Journal of Medical Microbiology (2013), **62**, 637–649 DOI 10.1099/jmm.0.049965-0

In vitro characterization of aggregation and adhesion properties of viable and heat-killed forms of two probiotic *Lactobacillus* strains and interaction with foodborne zoonotic bacteria, especially *Campylobacter jejuni*

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Received 17 July 2012
Accepted 11 January 2013

Bacterial aggregation and/or adhesion are key factors for colonization of the digestive ecosystem and the ability of probiotic strains to exclude pathogens. In the present study, two probiotic strains, *Lactobacillus rhamnosus* CNCM-I-3698 and *Lactobacillus farciminis* CNCM-I-3699, were evaluated as viable or heat-killed forms and compared with probiotic reference *Lactobacillus* strains (*Lb. rhamnosus* GG and *Lb. farciminis* CIP 103136). The autoaggregation potential of both forms was higher than that of reference strains and twice that of pathogenic strains. The coaggregation potential of these two beneficial micro-organisms was evaluated against several pathogenic agents that threaten the global safety of the feed/food chain: *Escherichia coli, Salmonella* spp., *Campylobacter* spp. and *Listeria monocytogenes*. The strongest coaggregative interactions were demonstrated with *Campylobacter* spp. by a coaggregation test, confirmed by electron microscopic examination for the two forms. Viable forms were investigated for the nature of the bacterial cell-surface molecules involved, by sugar reversal tests and chemical and enzymic pretreatments. The results suggest that the coaggregation between both probiotic strains and *C. jejuni* CIP 70.2^T is mediated by a carbohydrate–lectin interaction. The autoaggregation potential of the two probiotics decreased upon exposure to proteinase, SDS or LiCl, showing that proteinaceous components on the surface of the two lactobacilli play an important role in this interaction. Adhesion abilities of both *Lactobacillus* strains were also demonstrated at significant levels on Caco-2 cells, mucin and extracellular matrix material. Both viable and heat-killed forms of the two probiotic lactobacilli inhibited the attachment of *C. jejuni* CIP 70.2^T to mucin. In conclusion, *in vitro* assays showed that *Lb. rhamnosus* CNCM-I-3698 and *Lb. farciminis* CNCM-I-3699, as viable or heat-killed forms, are adherent to different intestinal matrix models and are highly aggregative *in vitro* with pathogens, especially *Campylobacter* spp., the most commonly reported zoonotic agent in the European Union. This study supports the need for further *in vivo* investigations to demonstrate the potential food safety benefits of *Lb. rhamnosus* CNCM-I-3698 and *Lb. farciminis* CNCM-I-3699, live or heat-killed, in the global feed/food chain.

INTRODUCTION

Food safety is of fundamental importance to the consumer, the food industry and the economy. Despite significant investment in this field, the incidence of food-borne diseases is still on the rise in the European Union (Hugas *et al.*, 2009; Smulders *et al.*, 2008). Several food-borne diseases are caused by the growth of pathogenic micro-organisms in the food; among them, zoonoses are infections which are transmitted from animals to humans either directly or via the food chain. Contaminated animals can propagate pathogenic strains via their faecal secretions

Abbreviation: EPS, exopolysaccharides.

through the herd and all along the food chain (Fosse *et al.*, 2009). Raw material, especially of animal origin, is one of the main sources of microbial contamination in the food sector. In spite of the decline observed between 1999 and 2001 in the European Union, the majority of the cases of food-borne diseases are associated with *Salmonella* and *Campylobacter* (O' Brien & de Valk, 2003; Crushell *et al.*, 2004).

In order to protect human health, it is important to develop appropriate intervention strategies to prevent zoonotic diseases from occurring. In recent years more consideration has been given to reducing the risk of infection by applying preventive measures, and the use of subtherapeutic doses of antibiotic in animal feed. The emergence of antibiotic-resistant strains highlights the necessity for developing novel alternative approaches (Alfredson & Korolik, 2007). Furthermore, prevention of disease in humans and a reduction of the pathogen reservoir in farm animals, without the need for antibiotics, are of both ecological and financial benefit to society. In recent years, the beneficial effects of probiotic use on animal health, on the reduction of pathogens in the food chain, and on growth performance have increasingly been highlighted (Wine *et al.*, 2009; Modesto *et al.*, 2009; Patterson & Burkholder, 2003).

Multiple mechanisms of action for the beneficial effect of probiotics have been proposed (Ng *et al.*, 2009; Sherman *et al.*, 2009; Lebeer *et al.*, 2008). The ability to adhere to epithelial cells and mucosal surfaces has been suggested as being an important property of many bacterial strains used as probiotics. Adherence is an important prerequisite for the colonization of probiotics in the intestinal cavity, providing a competitive advantage in this ecosystem (Lebeer *et al.*, 2008). Several workers have suggested that the ability of beneficial micro-organisms to aggregate and adhere aids in colonization of the gut and in the establishment of a barrier which prevents enteropathogens from establishing an infection. Factors which prevent infection include the physical presence of beneficial micro-organisms and modulation of the gut immune system by these organisms (Cesena *et al.*, 2001; Voltan *et al.*, 2007; Collado *et al.*, 2008; Sherman *et al.*, 2009).

In this study we evaluated qualitatively and quantitatively the autoaggregation and coaggregation capacities of two probiotic strains, *Lactobacillus rhamnosus* CNCM-I-3698 and *Lactobacillus farciminis* CNCM-I-3699, with model enteropathogens (*Salmonella*, *Campylobacter*, *Listeria* and *Escherichia coli*) focusing on their exclusion abilities against *Campylobacter jejuni*. The probiotic strains were also evaluated for their ability to adhere to mucin, extracellular matrix material and Caco-2 cells. These experiments were also conducted with heat-killed forms of *Lb. rhamnosus* CNCM-I-3698 and *Lb. farciminis* CNCM-I-3699 to check their functional interest.

METHODS

Bacterial strains and culture conditions. *Lactobacillus* strains were stored in de Man Rogosa Sharpe (MRS) broth (AES Chemunex) containing 30 % glycerol at −80 °C. Origins, details, culture and growth conditions of the strains investigated in this study are detailed in Table 1. Before analysis, strains were subcultured three consecutive times in appropriate broth and incubated at 37 °C.

For the preparation of heat-killed bacteria, washed bacteria resuspended in phosphate-buffered saline (PBS) and adjusted to OD_{600} 0.3 ± 0.05 (equivalent to 10^8 cells ml^{-1}) (Spectronic Genesys 5, Bioblock Fisher Scientific) were autoclaved at 120 °C for 15 min.

Auto- and coaggregation assays. The ability of bacteria to autoaggregate and coaggregate was assessed according to the method described by Collado *et al.* (2008) and expressed as a percentage of the total number of bacteria present. Briefly, stationary-phase cells from batch cultures were centrifuged, washed with PBS and resuspended in PBS to OD_{600} 0.3 ± 0.05 (equivalent to 10^8 c.f.u.ml^{-1}).

To determine percentage autoaggregation, bacterial suspensions were incubated in aliquots at room temperature (20 ± 1 °C) and were monitored at different times (0, 4, 20 and 24 h). To determine the ability of bacteria to coaggregate, equal volumes of cells (500 µl) of *Lb. farciminis* 3699 or *Lb. rhamnosus* 3698 and pathogenic strains were mixed together and incubated at room temperature (20 ± 1 °C) without agitation for 24 h. The OD_{600} of the bacterial suspensions was monitored at 4, 20 and 24 h and the percentage autoaggregation was expressed as follows: $(1-OD_{Time}/OD_{T0}) \times 100$, where OD_{Time} represents the optical density of the mix at a particular time (4, 20 or 24 h) and OD_{T0} the optical density at time zero.

Percentages of coaggregation were determined as $[(OD_{Patho}+OD_{Lb})/2-(OD_{mix})/(OD_{Patho}+OD_{Lb})/2] \times 100$, where OD_{Patho} and OD_{Lb} represent the OD_{600} in control tubes containing only the pathogen or the *Lactobacillus* strain respectively and OD_{mix} represents the optical density of the mixed culture after different periods of incubation. The standard deviations were derived from the coaggregation values of three independent experiments. After 24 h coincubation, bacterial aggregates of *Lactobacillus* and *C. jejuni* CIP 70.2^T were observed by scanning electron microscopy.

Preliminary characterization of molecules involved in aggregation. The surface-associated molecules involved in aggregation of *Lb. rhamnosus* 3698 and *Lb. farciminis* 3699 were investigated by sugar reversal tests and by chemical and enzymic pretreatments. The protocol described above to assess coaggregation was used with some modification. Filter-sterilized solutions of sugars [(+)-D-glucose, *N*-acetyl-D-glucosamine, methyl α-D-glucopyranoside, (+)-D-galactose, *N*-acetyl-D-galactosamine, methyl α-D-galactopyranoside, (+)-D-mannose and lactose] were added to the PBS during the aggregation assay at a final concentration of 100 mM. Mixtures were then vortexed and the OD_{600} was determined using a spectrophotometer.

Lactobacillus suspensions were also pretreated under agitation (120 r.p.m.) with 2 % SDS (1 h at 37 °C), 5 mol LiCl ml^{-1} (30 min at 4 °C) or 1 mg proteinase K ml^{-1} in PBS (pH 7.2) (30 min at 37 °C). Cells were harvested by centrifugation (10 min, 10 000 ***g***) and washed three times with PBS. The bacterial suspensions were adjusted to OD_{600} 0.3 ± 0.05 (equivalent to 10^8 c.f.u. ml^{-1}). Cells were mixed as above and their ability to aggregate was determined using the spectrophotometric assay at 600 nm.

Assay for *in vitro* adhesion to extracellular matrix material and mucin. The extracellular matrix material and mucin were prepared essentially as described previously (Tallon *et al.*, 2007; Goh & Klaenhammer, 2010). Matrigel is a commercial preparation of extracellular matrix material of rat origin containing laminin and type IV collagen as major components, followed by heparan sulphate proteoglycans, entactin and nidogen. Matrigel is used to form a gel-like structure resembling basement membranes (Kleinman *et al.*,

Table 1. Details of microbial strains used in this study

Strain*	Culture collection†	Culture medium and growth conditions	Origin of isolate when known
***Lactobacillus* strains**			
Lb. acidophilus DSM 9126	DSMZ	MRS broth (AES Chemunex) for 24 h at 37 °C	–
Lb. alimentarius CIP 102986^T	CIP		Marinated fish product
Lb. farciminis CNCM-I-3699	DGCC		Goat rumen isolate
Lb. farciminis CIP 103136	CIP		Sausage
Lb. plantarum DSM 211003	DSMZ		–
Lb. rhamnosus CNCM-I-3698	DGCC		Goat rumen isolate
Lb. rhamnosus GG (ATCC 53103)	Valio Finland		Intestinal tract of healthy human
Pathogenic models			
Salmonella Typhimurium PS1	LDA22	Columbia agar (bioMérieux) with 5 % horse blood at 37 °C	Pig isolate
Salmonella Enteritidis VS2	LDA22		Poultry isolate
Escherichia coli K88	LDA22		Pig isolate
Campylobacter jejuni CIP 70.2^T	CIP	Columbia agar with 5 % horse blood under microaerophilic conditions (GENbox microaer, bioMérieux) at 42 °C	Poultry isolate
Campylobacter jejuni CIP 70.80^T			Poultry isolate
Listeria monocytogenes WLSC 1685 (Scott A-Serovar 1/2a)	WSLC	Brain Heart Infusion (AES Chemunex) at 37 °C	Milk poisoning, human isolate

*^{T}, Type strain.
†DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; CIP, Collection Institut Pasteur, Paris, France; DGCC, Danisco Culture Collection; LDA, Laboratoire Départemental d'Analyses, Ploufragan, 22 Côtes d'Armor, France; WLSC, Weihe Stephan Listeria Collection, University of Munich, Germany.

1986). Briefly, Matrigel (Sigma-Aldrich) was diluted 1/20 in ice-cold PBS. Nunc Maxisorp 96-well microplate wells were coated with the Matrigel solution (100 µl per well) by incubation at 37 °C for 1.5 h at 4 °C overnight. Mucin is the main constituent of mucus present in the gastrointestinal tract. Mucin (type III from porcine stomach; Sigma) was dissolved in PBS at 10 mg ml^{-1}. Nunc Maxisorp 96-well microplate wells were coated with the protein solution (100 µl per well) by incubation at 4 °C overnight. The wells were then washed three times for 5 min with PBS containing 0.1 % BSA, then quenched for 2 h at room temperature with 2 % BSA in PBS.

The ability of *Lactobacillus* strains to adhere to mucin and Matrigel was determined as described previously (Goh & Klaenhammer, 2010). Adhered viable cells were enumerated by plate counts on MRS agar. Adhesion abilities of viable and heat-killed forms of *Lb. farciminis* 3699 or *Lb. rhamnosus* GG or *Lb. rhamnosus* 3698 were determined by quantitative PCR (qPCR) as follows: after incubation and recovery of the cells and matrix, DNA was isolated by the FTA Elute method (Whatman). Real-time PCR was performed in a Bio-Rad CFX96 real-time PCR detection system using optical-grade 96-well plates with the specific primers listed in Table 2. The PCR amplification was performed in a total volume of 25 µl using IQ SYBR Green Supermix (Bio-Rad). Each reaction included 1 × SYBR Green Supermix, 0.2 µM of each primer and 10 µl template. The reaction conditions for amplification were as follows: (i) preincubation at 95 °C for 3 min; (ii) amplification consisting of 40 cycles of 95 °C for 10 s, annealing at the appropriate temperature (Table 2) for 20 s, and extension at 72 °C for 15–20 s. Internal standards were prepared by amplification of serial dilutions of the respective bacteria in PBS ranging from 1×10^8 to 1×10^4 c.f.u. ml^{-1}. For the 100 % values, aliquots of each strain used were prepared and quantified separately. All adherence experiments were conducted in triplicate and bacterial adhesion was expressed as the percentage adhesion.

Inhibition of binding of *C. jejuni* CIP 70.2^T to mucin by *Lactobacillus* strains. The ability of probiotic strains to inhibit the binding of *C. jejuni* CIP 70.2^T to mucin was assessed by two tests corresponding either to preincubation of probiotic strains before addition of the pathogen or to coincubation of probiotic strains with the pathogen at the same time. In the first test, 100 µl of a viable or heat-killed probiotic suspension (10^8 c.f.u.ml^{-1}) was added to the wells coated with mucin and incubated for 1 h at 37 °C. Then unattached cells of the probiotic strain were removed by washing five times with PBS, and 100 µl of a 10^8 c.f.u. ml^{-1} suspension of *C. jejuni* CIP 70.2^T was added to the wells and incubated for 1 h at 37 °C. In the second test, 100 µl of each bacterial suspension was added at the same time and incubated in the same conditions as described above. Then for both tests, the wells were washed five times with PBS buffer and adhered cells were recovered by treating each well with 100 µl 0.05 % Triton X-100 solution for 15 min with agitation. Cell suspensions were diluted and plated on Campylosel agar (bioMérieux) to enumerate adhered cells. The percentage inhibition was calculated as the difference between the adhesion of the pathogen in the absence and presence of a *Lactobacillus* strain.

Caco-2 cell culture. Human colon adenocarcinoma Caco-2 cells ref. 860.102.02 (ECACC) were used from passages 43 to 57. Cells were

Table 2. Primers used in this study

Strain	Primer	Sequence (5′→3′)	Annealing temp. (°C)
Lb. farciminis 3699	FAR-1	ATC TCA TGC GTT TGT TGG TG	56.2
	FAR-2	TCG TTT GGT TTT GGC TCT TC	
Lb. rhamnosus 3698	F-A08rha	TTC AAC CAC CAG TGT GTC CT	57.2
	R-A08rha	TCT TCC GTC CGG AAC TAA TC	
Lb. rhamnosus GG	F-GG	TGA CGG TAT CCA ACC AGA AA	57.0
	R-GG	AAG TTT CCC AGT TTC CGA TG	

routinely grown in DMEM (BioMedia), supplemented with 10 % of the serum substitute Prolifix S6, 5 % L-glutamine (200 mM), 5 % Peni/Strepto and 5 % pyruvate (100 mM) (all from BioMedia). Cells were seeded at a concentration of 9.5×10^5 cells cm^{-2} in six-well culture plates. All experiments and maintenance were carried out at 37 °C in a 5 % CO_2/95 % air atmosphere. The culture medium was changed daily. Unless otherwise stated, cultures were used at post-confluence after 15 days of culture (differentiated cells). To determine the number of Caco-2 cells in a monolayer, cells were detached for 2 min with Splittix and Splitstop (Bio Media) at ambient temperature and counted using a Thoma cell. The protocol to assess adherence of *Lactobacillus* strains to Caco-2 cells was adapted from Chauvière *et al.* (1992). After incubation of the cells with lactobacilli, monolayers were washed three times with sterile PBS, then fixed with glutaraldehyde (2.5 %) for 30 min, Gram-stained and examined microscopically under oil immersion. Each adhesion assay was conducted in triplicate with cells from three successive passages. For each well, the number of adherent bacteria was counted in 10 random microscope fields. Adhesion of lactobacilli was expressed as the number of bacteria adhering to 100 Caco-2 cells.

Electron microscopy. Aliquots of bacterial aggregates were fixed overnight at 4 °C with a cacodylate buffer (0.1 M cacodylate and 0.1 M sucrose) containing 2.5 % (v/v) glutaraldehyde. After washing with PBS, the cells were post-fixed for 30 min in the dark at room temperature with cacodylate buffer containing 1 % osmium tetroxide. Cells were then washed three times with the same mixture and dehydrated in a concentration series (30 %, 50 % and 70 %) of ethanol solutions for 10 min each. The cells were then washed in 100 % ethanol for 10 min before being dried in a critical-point dryer (Balzers CPD 020) and coated with gold. All preparations were observed under a JEOL 6400F scanning electron microscope.

Statistics. The results for aggregation, coaggregation, inhibition tests and preliminary characterization are expressed as the mean ± SD of three independent experiments. Statistical analysis was done by StatGraphic*Plus* software. Data were subjected to a one-way analysis of variance (ANOVA).

RESULTS

Auto- and coaggregation assays

The *Lactobacillus* strains and pathogenic strains tested could all autoaggregate. Autoaggregation increased as a function of time and was highest at the 24 h time point (Table 3). The autoaggregative percentages of all pathogenic isolates ranged between 19 and 29 %, which is 2.5-fold below the range for the *Lactobacillus* isolates (between 48 % and 69 %) after 24 h in the same conditions. Among the *Lactobacillus* strains tested, *Lb. acidophilus* DSM 9126 exhibited the highest percentage of autoaggregation after 24 h (69 %). *Lb. farciminis* CIP 103136 and *Lb. rhamnosus* GG are well-known human probiotics so they were used here as reference strains. They showed autoaggregation abilities close to 48 % whereas the two tested probiotic strains *Lb. farciminis* 3699 and *Lb. rhamnosus* 3698 both showed significantly higher autoaggregation: 55.4 % ($P<0.05$) and 66.4 % ($P<0.05$), respectively. The autoaggregative properties of *Lb. farciminis* 3699 and *Lb. rhamnosus* 3698 were significantly reduced, by 18 % ($P<0.05$) and 11.7 % ($P<0.05$) respectively, when the cells were heat-treated (Table 3) but the values remained within the range obtained for the reference strains.

Table 3. Autoaggregation percentages as a function of time for *Lactobacillus* and gastrointestinal pathogen strains determined by spectrophotometric assay

Strain	Autoaggregation (%)*		
	4 h	20 h	24 h
***Lactobacillus* strains**			
Lb. farciminis CNCM-I-3699	11.7 ± 0.8	35.0 ± 4.8	55.4 ± 0.7
Lb. farciminis CIP 103136	15.6 ± 2.6	31.8 ± 4.9	48.6 ± 0.7
Lb. rhamnosus CNCM-I-3698	22.1 ± 1.4	38.9 ± 0.7	66.4 ± 2.2
Lb. rhamnosus GG	14.2 ± 4.4	28.5 ± 7.3	48.2 ± 3.5
Lb. acidophilus DSM 9126	31.7 ± 3.7	50.4 ± 5.8	69 ± 5.4
Lb. alimentarius CIP 102986^T	13.4 ± 2.7	32.3 ± 12.4	49.5 ± 7.7
Lb. plantarum DSM 211003	14.2 ± 4.4	28.5 ± 7.3	48.2 ± 3.5
***Lactobacillus* strains (heat-killed)†**			
Lb. farciminis CNCM-I-3699	4.2 ± 0.7	ND	45.4 ± 1.0
Lb. rhamnosus CNCM-I-3698	13.2 ± 2.2	ND	58.6 ± 4.1
Pathogenic strains			
Salmonella Typhimurium PS1	2.9 ± 1.2	14.6 ± 11.5	26.6 ± 2.9
Salmonella Enteritidis VS2	2.9 ± 1.0	13.5 ± 6.8	18.9 ± 4.6
Escherichia coli K88	6.9 ± 1.6	17.7 ± 7.2	27.4 ± 1.4
Campylobacter jejuni CIP 70.2^T	9.1 ± 1.8	19.3 ± 1.5	22.6 ± 1.8
Campylobacter coli CIP 70.80^T	6.9 ± 1.0	16.8 ± 4.2	22.8 ± 5.3
Listeria monocytogenes WLSC 1685	8.2 ± 2.8	23.8 ± 5.9	29.2 ± 6.5

*Values are means ± SD; ND, not determined.
†Heat-killed at 120 °C for 15 min.

Viable forms of *Lb. rhamnosus* 3698 and *Lb. farciminis* 3699 exhibited coaggregative properties with all pathogenic strains tested after 24 h incubation at 20 °C (Table 4). The ability of *Campylobacter* strains to coaggregate was significantly better than that of the other pathogenic strains tested ($P<0.05$), regardless of the strain of *Lactobacillus* used in the assay.

Whatever the physiological status (dead or alive) of *Lb. rhamnosus* 3698 and *Lb. farciminis* 3699, the coaggregative abilities remained similar (Fig. 1). The highest coaggregation of the heat-killed forms of both lactobacilli was again seen with *C. jejuni* CIP 70.2^T. When viable *Lb. rhamnosus* 3968 and *Lb. farciminis* 3969 bacteria were used, the percentage coaggregation was 61.2 % ± 2.5 and 53.6 % ± 2.9 respectively, compared to 59.7 % ± 2.5 and 51.1 % ± 2.6 respectively when heat-killed cells of *Lb. rhamnosus* 3968 and *Lb. farciminis* 3969 were used. *C. jejuni* CIP 70.2^T was chosen for further experiments. A high level of coaggregative properties of *Lb. farciminis* 3699 and *Lb. rhamnosus* 3698 was also illustrated by qualitative scanning electron microscopy observations (Fig. 2). Micrographs showed the spatial arrangement of microbial coaggregates and fine interactions between the two types of cells. They also highlighted the presence of exopolymeric filaments which probably act as cement between cells.

Table 4. Coaggregation percentages at different times for viable *Lb. rhamnosus* 3698 and *Lb. farciminis* 3699 with gastrointestinal pathogen strains, determined by spectrophotometric assay

Strain combination	Coaggregation (%)*		
	4 h	20 h	24 h
***Lb. rhamnosus* CNCM-I-3698 with:**			
Salmonella Typhimurium PS1	4.5 ± 0.9	26.8 ± 10.9	42.9 ± 4.2
Salmonella Enteritidis VS2	3.9 ± 2.1	19.3 ± 1.4	24.6 ± 0.2
Escherichia coli K88	7.9 ± 1.8	27.0 ± 6.0	38.2 ± 3.4
Campylobacter jejuni CIP 70.2^T	21.2 ± 1.0	49.6 ± 4.9	61.2 ± 2.5
Campylobacter coli CIP 70.80^T	17.5 ± 5.9	47.0 ± 4.9	57.9 ± 1.8
Listeria monocytogenes WLSC 1685	13.3 ± 1.7	33.5 ± 7.4	43.7 ± 4.3
***Lb. farciminis* CNCM-I-3699 with:**			
Salmonella Typhimurium PS1	4.8 ± 1.3	18.6 ± 1.7	30.6 ± 2.7
Salmonella Enteritidis VS2	4.0 ± 1.9	23.4 ± 4.5	25.8 ± 3.8
Escherichia coli K88	8.0 ± 1.2	37.0 ± 2.9	34.5 ± 3.0
Campylobacter jejuni CIP 70.2^T	23.3 ± 0.6	42.9 ± 5.9	53.6 ± 2.9
Campylobacter coli CIP 70.80^T	12.9 ± 0.9	37.0 ± 2.9	50.8 ± 0.4
Listeria monocytogenes WLSC 1685	10.4 ± 1.6	31.5 ± 1.7	37.4 ± 0.8

*Values are means ± SD.

Putative molecules involved in auto- and coaggregation

The surface-associated molecules involved in autoaggregation and coaggregation of *Lb. rhamnosus* 3698 and *Lb. farciminis* 3699 with *C. jejuni* CIP 70.2^T were investigated by pretreatments with proteinase and chemical agents and by sugar reversal tests. Inhibition data for both strains with *C. jejuni* CIP 70.2^T are presented in Fig. 3(a, b). The results for inhibition of autoaggregation and coaggregation are presented in Table 5. The addition of simple sugars was expected to reverse lectin–saccharide (protein–carbohydrate)-like interactions. Here, all sugars used showed a high power of inhibition of the autoaggregation capability of the two probiotic *Lactobacillus* strains, except in the case of (+)-D-glucose, which did not affect the autoaggregation ability of *Lb. rhamnosus* 3698 in comparison with the control (Table 5).

Investigation of the ability of sugars to reverse coaggregation of *Lb. farciminis* 3699 and *Lb. rhamnosus* 3698 with the enteropathogen *C. jejuni* CIP 70.2^T showed that their ability to coaggregate was similarly and significantly reduced by (+)-D-glucose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, lactose and (+)-D-galactose but in this last case only for *Lb. rhamnosus* 3698 (Table 5, Fig. 3a, b).

The pretreatment of the cell walls of the two *Lactobacillus* strains with proteinase K, LiCl or SDS (Fig. 3a, b), significantly reduced their ability to autoaggregate (Table 5). These agents also significantly reduced the coaggregation potential of *Lb. rhamnosus* 3698 (Fig. 3b), while no significant reduction was observed for *Lb. farciminis* 3699, with any of the pretreatments (Fig. 3a).

Thus sugar and anti-protein reversion of the autoaggregation abilities of *Lb. rhamnosus* 3698 and *Lb. farciminis* 3699 was observed and the effects were very similar. The results for coaggregation were similar for sugar reversion but no anti-protein reversion was observed for *Lb. farciminis*, in contrast to *Lb. rhamnosus*. The autoaggregation potential of the two probiotics seems to be mediated by carbohydrate–lectin interactions and/or proteinaceous components. Coaggregation of *Lb. rhamnosus* 3698 with *C. jejuni* CIP 70.2^T involves carbohydrate–lectin interactions and/or proteinaceous components.

In vitro adhesion of viable and heat-killed forms of *Lactobacillus* strains to different intestinal matrix models

Adhesion properties of viable and heat-killed forms of three *Lactobacillus* strains on Caco-2 cells have been determined by microscopic counting after Gram staining in our laboratory previously. *Lb. rhamnosus* GG, considered here as a positive control, exhibited a strong adhesion ability, with 2000 cells per 100 Caco-2 cells (Fig. 4). In comparison, viable forms of *Lb. rhamnosus* 3698 and *Lb. farciminis* 3699 had very limited ability to adhere to Caco-2 cells: respectively 74 and 590 cells per 100 Caco-2 cells. The

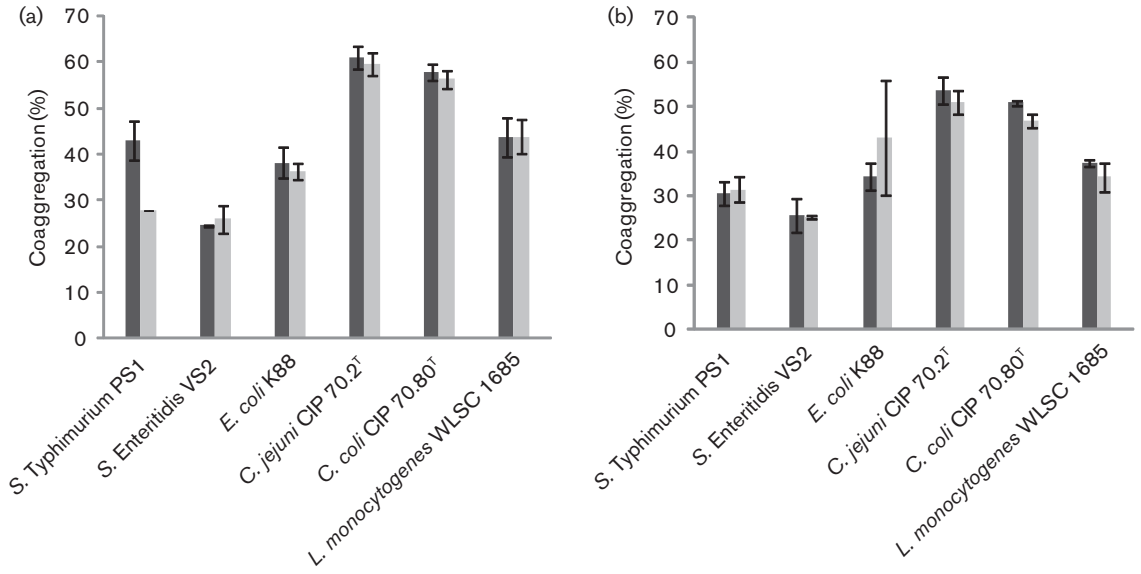

Fig. 1. Coaggregation abilities of viable forms (dark-grey bars) and heat-killed (120 °C, 15 min) forms (light-grey bars) of (a) *Lb. rhamnosus* CNCM-I-3698 and (b) *Lb. farciminis* CNCM-I-3699 after 24 h contact with different pathogenic isolates.

inactivation of the cells by a heat treatment had a marked effect on the adhesion ability of the cells: heat-killed cells of *Lb. rhamnosus* GG became non-adherent (48 cells per 100 Caco-2 cells) whereas *Lb. rhamnosus* 3698 and *Lb. farciminis* 3699 became more adherent, respectively 1472 and 1557 cells per 100 Caco-2 cells (Fig. 4).

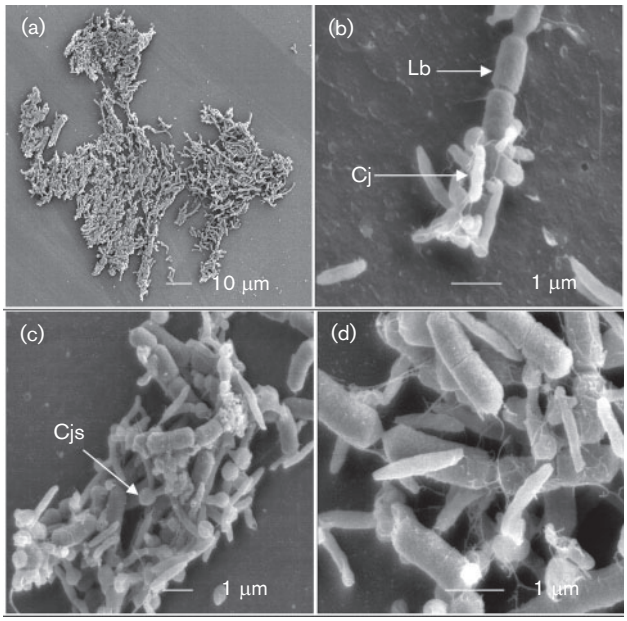

Fig. 2. Scanning electron microscopic observation of *Lb. rhamnosus* CNCM-I-3698 (Lb) coaggregating with *Campylobacter jejuni* CIP 70.2^T (Cj) and forming cell clusters (a, b), with synthesis of exopolymeric filaments (c, d). Cjs, stress form of *C. jejuni* CIP 70.2^T (coccoid).

The effect of heat treatment on the ability to adhere to mucin was determined by qPCR to get a more accurate and specific count of adherent cells on the matrix (Fig. 5). Adhesion of viable forms of *Lb. rhamnosus* GG was the highest (18 %) compared to *Lb. rhamnosus* 3698 (9 %) and *Lb. farciminis* 3699 (5.6 %). Heat-inactivation of the cells induced a reverse phenomenon, i.e. heat-killed cells of *Lb. rhamnosus* GG lose adhesion ability (reduced by 6.4-fold) whereas heat-killed forms of *Lb. rhamnosus* 3698 and *Lb. farciminis* 3699 increase their adhesion abilities, by 2- and 7.8-fold, respectively. These results are in accordance with the above data obtained on Caco-2 cells.

The adhesion properties on Matrigel were determined with viable forms of the two tested *Lactobacillus* strains and reference strains: *Lb. farciminis* CIP 103136 (Mercier-Bonin *et al.*, 2011) and *Lb. rhamnosus* GG, known as mucus-binding strains (von Ossowski *et al.*, 2010, 2011). Compared to the reference strains, the two tested lactobacilli showed lower ability to adhere to mucin, especially for *Lb. farciminis* 3699. On Matrigel matrix, all four strains had higher adhesion ability and strains of the same species showed similar adhesion (Fig. 6).

Inhibition of binding of *C. jejuni* CIP 70.2^T to mucin by viable and heat-killed forms of *Lb. rhamnosus* 3698 and *Lb. farciminis* 3699

The ability of viable and heat-killed forms of *Lb. rhamnosus* 3698 and *Lb. farciminis* 3699 to prevent binding of *C. jejuni* CIP 70.2^T to mucin was assessed in

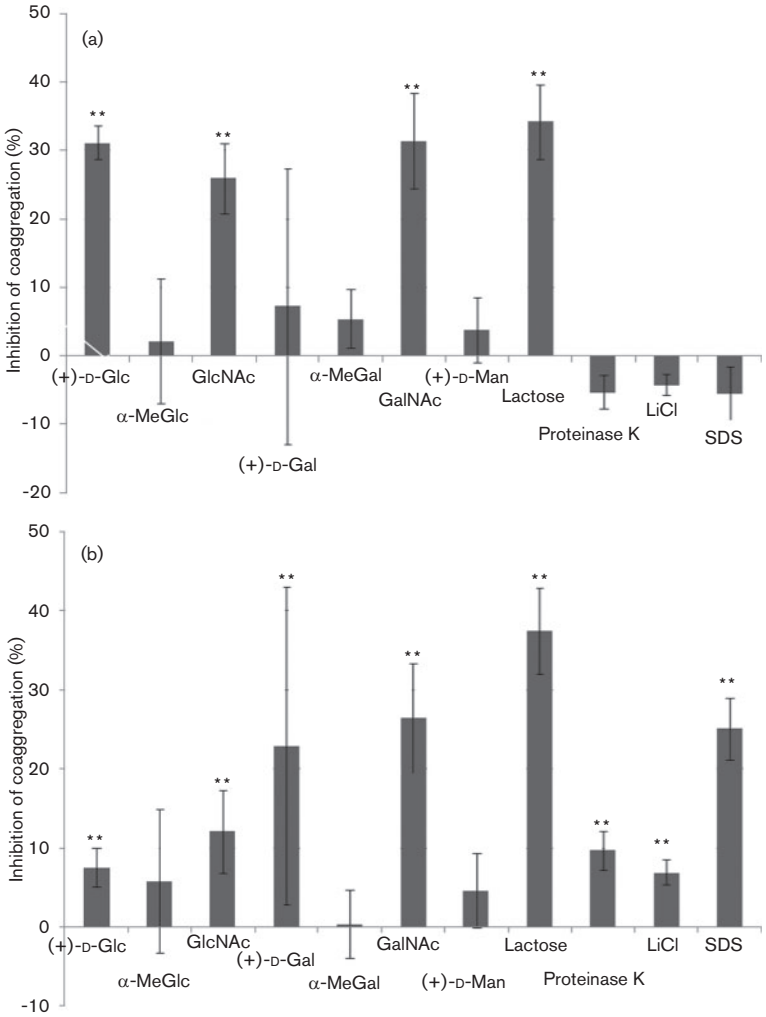

Fig. 3. Percentage of coaggregation inhibition between (a) *Lb. farciminis* CNCM-I-3699 or (b) *Lb. rhamnosus* CNCM-I-3698 and *Campylobacter jejuni* CIP 70.2^T in the presence of sugars or after cell surface pretreatments. Glc, glucose; α-MeGlc, methyl α-D-glucopyranoside; GlcNAc, *N*-acetyl-D-glucosamine; Gal, galactose; α-MeGal, methyl α-D-galactopyranoside; GalNAc, *N*-acetyl-D-galactosamine; Man, mannose. **, Significant at $P<0.05$.

two ways. In the first method the ability of *C. jejuni* to adhere to mucin that had been preincubated with either *Lb. rhamnosus* 3698 or *Lb. farciminis* 3699 was assessed (Fig. 7a). The results demonstrated that the viable form of *Lb. rhamnosus* 3698 was able to cause 50 % inhibition of pathogen binding to mucin whereas for viable form of *Lb. farciminis* 3699, inhibition was only 15 % (Fig. 7a). When heat-killed forms were tested, exclusion of *C. jejuni* CIP 70.2^T was improved, especially for heat-killed *Lb. farciminis* 3699 (from 15 to 70 %) (Fig. 7a). In the second method either *Lb. rhamnosus* 3698 or *Lb. farciminis* was added to the mucin at the same time as *C. jejuni* (Fig. 7b). Results of inhibition of the binding of *C. jejuni* CIP 70.2^T to mucin by competition with *Lactobacillus* strains demonstrated that heat-treated cells of lactobacilli are more efficient than viable cells (Fig. 7b). Whatever the mechanism of exclusion, heat-killed forms of both *Lactobacillus* strains efficiently prevent the adhesion of the pathogen to mucin.

DISCUSSION

In previous papers, we have demonstrated that *in vitro* antagonistic activities of viable and heat-killed forms of *Lb. rhamnosus* 3698 and *Lb. farciminis* 3699 towards *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* (Bernardeau *et al.*, 2009) involve coaggregation properties and production of antimicrobial substances. Those results support previous *in vivo* observation of the preventive effect of the heat-killed forms of both strains in pigs (Bernardeau & Guilmoto, 2008). In this study, we demonstrate that coaggregation properties of those two probiotic strains are not species specific since coaggregation effects were registered towards

Table 5. Inhibition of autoaggregation of viable forms of *Lb. farciminis* 3699 or *Lb. rhamnosus* 3698 and of their coaggregation with *C. jejuni* CIP 70.2^T by adding specific sugars or by the pretreatment of the cell surface proteins

++, Significantly different from the control (P>0.05, ANOVA); +, significantly different (P>0.10); NS, not significantly different; ND, not determined. Glc, glucose; α-MeGlc, methyl α-D-glucopyranoside; GlcNAc, *N*-acetyl-D-glucosamine; Gal, galactose; α-MeGal, methyl α-D-galactopyranoside; GalNAc, *N*-acetyl-D-galactosamine; Man, mannose.

	Lb. farciminis 3699		*Lb. rhamnosus* 3698	
	Autoaggregation	**Coaggregation**	**Autoaggregation**	**Coaggregation**
Sugar				
(+)-D-Glc	++	++	NS	++
α-MeGlc	++	NS	++	NS
GlcNAc	++	++	++	++
(+)-D-Gal	++	NS	++	++
α-MeGal	++	NS	++	NS
GalNAc	++	++	++	++
(+)-D-Man	++	NS	++	NS
Lactose	ND	++	ND	++
Pretreatment				
Proteinase K	++	NS	++	++
LiCl	++	NS	++	++
SDS	++	NS	+	++

representative strains of *Salmonella*, *E. coli*, *Listeria monocytogenes* and *Campylobacter* spp. Among those pathogens, both lactobacilli exhibited a strong interaction with *C. jejuni* CIP 70.2^T. This pathogen is recognized as the main cause of foodborne gastrointestinal diseases worldwide (EFSA, 2012). Additionally, *Campylobacter* can cause invasive disease and extra-intestinal sequelae including bacteraemia and Guillain–Barré syndrome (Byrne *et al.*, 2007). *C. jejuni* is sensitive to lactic acid (van Netten *et al.*, 1995; Byrd *et al.*, 2001; Dibner & Buttin, 2002). The incorporation of lactic acid in the drinking water of animals has been used successfully to reduce *Salmonella* and *Campylobacter* contamination but side effects result in the reduction of water consumption (Byrd *et al.*, 2001). It is also well known that organic acids are buffered by the feed and are less efficient in the gut. The coaggregation of *Lb. rhamnosus* 3698 and *Lb. farciminis* 3699 with *C. jejuni* may constitute a more efficient host defence mechanism against infection compared to organic acid administrated as chemical agent in feed, as both strains produce lactic acid. Furthermore, the coaggregation phenomenon reduces the cell–cell distances between probiotics and pathogens, increasing the efficiency of antimicrobial metabolites produced by viable probiotics (organic acids, bacteriocins, etc.).

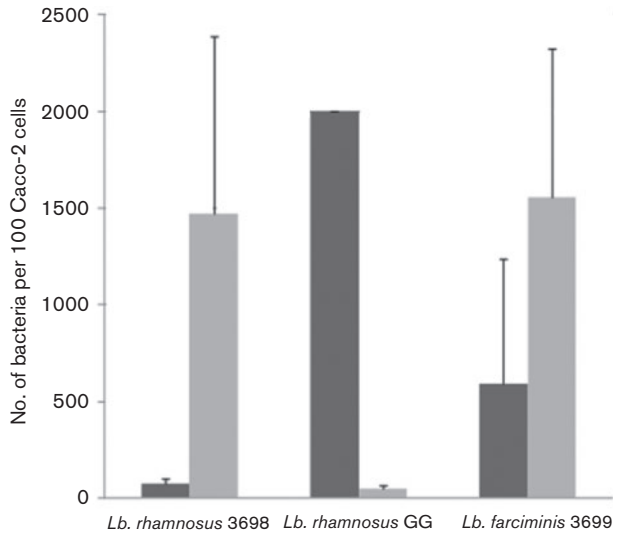

Fig. 4. Adhesion of viable (dark-grey bars) and heat-killed (120 °C, 15 min) forms (light-grey bars) of three probiotic *Lactobacillus* strains in the Caco-2 cell line model (n=3; three different passages of Caco-2 cells) determined by Gram staining and optical microscopic counting.

Electron microscopic pictures of coaggregates suggest the putative role of exopolymers such as exopolysaccharides (EPS) and this is reminiscent of work by Bernardeau *et al.* (2009). EPS are produced by many bacterial species from various ecological niches and have been described as cement for biofilm formation (Vu *et al.*, 2009). Data obtained in this study suggest that autoaggregation of *Lb. farciminis* 3699 with *C. jejuni* CIP 70.2^T seems to be due only to carbohydrate–lectin interactions whereas for *Lb. rhamnosus* 3698, both carbohydrate–lectin interactions and proteinaceous components are involved. The galactosides and the glucosides are sugars that are most commonly recognized by lectins from bacteria of the oral cavity (Kolenbrander, 2000) or aquatic biofilms (Rickard *et al.*, 2000, 2003, 2004). It is well established that these sugars

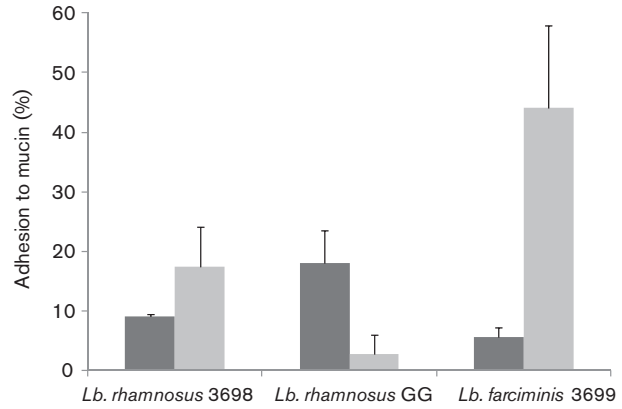

Fig. 5. Adhesion of viable (dark-grey bars) and heat-killed (120 °C, 15 min) forms (light-grey bars) of three probiotic *Lactobacillus* strains to a mucin layer (n=3) determined by qPCR.

enter into the composition of the different structures present at the bacterial surface (teichoic acids, neutral polysaccharides, glycoproteins and EPS). The main cell–cell interactions described among lactobacilli are between proteins, lipoteichoic acid, glycoproteins and carbohydrates of the bacterial cell wall (Boris *et al.*, 1998; Marcotte *et al.*, 2004; Ocaña & Nader-Macías, 2002; Golowczyc *et al.*, 2009; Lebeer *et al.*, 2008; Mackenzie *et al.*, 2010).

Both probiotic strains tested showed higher autoaggregating phenotypes compared to the probiotic reference strains *Lb. rhamnosus* GG and *Lb. farciminis* 103136 or to the pathogens tested, and this is consistent with previous studies (Collado *et al.*, 2007, 2008). Our data suggest that autoaggregation of *Lb. rhamnosus* 3698 and *Lb. farciminis* 3699 involves carbohydrate–lectin interactions and/or proteinaceous components present on the surface. Autoaggregation is a known property of some *Lactobacillus* isolates (Collado *et al.*, 2007, 2008) and allows the formation of biofilm in the gastrointestinal tract which contributes, as a physico-chemical barrier, to the preservation of the intestinal wall, along with the stimulation of the immune system (Jankovic *et al.*, 2003; Castagliuolo *et al.*, 2005; Voltan *et al.*, 2007; Turpin *et al.*, 2010), and to protection against colonization by pathogenic bacteria (Collado *et al.*, 2007, 2008). Furthermore, bacteria with a high autoaggregation potential will more easily colonize the intestinal and urogenital tracts (Boris *et al.*, 1998; Cesena *et al.*, 2001; Collado *et al.*, 2005, 2007, 2008).

Lb. farciminis 3699 and *Lb. rhamnosus* 3698 adhered well to mucin and extracellular material matrix models, which correlates with their ability to autoaggregate. Adhesion abilities exhibited by the two tested strains are in a range comparable with those of the probiotic muco-adhesive strain of *Lb. farciminis* CIP 103136 (Mercier-Bonin *et al.*, 2011) and probiotic reference strain *Lb. rhamnosus* GG (Kirjavainen *et al.*, 1998; von Ossowski *et al.*, 2011; Van den Abbeele *et al.*, 2012). Mucin is a glycosylated protein and attachment of bacteria to mucin involves specific bacterial proteins such as mucus-binding proteins and other lectins or adhesins. A majority of the aggregation interactions between gut isolates are mediated by lectin–carbohydrate interaction (Ledder *et al.*, 2008). In *Lactobacillus reuteri* strains, mucus binding showed excellent correlation with the presence of cell-surface mucus-binding protein and was further highly associated with the autoaggregation properties of *Lb. reuteri* strains in washed cell suspensions (Mackenzie *et al.*, 2010). Similarly, EPS, which contribute to cell-surface physico-chemical properties, have been implicated in the *Lb. reuteri* aggregation phenotype as supported by the implication of EPS-producing enzymes in cell aggregation and biofilm formation (Walter *et al.*, 2008). Furthermore the aggregating strain *Lactobacillus crispatus* M 247 was recovered from the faeces and colonic mucosa of mice after 14 days of diet supplementation but an

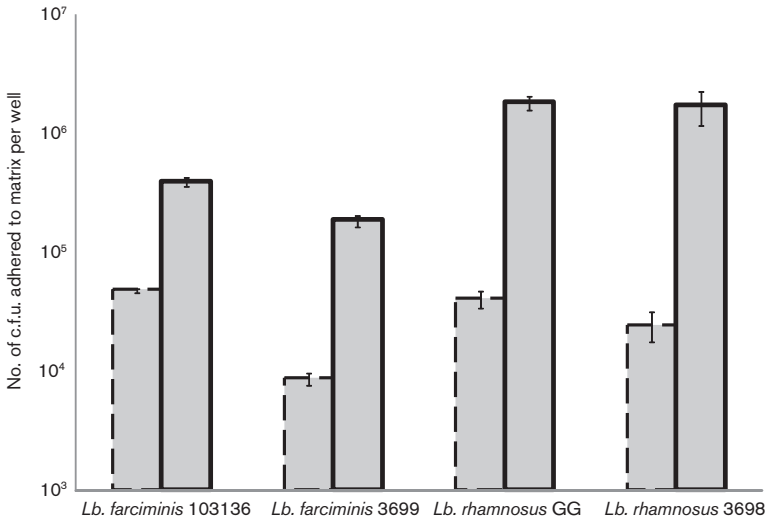

Fig. 6. Adhesion of viable *Lb. farciminis* CIP 103136 and CNCM-I-3699 and viable *Lb. rhamnosus* GG and CNCM-I-3698 to mucin (bars with dashed outline) and Matrigel matrix (bars with solid outline) (n=3) and determined by plate counts on agar.

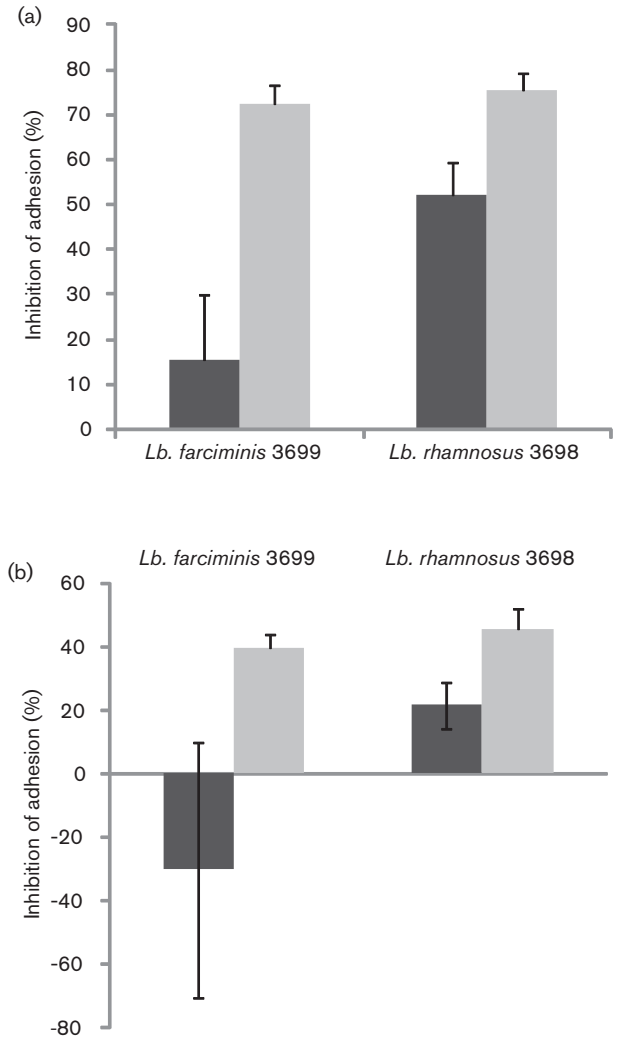

Fig. 7. Evaluation of the prevention of attachment of *C. jejuni* CIP 70.2^T to a mucin layer by viable (dark-grey bars) and heat-killed forms (light-grey bars) of *Lb. farciminis* 3699 and *Lb. rhamnosus* 3698 ($n=3$). Inhibition of pathogen adhesion was determined after preincubation of lactobacilli (a) or after coincubation (b). In the absence of probiotic a value of 0 % was assigned for inhibition of binding.

aggregation-deficient isogenic mutant MU5 was absent, leading to the conclusion that the aggregation phenotype in *Lb. crispatus* determines intestinal colonization in murine colonic mucosa (Voltan *et al.*, 2007). These *in vivo* studies indicate that bacteria with aggregating phenotypes persist longer in the gastrointestinal tract compared with non-aggregating phenotype and that adhesion and aggregation appear to be correlated. Adhesion to the intestinal mucosa could confer a competitive advantage important for maintenance of bacteria in the gastrointestinal tract and it is generally accepted that adhesion properties contribute to the efficacy of probiotic strains (Hudault *et al.*, 1997; Servin & Coconnier, 2003).

The inhibition of adhesion of pathogens in the intestine could prevent translocation and subsequent infection. Mucin is a powerful attractant for *Brachyspira*, *Campylobacter* and some *Vibrio cholerae* (Witters & Duhamel, 1999; Tu *et al.*, 2008). Alemka *et al.* (2010) demonstrated that mucus supports *C. jejuni* reproduction and enhances adhesion to and invasion of the underlying epithelium. *C. jejuni* replication in mucus may ensure bacterial persistence in the host since this substrate is rich in nutrients and is essentially microaerophilic. Several probiotic bacteria including bifidobacteria and lactobacilli adhere to mucosal tissue in a strain-specific manner (O'Hara & Shanahan, 2007). Selected probiotic strains should be able to compete with pathogens for the same receptors and to occupy their potential binding sites in the gut, thus representing a potential therapeutic strategy (Bouzaine *et al.*, 2005; O'Hara & Shanahan, 2007). This enhances the intestinal persistence of the probiotic bacteria, and limits pathogen access to the epithelium (O'Hara & Shanahan, 2007). In general, the incubation of enteropathogens with probiotic strains having a high coaggregation potential decreases the adhesion of the pathogen to the intestinal mucus (Tuomola *et al.*, 1999), as well as the invasive capacity of pathogens on enterocytes (Golowczyc *et al.*, 2007).

As adhesion and coaggregation properties involve cell-surface properties, we considered the question of whether viability of the probiotic strains was essential. Most studies published on heat-killed bacteria have focused on the evidence of conservation of immunomodulatory functions in a range of killing temperatures from 60 °C to 121 °C (Ishikawa *et al.*, 2010; Ou *et al.*, 2011). However, some papers deal with the effect of heat treatment on adhesion. It was observed for example that there was a dramatic decrease in the adhesive ability of *Lb. rhamnosus* GG whatever the intestinal matrix model (Tuomola *et al.*, 1999) or on Caco-2 cells (Ouwehand *et al.*, 2000). The reduction of adhesion can be explained by the heat-sensitive proteinaceous nature of the molecules involved in *Lb. rhamnosus* GG adhesion (von Ossowski *et al.*, 2010, 2011). Interestingly, heat-killed forms of both *Lb. rhamnosus* 3698 and *Lb. farciminis* 3699 exhibited higher adhesion potential to the different intestinal matrix models and higher exclusion potential against binding of *C. jejuni* CIP 70.2^T to mucin. Both probiotic strains studied in this work displayed similar coaggregation with the pathogen *C. jejuni* CIP 70.2^T, regardless of whether they had been heat-treated. Inactivation by heat has been shown previously to generally decrease the adhesion of probiotic bacteria (Ouwehand *et al.*, 2000) but a similar improvement of adhesion to that described in our study has also been demonstrated for a strain of *Propionibacterium freudenreichii* (Ouwehand *et al.*, 2000). The reason why adhesion is improved after heat treatment has not yet been elucidated but it may be due to the nature of the molecules involved. One hypothesis could be that non-proteinaceous molecules like EPS could be produced by the cells as a protective barrier against heat before they die and that the overexpression of

EPS mediates the greater adhesion of heat-killed probiotic forms. The high adhesion potential of heat-killed *Lb. rhamnosus* 3698 and *Lb. farciminis* 3699 also correlated with an increase of their potential for exclusion against *C. jejuni* CIP 70.2^T. These data are in accordance with the study of Ostad *et al.* (2009), who demonstrated that the inhibition of attachment of the pathogenic bacteria by inactivated cells of faecal *Lb. acidophilus* was similar for live and heat-inactivated forms. Based on an *in vivo* approach, oral administration of heat-killed *Lb. plantarum* strain b240 to infected mice with *Salmonella* Typhimurium was found to inhibit adhesion and invasion of intestinal cells by the pathogen, which normally leads to systemic infection associated with multiple organ failure (Ishikawa *et al.*, 2010). The capacity of the heat-killed probiotic cells to retain their aggregative and adhesion abilities could be considered a selective advantage, enabling these strains to remain functional in harsh conditions such as those related to drying processes. Heat-killed probiotic strains exhibiting such properties would have the notable advantage over viable forms of allowing for the generation of more stable products with limited constraints regarding regulatory requirements, technological process and storage.

All these characteristics of *Lb. farciminis* 3699 and *Lb. rhamnosus* 3698 suggest their ability to establish a gut barrier against pathogens, especially *Campylobacter*, and thus it should be interesting to investigate those two strains, live or heat-killed, in a pre-harvest strategy to reduce pathogen loading in animals, considered a major reservoir of pathogens that threaten the food chain.

ACKNOWLEDGEMENTS

The authors thank Dr Didier Goux from the Microscopic Centre of the University of Caen for his technical help with scanning electron microscopy.

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