF, Salvadori M, Collins SM (2006) Incidence and epidemiology of irritable bowel syndrome after a large waterborne outbreak of bacterial dysentery. Gastroenterology 131:445–450
26. Santini C, Baffoni L, Gaggia F, Granata M, Gasbarri R, Di Gioia D, Biavati B (2010) Characterization of probiotic strains: an application as feed additives in poultry against *Campylobacter jejuni*. Int J Food Microbiol 141:S98–S108
27. Searle LE, Best A, Nunez A, Salguero FJ, Johnson L, Weyer U, Dugdale AH, Cooley WA, Carter B, Jones G, Tzortzis G, Woodward MJ, La Ragione RM (2009) A mixture containing galactooligosaccharide, produced by the enzymic activity of *Bifidobacterium bifidum*, reduces *Salmonella enterica* serovar *Typhimurium* infection in mice. J Med Microbiol 58:37–48
28. Servin AL (2004) Antagonistic activity of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol Rev 28:405–440
29. Sheehan VM, Sleator RD, Hill C, Fitzgerald GF (2007) Improving gastric transit, gastrointestinal persistence and therapeutic efficacy of the probiotic strain *Bifidobacterium breve* UCC2003. Microbiology 53:3563–3571
30. Sherman PM, Ossa JC, Johnson-Henry K (2009) Unravelling mechanisms of action of probiotics. Nutr Clin Pract 24:10–14
31. Sherman PM, Ossa JC, Wine E (2010) Bacterial infections: new and emerging enteric pathogens. Curr Opin Gastroenterol 26:1–4
32. Teneberg S, Angstrom J, Ljungh A (2004) Carbohydrate recognition by enterohemorrhagic *Escherichia coli*: characterization of a novel glycosphingolipid from cat small intestine. Glycobiology 14:187–196
33. Thakur S, Zhao S, McDermott PF, Harbottle H, Abbott J, English L, Gebreyes WA, White DG (2010) Antimicrobial resistance, virulence, and genotypic profile comparison of *Campylobacter jejuni* and *Campylobacter coli* isolated from humans and retail meats. Foodborne Pathog Dis 7:835–844
34. Ventura M, O'Flaherty S, Claesson MJ, Turroni F, Klaenhammer TR, van Sinderen D, O'Toole PW (2009) Genome-scale analyses of health-promoting bacteria: probiogenomics. Nat Rev Microbiol 7:61–71
35. Wine E, Gareau MG, Johnson-Henry K, Sherman PM (2009) Strain-specific probiotic (*Lactobacillus helveticus*) inhibition of *Campylobacter jejuni* invasion of human intestinal epithelial cells. FEMS Microbiol Lett 300:146–152
36. Yabe S, Higuchi W, Iwao Y, Takano T, Razvina O, Reva I, Nishiyama A, Yamamoto T (2010) Molecular typing of *Campylobacter jejuni* and *C. coli* from chickens and patients with gastritis or Guillain–Barré syndrome based on multilocus sequence types and pulsed-field gel electrophoresis patterns. Microbiol Immunol 54:362–367
37. Young KT, Davis LM, Di Rita VJ (2007) *Campylobacter jejuni*: molecular biology and pathogenesis. Nat Rev Microbiol 5:665–679
38. Zhang M, He L, Li Q, Sun H, Gu Y, You Y, Meng F, Zhang J (2010) Genomic characterization of the Guillain-Barré syndrome-associated *Campylobacter jejuni* ICDCCJ07001 isolate. PloS one 5:e15060