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Feruloyl esterase activity is influenced by bile, probiotic intestinal adhesion and milk fat

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RESEARCH ARTICLE

Abstract

Cinnamoyl esterases (CE) are microbial and mammalian intestinal enzymes able to release antioxidant hydroxycinnamic acids from their non-digestible ester-linked forms naturally present in vegetable foods. Previous findings showed that oral administration of *Lactobacillus fermentum* CRL1446 increased intestinal CE activity and improved oxidative status in mice. The aim of this work was to evaluate the *in vitro* CE activity of *L. fermentum* CRL1446 and the effect of bile on this activity, as well as strain resistance to simulated gastrointestinal tract (GIT) conditions and its ability to adhere to intestinal epithelium and influence its basal CE activity. *L. fermentum* CRL1446 and *L. fermentum* ATCC14932 (positive control for CE activity) were able to hydrolyse different synthetic hydroxycinnamates, with higher specificity toward methyl ferulate (3,853.73 and 899.19 U/g, respectively). Feruloyl esterase (FE) activity was mainly intracellular in *L. fermentum* CRL1446 and cell-surface associated in *L. fermentum* ATCC14932. Both strains tolerated simulated GIT conditions and were able to adhere *ex vivo* to intestinal epithelium. Pre-incubation of *L. fermentum* strains with bile increased FE activity in both whole cells and supernatants (~2-fold), compared to controls, suggesting that cells were permeabilised by bile, allowing more substrate to enter the cell and/or leakage of FE enzymes. Three-fold higher FE activities were detected in intestinal tissue fragments with adhered *L. fermentum* CRL1446 cells compared to control fragments (without bacteria), indicating that this strain provides exogenous FE activity and could stimulate esterase activity in the intestinal mucosa. Finally, we found that milk fat had a negative effect on FE activity of intestinal tissue, in absence or presence of adhered *L. fermentum*. These results help explaining the increase in intestinal FE activity previously observed in mice fed with *L. fermentum* CRL1446, and support the potential use of this strain for the development of new functional foods directed to oxidative stress-related ailments.

Keywords: intestinal esterase activity, *Lactobacillus fermentum,* antioxidant hydroxycinnamic acids, probiotics, functional foods

1. Introduction

Cinnamoyl esterases (CE) are carboxyl ester hydrolases that catalyse the hydrolysis of hydroxycinnamate esters, which are commonly found in cereals, fruits and vegetables, releasing hydroxycinnamic acids (ferulic, sinapic, caffeic, *p*-coumaric acids) (Fazary and Ju, 2007). CE that hydrolyse mainly esters of ferulic acid are known as feruloyl esterases (FE). Hydroxycinnamic acids (HA) exhibit both *in vitro* and *in vivo* chemoprotective and antioxidant properties (Srinivasan *et al.*, 2007; Zhao and Moghadasian, 2010); therefore, the ability of CE to release HA has attracted growing interest due to their potential beneficial effects on human and animal health (Faulds, 2010). In this sense, it is suspected that they may contribute toward the beneficial effects of a bran-rich diet, evidenced by a lower incidence of oxidative stress-related ailments like cancer, diabetes, cardiovascular and neurodegenerative diseases, and ageing (Srinivasan *et al.*, 2007; Vitaglione *et al.*, 2008).

The hydrolysis of ester bonds and subsequent release of HA in the gut is the first step required for the bioavailability and metabolism of hydroxycinnamates. Andreasen *et al.* (2001a,b) reported that intestinal CE activity in rats and humans has an epithelial and a microbial origin. This enzymatic activity is commonly found in different bacterial

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genera present in the human and animal gut (Couteau *et al.*, 2001; Lai *et al.*, 2009; Wang *et al.*, 2004), and it has been reported in very few strains isolated from food (Abeijón Mukdsi *et al.*, 2012; Donaghy *et al.*, 1998; Guglielmetti *et al.*, 2008). The levels and specificity of these enzymes are critical factors influencing the bioavailability of HA (Faulds, 2010).

Several studies demonstrated that the incorporation of probiotic bacteria (mainly lactobacilli and bifidobacteria) into functional foods has beneficial effects on human health. Lactic acid bacteria (LAB) with FE activity may be proposed as probiotics for their ability to contribute to HA release in the gut. At present, there is little evidence of probiotic LAB with FE activity. Some studies reported that the administration of FE-producing *Lactobacillus fermentum* strains was useful to prevent or treat hypercholesterolemia and metabolic syndrome in hamsters and rats (Bhathena *et al.*, 2009; Tomaro-Duchesneau *et al.*, 2014). Nevertheless, these authors did not evaluate intestinal FE activity of the host.

In this context, we demonstrated that in mice fed with a conventional balanced diet (containing hydroxycinnamates mainly from maize bran), the administration of the FE-producing *L. fermentum* CRL1446 in the drinking water (dose 10^7 cells/day for 7 days), produced a 2-fold increase in total intestinal FE activity compared to non-treated mice, enhancing the bioavailability of ferulic acid (FA), thus improving oxidative status (Abeijón Mukdsi *et al.*, 2012). This activity increase was similar to that observed when administering cheese as a vehicle for the probiotic strain (Abeijón Mukdsi *et al.*, 2013). When evaluating FE activity in each intestinal fraction, the highest increase (~4-fold) was observed in large intestine content and small intestine mucosa (SIM) (~3-fold), when CRL1446 strain was administered in drinking water (Abeijón Mukdsi *et al.*, 2012), and in mucosa from both small and large intestine (~2-fold), when it was administered in goat milk cheese (Abeijón Mukdsi *et al.*, 2013). It has been reported that food ingredients used as carriers of probiotic strains can interact with them, altering some of their properties (Ranadheera *et al.*, 2010).

Probiotic strains must resist the passage through the gastrointestinal tract (GIT), where adverse conditions of pH and the presence of bile salts can affect their viability. Moreover, to exert their *in vivo* beneficial effect, FE must remain active in the GIT where emulsifying compounds, such as bile salts can alter their activity. The location of FE enzyme(s) in the bacterial cell is crucial for their stability in the gut environment, as well as for their accessibility to the substrate. At present, the effect of bile on bacterial FE activity has not been evaluated.

Another important trait of probiotic strains is their ability to adhere to the host gut, which is presumed to be a requisite for sufficient host-interaction to confer health benefits (Van Tassel and Miller, 2011). Moreover, it is not known whether adhesion of FE-producing bacteria to intestinal cells could affect the intestinal FE activity of the host.

In the present work, we performed *in vitro* and *ex vivo* assays aimed to help understanding the effects of *L. fermentum* CRL1446 administration on intestinal FE activity previously observed *in vivo* (Abeijón Mukdsi *et al.*, 2012, 2013). Thus, the goal of this work was to evaluate *in vitro* the CE activity of *L. fermentum* CRL1446 and the effect of bile on this activity, as well as strain resistance to simulated GIT conditions and its ability to adhere to intestinal epithelium and influence on its basal FE activity. Finally, the effect of goat milk fat on FE activity of intestinal tissue was also evaluated.

2. Materials and methods

Microorganisms, media and culture conditions

L. fermentum strain CRL1446, isolated from Argentinean goat milk cheese, was obtained from the Culture Collection of the Centro de Referencia para Lactobacilos (CERELA, Tucumán, Argentina). *L. fermentum* strain ATCC14932, isolated from human saliva, was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), and used as a positive control of CE activity (Bhathena *et al.*, 2007). *L. fermentum* CRL1446 was preliminary selected for its *in vitro* CE activity, evidenced by the formation of clear zones of hydrolysis around colonies grown in De Man-Rogosa-Sharpe (MRS; Britania, Buenos Aires, Argentina) agar supplemented with ethyl ferulate (Sigma, St. Louis, MO, USA) (Abeijón Mukdsi, 2009), and because it increased intestinal FE activity when administered to mice (Abeijón Mukdsi *et al.*, 2012, 2013).

Determination of cinnamoyl esterase activity in cell suspension

Cells were grown in MRS broth at 37 °C and harvested after 16 h (late-log phase) by centrifugation (10,000×*g*, 10 min, 4 °C). The pellet was washed twice and resuspended in phosphate buffered saline (PBS) pH 7 to an OD_{560} of ~1. Hydroxycinnamate solution (methyl ferulate (MtF), methyl caffeate (MtC) or chlorogenic acid (ChA); Sigma) was added to give a final concentration of 5 mM and cell suspensions were incubated at 37 °C for 18 h. Reactions were stopped by the addition of glacial acetic acid (2 mM, pH 2.5). Cells were harvested by centrifugation (13,000×*g*, 10 min, 4 °C), and the supernatants were filtered (0.22 µm, white GSWP, 25 mm; Millipore Corp., Bedford, MA, USA) prior to high performance liquid chromatography (HPLC) analysis of released HA. Controls containing the reaction mixture plus glacial acetic acid were also incubated to test for the presence of background peaks. Results were expressed as units (U) of CE activity per gram of cell dry weight. One U was defined as the amount of enzyme releasing 1 µmol of HA (ferulic or caffeic acid) per h.

Subcellular fraction preparation

Subcellular fractions were obtained according to the method described by Abeijón Mukdsi *et al.* (2009). Cells cultured in 400 ml of MRS broth were harvested at late-log phase by centrifugation (10,000×*g*, 10 min, 4 °C), washed twice with 100 mM sodium phosphate buffer pH 7 and resuspended at 50% (w/v) in the same buffer. Cell suspensions were disrupted by three successive passes through a French pressure cell at 1000 psi (Thermo Spectronic, Rochester, NY, USA). Cellular debris was removed by centrifugation (20,000×*g*, 30 min, 4 °C) and the supernatant (cell-free extract) was ultracentrifuged (45,000×*g*, 30 min, 4 °C) to separate the intracellular and the cell surface-associated fractions. The extracellular fraction was obtained by means of the supernatant of the growth medium. The supernatant was sterilised by filtration (0.22 µm). Protein concentrations in each subcellular fraction were measured according to the method of Bradford (1976), using a commercial kit (Bio-Rad, Hercules, CA, USA) and bovine serum albumin (Sigma) as standard.

Subcellular location of feruloyl esterase activity

FE activity was determined by incubation of supernatant, intracellular fraction and cell surface-associated fraction in PBS pH 7 containing 1 mM MtF at 37 °C for 18 h. Reactions were stopped by the addition of acetic acid and released FA was determined by HPLC. Results were expressed as units (U) of FE activity per milligram of protein. One U was defined as the amount of enzyme releasing 1 µmol of FA per h.

Determination of hydroxycinnamic acids by HPLC

Separations were performed on a Knauer system (Berlin, Germany) equipped with an UV detector, using a reverse-phase C-18 column (Varian Pursuit XRs-C18, 5 µm, 250 x 4.6 mm; Varian, Lake Forest, CA, USA). A 20 µl sample was injected and an isocratic linear solvent gradient of water:acetonitrile:acetic acid (69:30:1, v/v/v) was run as eluent at a flow rate of 1 ml/min. Compounds were monitored by absorbance at 320 nm. Released HA was quantified from the regression curve (R^2 >98%) of the corresponding standard (Apin Chemicals, Abingdon, OK, UK), using external standard calibration.

Evaluation of bacterial resistance to sequential exposition to simulated gastric and intestinal juices

Resistance to GIT conditions was evaluated according to the protocol of Zárate *et al.* (2000). Briefly, 100 µl of bacterial cell suspension containing ~10^9-10^{10} cfu/ml were transferred to 5 ml of simulated gastric juices at pH 3 and 4. Cells were incubated at 37 °C and harvested by centrifugation after 2 h of incubation. Subsequently, cells were washed twice, resuspended in simulated intestinal juice and further incubated for 2 h at 37 °C. The number of viable cells after the gastric treatment and after the sequential gastric plus intestinal treatments was determined by plating onto MRS agar. Plates were incubated under microaerophilic conditions for 72 h and results were expressed as logarithm_{10} of cfu/ml. Simulated gastric juice composition was 125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$ and 3 g pepsin/l. The pH was adjusted to 3 and 4 with 100 mM HCl. Simulated intestinal juice consisted of 0.3% (w/v) oxgall (dehydrated fresh bile; Sigma) and 0.1% (w/v) pancreatin (ICN Biomedicals, Aurora, OH, USA). The pH was adjusted to 8 with 5 mM NaOH.

Effect of bile on feruloyl esterase activity

The effect of bile on enzyme activity was evaluated according to Noh and Gilliland (1993). Cells at late-log phase were harvested, washed with PBS pH 7, and resuspended in the same buffer to reach an OD_{560} of ~1. This cell suspension (5 ml) was added to 5 ml of PBS containing 0.6% (w/v) oxgall, to a final concentration of 0.3% (w/v). Cell suspension added to PBS without oxgall was used as control. Cells were incubated at 37 °C for 10 min, harvested by centrifugation (10,000×*g*, 10 min, 4 °C), and resuspended in 10 ml of PBS. FE activity was determined in supernatants and cell suspensions by incubation (37 °C, 18 h) in PBS containing 1 mM MtF as substrate. Released FA was determined by HPLC. Results were expressed as units (U) of FE activity per mg of protein (supernatant) or U per mg of cell dry weight (cell suspension). One U was defined as mentioned above. Protein concentrations were determined as described above.

Effect of bile on cellular integrity

The effect of bile on bacterial integrity was assessed using the protocol described by Noh and Gilliland (1993). Test tubes with 3 ml PBS pH 7 and another with 3 ml of PBS supplemented with 0.6% (w/v) oxgall were prepared for each strain. 3 ml of washed cell suspension (~10^9-10^{10} cfu/ml) were added in each tube, so that the final concentration of oxgall was 0.3% (w/v). Cell suspensions were incubated at 37 °C and OD_{560} was determined every 10 min for 1 h.

Animals

Six-week-old male Swiss albino mice were used as a source of intestinal tissue fragments and intestinal epithelial cells (IEC) for *ex vivo* adhesion assays. The animals were obtained from the closed random-bred colony maintained at CERELA, housed in metal cages and acclimated to 22±2 °C with a 12 h light/dark cycle. They received a conventional balanced diet (60.8% carbohydrates, 25.5% proteins, 3.8% fats, 3.4% raw fibre, 6.5% total minerals; Asociación de Cooperativas Argentinas, Buenos Aires, Argentina) and drinking water *ad libitum.* Animals were fasted for 16 h before sacrifice. Experimental procedures were approved by the Animal Protection Committee of CERELA, and complied with current Argentinean laws.

Ex vivo bacterial adhesion to intestinal tissue fragments

Adhesion assays were performed according to Babot *et al.* (2014) with some modifications. Briefly, animals were sacrificed by cervical dislocation and immediately eviscerated for collection of ileum, which was in turn rinsed repeatedly with ice-cold PBS pH 7 to eliminate the digesta content. Tissues were cut lengthwise, washed again with cold PBS and then immersed into RPMI 1640 medium supplemented with 100 μg/ml streptomycin and 100 IU/ml penicillin (Gibco, Grand Island, NY, USA) for 30 min at 37 °C. After this, tissue samples were repeatedly washed with fresh medium to remove antibiotics and cut in 100 mm^2 pieces. Each ileum piece was immersed into RPMI supplemented with 1% (v/v) foetal bovine serum (FBS; Gibco) containing 1×10^8 cfu/ml *L. fermentum* and incubated at 37 °C for 1 h in a humid chamber gassed with a mixture of 5% CO_2 and 95% air (Nuaire Co., Plymouth, MN, USA). Finally, tissue pieces were repeatedly rinsed with ice-cold PBS/FBS to remove non-adhered cells, homogenised in the same fresh solution and plated onto MRS agar. The number of cfu per mm^2 of tissue was determined after 72 h incubation at 37 °C. Tissue pieces without inoculation were also incubated to control the sterility of the tissue used. Adhesion results were admissible when viable cell counts in controls were negative or lower than 10^1 cfu/mm^2. According to Babot *et al.* (2014), strains were classified as: adherent (>1×10^3 cfu/mm^2); weakly adherent (10^1-10^3 cfu/mm^2); and non-adherent (<1×10^1 cfu/mm^2).

Ex vivo bacterial adhesion to isolated intestinal epithelial cells

IEC of the distal portion of the ileum were gently scraped off with the edge of a microscope slide. The cells were suspended and washed twice with PBS pH 7 with 1% (v/v) FBS. The cells were collected (800×*g*, 5 min, 4 °C) and their concentration adjusted to 1×10^6 cells/ml in RPMI/FBS. Cell counting was carried out in a Neubauer cell chamber at 40× magnification in a conventional light microscope (Zeiss-Axiolab; Carl Zeiss, Jena, Germany). Suspensions of 1×10^8 cfu/ml *L. fermentum* and recently obtained IEC were mixed (1:4) and incubated for 1 h at 37 °C with a mixture of 5% CO_2 and 95% air (Nuaire Co.). After incubation, the mixtures were centrifuged (120×*g*, 5 min, 4 °C), washed twice with RPMI and suspended in the initial volume of RPMI/FBS. Adhesion to IEC was examined by counting adhered bacteria in 30 IEC, using phase-contrast microscopy. Results were expressed as the percentage of IEC with adhered bacteria (adhesion percentage) and mean number of bacteria adhered per IEC (adhesion index).

Effect of bacterial adhesion and milk fat on feruloyl esterase activity of intestinal tissue

Ileum pieces with adhered *L. fermentum* CRL1446 cells were obtained as described above. Sterile pieces (with no adhered bacteria) were used as controls of basal FE activity. Each piece was immersed into 2 ml PBS or PBS supplemented with 0.4% (v/v) goat milk fat emulsion and incubated during 3 h at 37 °C. After that, intestinal pieces were transferred into tubes containing fresh PBS, 1 mM MtF was added as substrate and tubes were incubated at 37 °C for 18 h. Reactions were stopped by addition of acetic acid. Supernatants were recovered (12,000×*g*, 5 min, 4 °C) for free FA determination by HPLC. Results were expressed as units (U) of FE activity per g of intestinal fragment. One U was defined as mentioned above.

Statistical analysis

Results are means ± standard deviation from three independent experiments. After the analyses of variance (ANOVA), Tukey's test was used to identify statistically significant differences ($P<0.05$). These analyses were carried out using statistical software (Minitab1.5, State College, PA, USA).

3. Results

Cinnamoyl esterase activity in *Lactobacillus fermentum* cells

CE activities were quantified by incubation of cell suspensions in presence of MtF, MtC and ChA. Both strains hydrolysed all substrates and showed higher activity on MtF (Table 1), displaying *L. fermentum* CRL1446 an activity 4-fold higher than ATCC14932. In presence of MtC, *L. fermentum* CRL1446 presented activity levels 16-fold higher than ATCC14932, whereas in presence of ChA, *L. fermentum* ATCC14932 cells showed an activity 5-fold higher than CRL1446.

Table 1. Cinnamoyl esterase activity in *Lactobacillus fermentum* strains.[1]

Strain	**Cinnamoyl esterase activity**		
	Methyl ferulate	**Methyl caffeate**	**Chlorogenic acid**
CRL1446	$3,853.73 \pm 234.14^a$	11.42 ± 0.05^a	0.60 ± 0.04^b
ATCC14932	899.19 ± 123.20^b	0.70 ± 0.04^b	3.00 ± 0.03^a

[1] Results are expressed as Units/g of cell dry weight (U = μmol of ferulic or caffeic acid released per h). Data are presented as mean ± standard deviation from three independent experiments. Means in the same column with different superscript letters differ significantly ($P<0.05$).

Subcellular location of feruloyl esterase activity

To ascertain the subcellular distribution of FE, this activity was determined in the extracellular (culture supernatant), intracellular and cell surface-associated fractions of *L. fermentum* CRL1446 and ATCC14932; both strains expressing strong FE activity (Table 2). *L. fermentum* ATCC14932 showed the highest levels of FE activity located in the cell surface-associated and intracellular fractions. However, FE activity in *L. fermentum* CRL1446 was mainly intracellular. In this strain, intracellular FE activity was 2-fold higher than that associated with the cell envelopes, and considerably higher (3.4-fold) than that observed in ATCC14932 strain. In both strains, the extracellular FE activity was negligible compared to that detected in the other subcellular fractions.

Bacterial resistance to sequential exposition to simulated gastric and intestinal juices

Strain resistance to GIT conditions was determined in an *in vitro* model, and the results are shown in Table 3. Assays with simulated gastric juices showed that both strains survived after 2 h incubation at pH 3 and pH 4. *L. fermentum* CRL1446 showed decreases of viable cell numbers of ~0.5 and 2 log units at pH 4 and pH 3, respectively. However, ATCC14932 strain showed viability decreases of ~4 log units at both pHs evaluated. In both strains, no significant changes in cell viability were observed after incubation in simulated intestinal juices.

Effect of bile on feruloyl esterase activity

To exert their *in vivo* beneficial effect, FE enzymes must remain active in the GIT, where bile can affect their activity. Table 4 shows FE activity determined in cell suspensions and supernatants of *L. fermentum* CRL1446 and ATCC14932 pre-incubated in the presence of 0.3% (w/v) oxgall. In both strains, higher FE activity levels (~2-fold) were detected in both cells and supernatants of cell suspensions exposed to oxgall, compared to controls (non-exposed cells).

Table 2. Subcellular location of feruloyl esterase activity.[1]

Subcellular fraction	***Lactobacillus fermentum***	
	Strain CRL1446	**Strain ATCC14932**
Extracellular	9.36 ± 1.20^a	13.71 ± 2.10^a
Intracellular	217.88 ± 20.34^b	64.37 ± 8.45^b
Cell surface-associated	103.28 ± 12.65^c	94.03 ± 10.32^c

[1] Results are expressed as Units/mg of protein (U = μmol of ferulic acid released per h). Data are presented as mean ± standard deviation of three independent experiments. Means in the same column with different superscript letters differ significantly ($P<0.05$).

Effect of bile on cellular integrity

Cellular integrity of *L. fermentum* CRL1446 and ATCC14932 was determined by measuring the absorbance of cell suspensions incubated in the presence of 0.3% (w/v) oxgall. Cell suspensions in PBS (without oxgall) were used as controls. In both strains, no significant differences in absorbance measurements were observed in the presence and absence of oxgall (not shown).

Ex vivo bacterial adhesion to intestinal tissue and isolated intestinal epithelial cells

L. fermentum adhesion property was evaluated by interaction of cell suspensions with intestinal tissue fragments and with IEC exfoliated from the ileal tissue. Results are shown in Table 5. Both strains showed good ability to adhere to ileum mucosa with viable cell counts of ~10^6 cfu/mm^2 of tissue (~7 log cfu/mm^2). The highest adhesion percentage was observed for *L. fermentum* CRL1446 (16.6%). Similar adhesion indexes were obtained for both strains (one or two adhered bacteria per IEC).

Table 3. Cell viability of *Lactobacillus fermentum* strains after sequential incubation in simulated gastric and intestinal juices.

Conditions		Time of incubation (h)	*L. fermentum* strain (Log cfu/ml)[1]	
			CRL1446	ATCC14932
Gastric juice (GJ)	pH 3	0	9.33±0.14^{a}	10.58±0.18^{a}
		2	7.23±0.12^{b}	6.92±0.07^{b}
	pH 4	0	9.33±0.14^{a}	10.58±0.18^{a}
		2	8.88± 0.15^{b}	6.60±0.23^{b}
Intestinal juice (IJ)	(from GJ at pH 3)[2]	0	7.23±0.12^{a}	6.92±0.07^{a}
		2	7.17±0.11^{a}	6.87±0.10^{a}
	(from GJ at pH 4)[3]	0	8.88±0.15^{a}	6.60±0.23^{a}
		2	8.08±0.09^{a}	6.95±0.12^{a}

[1] Data are presented as mean ± standard deviation from three independent experiments. Means in the same column (under a same condition) with different superscript letters differ significantly ($P<0.05$).

[2] Cells pre-incubated 2 h in simulated GJ at pH 3 and then incubated in simulated IJ pH 8.

[3] Cells pre-incubated 2 h in simulated GJ at pH 4 and then incubated in simulated IJ pH 8.

Table 4. Effect of bile on feruloyl esterase activity of *Lactobacillus fermentum*.[1]

Enzyme source	Strain CRL1446		Strain ATCC14932	
	Control[2]	Bile[3]	Control	Bile
Supernatant	10.34±1.35^{a}	23.04±2.67^{b}	14.50±1.03^{a}	25.97±3.65^{b}
Cell suspension	4.13±0.90^{a}	9.67±1.12^{b}	1.10±0.45^{a}	2.54±1.03^{b}

[1] Results are expressed as Units/mg of protein (supernatants) or Units/mg cell dry weight (cell suspensions). U = µmol of ferulic acid released per h. Data are presented as mean ± standard deviation from three independent experiments. Means in the same row for each strain with different superscript letters differ significantly from control ($P<0.05$).

[2] Control = cells not exposed to oxgall.

[3] Cells exposed to 0.3% oxgall (w/v) for 10 min.

Table 5. Intestinal adhesion ability of *Lactobacillus fermentum* strains.

Strain	Log cfu/mm^{2} [1]	Adhesion percentage[2]	Adhesion index[3]
CRL1446	6.69±0.31^{a}	16.61±2.99^{a}	1.3±0.5^{a}
ATCC14932	6.57±0.10^{a}	6.53±2.02^{b}	1.8±0.8^{a}

[1] Results are expressed as log cfu/mm^{2} of intestinal tissue.

[2] Percentage of IEC with adhered bacteria.

[3] Mean number of bacteria adhered per IEC. Data are presented as mean ± standard deviation from three independent experiments. Means in the same column with different superscript letters differ significantly ($P<0.05$).

Effect of bacterial adhesion and milk fat on feruloyl esterase activity of intestinal tissue fragments

Intestinal tissue fragments, with or without adhered *L. fermentum* CRL1446 cells, were incubated in presence or absence of goat milk fat. Then, FE activity was determined and results are shown in Figure 1. In fragments with adhered *L. fermentum*, FE activity was ~3-fold higher than in control fragments (with no adhered bacteria). In presence of milk fat, FE activity was ~2-fold lower than in its absence, regardless of the presence of adhered *L. fermentum*.

4. Discussion

Hydroxycinnamates are normally present in several vegetable foods, ester-linked to cell wall polysaccharides. They cannot be absorbed in the gut until they reach the colon, where CE-producing microbiota can hydrolyse ester bonds and then, free HA become available for absorption (Faulds, 2010). These compounds have strong antioxidant properties and play a role in the prevention of several chronic diseases, such as cardiovascular diseases, diabetes, cancer, among others (Zhao and Moghadasian, 2010). Thus, probiotic bacteria with CE activity are a promising alternative for the prevention and/or treatment of many oxidative stress-related ailments.

In a previous study, we demonstrated that oral administration of the FE-producing strain *L. fermentum* CRL1446 to mice (10^7 cells/day/mouse) increased total intestinal FE activity from day 5. This activity increase was related to an enhancement of oxidative status, evidenced by a decrease in basal plasmatic thiobarbituric acid-reactive substance levels and an increase in plasmatic glutathione reductase activity. In addition, no significant changes in intestinal microbiota counts were observed in mice receiving *L. fermentum* CRL1446, via drinking water and goat milk cheese for 7 days (Abeijón Mukdsi *et al.*, 2012, 2013). The mechanisms by which *L. fermentum* CRL1446 administration increases total intestinal FE activity *in vivo*, still remain to be elucidated. Thus, in this work we performed *in vitro* and *ex vivo* assays aimed to find some possible explanations for the effects obtained *in vivo*.

First, we explored the CE activity of two *L. fermentum* strains (CRL1446 and ATCC14932) on different synthetic substrates (MtF, MtC and ChA). Esters of FA are highly abundant in cereal bran and whole grains. Esters of caffeic acid and ChA (ester of caffeic and quinic acid) are naturally present in high concentrations in coffee, fruits and vegetables (Zhao and Moghadasian, 2010). Both *L. fermentum* strains displayed the highest activity on MtF. Therefore, we referred to FE activity throughout the manuscript and MtF was used as substrate for further enzymatic determinations. Among several LAB tested for the presence of CE, Donaghy *et al.* (1998) observed the highest activity in *L. fermentum* NCFB 1751, when this strain was tested in culture and as crude enzyme preparation. Guglielmetti *et al.* (2008) found among 100 strains isolated from food and human gut, 12 lactobacilli strains belonging to the species *L. helveticus*, *L. acidophilus* and *L. fermentum*, active on ethyl ferulate and ChA. Similarly to our results, *L. fermentum* strains showed preference toward ferulate esters. Bhathena *et al.* (2007) reported the production of FA from ethyl ferulate using microencapsulated *L. fermentum* ATCC11976 and ATCC14932, strains with high levels of FE activity. Other authors reported that *L. fermentum* NCIMB 5221 had a greater FA production compared to other FE-producing lactobacilli (Tomaro-Duchesneau *et al.*, 2012).

FE-producing *L. fermentum* strains evaluated in this study did not seem to further metabolise free FA (or do so extremely slowly), due to its accumulation in the medium (not shown). Similar observations were reported by other

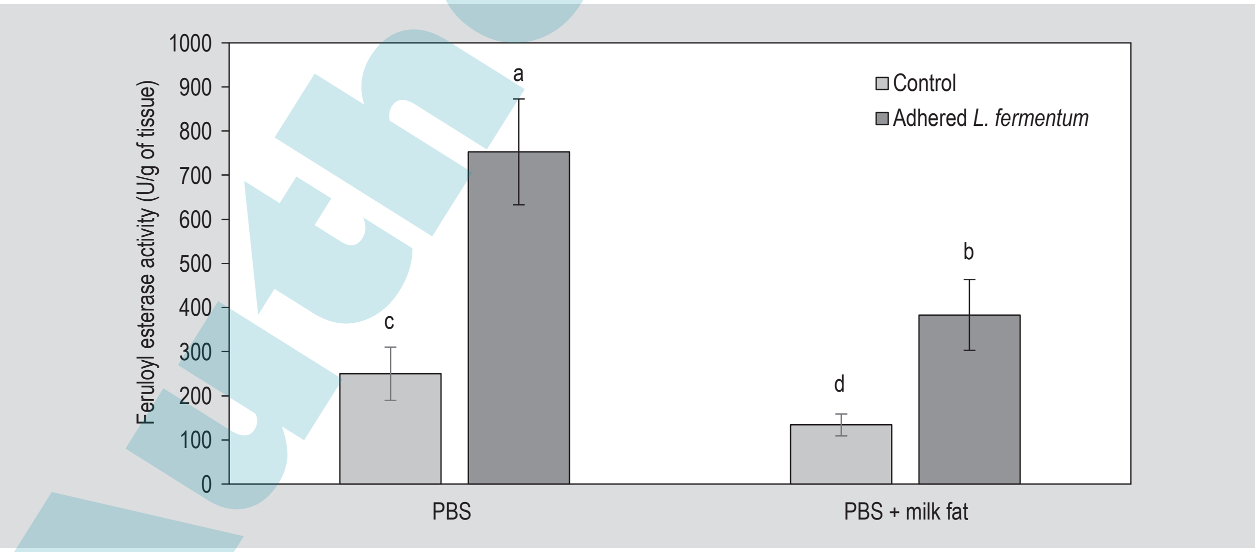

Figure 1. Feruloyl esterase activity in intestinal tissue fragments without and with adhered *Lactobacillus fermentum* CRL1446 (~10^6 cfu/mm^2) after pre-incubation in phosphate buffered saline (PBS) and PBS supplemented with milk fat. Results are expressed as Units/g of intestinal tissue (U = µmol of ferulic acid released per h). Data are presented as mean ± standard deviation from two independent experiments. Means with different letters (a-d) differ significantly from each other.

authors (Donaghy *et al.*, 1998; Tomaro-Duchesneau *et al.*, 2012). Therefore, FA released by FE-producing *L. fermentum* strains in the gut, would be available for absorption by epithelial cells, exerting its biological effects. Nevertheless, uptake and metabolism of free FA by other species or genera present in gut microbiota could not be discarded.

The location of FE enzymes in the bacterial cell is determinant for their accessibility to the substrate, as well as for their stability in harsh environments such as the GIT. Hydroxycinnamates normally ingested in the diet are much more complex substrates than MtF. However, in the gut, microbial FE act synergistically with xylanases and other plant cell wall polysaccharide-degrading enzymes for release of FA (Faulds, 2010).

FE activity was mainly intracellularly located in both *L. fermentum* strains evaluated, and also present in the cell-wall fraction. In accordance with our findings, other authors reported that CE activity in LAB is exclusively cell-associated (cytoplasmic and cell wall-anchored), whereas extracellular activity has not been observed (Couteau *et al.*, 2001; Donaghy *et al.*, 1998). The co-existence of two different esterases, one cytoplasmic and another cell surface-associated, has been previously described in *L. fermentum* (Abeijón Mukdsi, 2009; Gobbetti *et al.*, 1997). *L. fermentum* CRL1446, a FE-producing strain evaluated in this study, produces both an intracellular and a cell surface-associated esterase active on naphthyl esters of short-chain fatty acids (Abeijón Mukdsi, 2009). It is known that even though FE preferentially hydrolyse aromatic compounds, they can also act upon a wide range of substrates, including aliphatic esters, with a lower catalytic efficiency (Esteban-Torres *et al.*, 2015; Lai *et al.*, 2009).

Intracellular FE are protected from the adverse conditions of the GIT such as pH, salts, and denaturing agents. Nevertheless, it is not clear yet how substrate-enzyme interactions occur. At present, there is no evidence of any efficient functional hydroxycinnamate transporter in LAB. Other probable alternative is that FE could be released from the cell through cell lysis or permeabilisation. On the other hand, the presence of cell surface-associated FE could facilitate enzyme accessibility to the substrate. Moreover, cell-wall microenvironment would improve enzyme stability.

Before reaching the distal part of the intestinal tract and exerting their beneficial effect, probiotic bacteria must survive during transit through the stomach and the upper intestinal tract. Thus, we evaluated *L. fermentum* CRL1446 and ATCC14932 resistance to sequential exposition to simulated gastric and intestinal juices. Both pH of simulated gastric juice and bile concentration in simulated intestinal juice are among the value ranges found in the human GIT (Bao *et al.*, 2010). Our results indicated that the resistance to GIT conditions was strain-specific, being *L. fermentum* CRL1446 more resistant than *L. fermentum* ATCC14932. The strain-dependence of GIT resistance was also observed by Bao *et al.* (2010). These authors studied 90 strains of *L. fermentum* isolated from traditional Asian dairy products, for their tolerance to acid, simulated gastrointestinal juice and bile salts. Eleven strains showed good resistance to GIT and bile salt tolerance. Similarly to our observations for CRL1446 strain, Mikelsaar and Zilmer (2009) reported that by the co-action of pH and pepsin followed by bile and pancreatin, a decrease of 0.5 to 1.5 log of viable cell numbers was observed in the probiotic strain *L. fermentum* M-3.

Results obtained during the sequential incubation in simulated gastric and intestinal juices, allowed determining that slight decreases of *L. fermentum* cell viability were due to the deleterious effect of the acidic conditions of the stomach; however, the presence of bile did not affect strain viability. Similar results were reported by Bao *et al.* (2010) for the strain *L. fermentum* F6. Specific bile resistance mechanisms have been described in intestinal LAB, bile efflux and bile salt hydrolase activity being the most prevalent (Ruiz *et al.*, 2013). Several mechanisms of resistance to acid pH described in LAB were reviewed by Van de Guchte *et al.* (2002). Interestingly, genes that provide protection in bile stress also protect against acid stress in probiotic *L. reuteri* (Wall *et al.*, 2007; Whitehead *et al.*, 2008). This indicated that once cells experience acid stress in the stomach, many of the important pathways for dealing with bile stress in the small intestine will already be activated.

Due to its detergent-like properties, bile can cause protein denaturation (Begley *et al.*, 2005). To the best of our knowledge, there are no reports about the effect of bile on FE activity of bacterial cells. Therefore, we evaluated the effect of bile on the FE activity of *L. fermentum* CRL1446 and ATCC14932 by incubating cells in presence of oxgall, and determining the activity in cells themselves and in supernatants obtained from cell suspensions. Both cells as well as supernatants pre-incubated with oxgall showed higher FE activities than the controls (non-exposed to oxgall). The higher FE activity detected in oxgall-exposed cells would indicate that the permeability of *L. fermentum* cells increased, allowing more substrate to enter the cells. On the other hand, the increased FE activity in supernatants from oxgall-exposed cell suspensions would also be related to the permeabilising effect of bile. It is known that bile acids cause damage to cells that are considered to be bile resistant, most likely via disruption of the membrane and cell wall (Whitehead *et al.*, 2008). The identification of a putative cell wall-altering esterase as a key enzyme in responding to bile stress in probiotic *Lactobacillus reuteri* ATCC55730, suggested that cells experience cell envelope impairment upon exposure to bile (Whitehead *et al.*, 2008).

Thus, alteration of cell envelopes would allow the release of intracellular and/or cell wall-anchored FE to the medium, which are monomeric enzymes with a molecular mass of ~30 kDa (Esteban-Torres *et al.*, 2015; Lai *et al.*, 2009). The cell-wall associated FE detected in *L. fermentum* strains would be more likely released under bile exposure.

The fact that bile did not lyse *L. fermentum* CRL1446 and ATCC14932 cells (not shown) supports our hypothesis that once in the gut, *L. fermentum* cells are permeabilised by bile and, in spite of this permeabilisation, they remain viable. Begley *et al.* (2005) reported that many resistance mechanisms resulting in alteration of lactobacilli cell surface structures are common for bile and acid stress, contributing to maintain cell integrity.

Our results suggest that *L. fermentum* CRL1446 can provide meaningful FE activity by permeabilisation of the cells in the gut. This fact would be related to the increase of FE activity detected in intestinal content of mice receiving this strain in drinking water, compared to non-treated mice (Abeijón Mukdsi *et al.*, 2012). We previously demonstrated that administration of CRL1446 strain did not modify colonic microbiota counts (Abeijón Mukdsi *et al.*, 2012); nevertheless, it could stimulate FE activity of colonic luminal microbiota via an indirect effect. It is known that food matrix protects probiotic bacteria from the permeabilising effect of bile (Ranadheera *et al.*, 2010). This could partially explain the fact that mice receiving *L. fermentum* CRL1446 via goat milk cheese did not show significant activity increase in intestinal contents (Abeijón Mukdsi *et al.*, 2013).

It is considered that probiotic bacteria that reach the intestine alive and adhere to the intestinal epithelium may have higher possibilities to persist longer in the gut ecosystem, increasing the duration of their provision of beneficial effects in the host. Although lactobacilli have been isolated from all portions of the human GIT, the terminal ileum appears to be the preferential site of colonisation of lactobacilli (Plant and Conway, 2002).

In this study, we first evaluated adhesion properties of *L. fermentum* strains in intestinal tissue fragments obtained from the distal section of ileum. According to Babot *et al.* (2014), both strains evaluated were considered as adherent strains (>10^3 cfu/mm^2 of tissue). The number of adhered *L. fermentum* cells (~10^6 cfu/mm^2) was similar to that reported by Plant and Conway (2002). These authors also performed *ex vivo* adhesion assays using resected tissue pieces from mice intestine, and found that *L. fermentum* strains showed different patterns of adhesion to tissue from all regions of the GIT. *L. fermentum* KLD adhered in high numbers to small intestine tissue sections (4×10^6 cfu/mg of tissue), whereas *L. fermentum* 8896 showed negligible adhesion to all tissue types. We also evaluated adhesion properties by interaction of *L. fermentum* cell suspensions and IEC exfoliated from the ileal tissue without the mucus that covers the intestinal mucosa. This method was more sensitive than the tissue fragment assay and allowed the detection of differences in the adhesion ability among strains, displaying *L. fermentum* CRL1446 higher adhesion percentage than *L. fermentum* ATCC14932. Maragkoudakis *et al.* (2006) reported that lactobacilli isolated from dairy products had different rates of Caco-2 cell adherence, ranging from 0.2 to 25.5%. Most had a low adhesion rate (<4%). The probiotic *L. fermentum* RM28 strain isolated from fermented milk showed good adherence (7%) to Caco-2 cells (Thirabunyanon *et al.*, 2009). Even though experimental models were different (exfoliated normal IEC vs Caco-2 cell line), adhesion percentages found in our study were in between the aforementioned range.

There is some evidence that relates adhesion *in vitro* to temporal colonisation of the GIT by *L. fermentum* (Mikelsaar and Zilmer, 2009). Plant and Conway (2002) reported that persistence of *L. fermentum* KLD (human faeces isolate given oro-gastrically at dose 10^8 cells/mouse) within the faeces of mice may be a consequence of its capacity to adhere in high numbers through the mouse GIT.

The good adhesion ability of *L. fermentum* CRL1446 is in accordance with the higher FE activity detected in intestinal mucosa from mice receiving this strain, compared to control mice (Abeijón Mukdsi *et al.*, 2012; 2013). Thus, we hypothesised that *L. fermentum* adheres to intestinal epithelium, increasing its residence time in the gut, delivering FE activity *in situ*, whereas another possible explanation is that bacteria-IEC interaction stimulates the basal FE activity of epithelial cells. Therefore, we evaluated FE activity in ileum tissue fragments with and without adhered *L. fermentum* CRL1446. The 3-fold higher activity observed in fragments with adhered CRL1446 strain compared to controls (without bacteria), clearly demonstrated that this strain was able to increase basal intestinal FE activity of the host (via a direct and/or indirect effect).

Interestingly, FE activity increase observed in SIM of mice receiving *L. fermentum* CRL1446 in drinking water was higher than that observed in animals fed with functional cheese (3-fold vs 2-fold) (Abeijón Mukdsi *et al.*, 2012, 2013). We thought that since goat milk has a high content of fat (~4% v/v) (Park *et al.*, 2007), and esterases are also active on milk fat, this food component could influence on intestinal activity. Thus, we finally investigated the effect of milk fat on FE activity, by using the same system aforementioned (fragments with/without adhered CRL1446). A 0.4% (v/v) milk fat concentration was used taking into account a 1/10 dilution factor by GIT fluids during digestion, and 3 h incubation was estimated as intestinal transit time in mice. Two-fold lower FE activity was detected in tissue fragments pre-incubated in presence of milk fat, indicating that fat negatively affects FE activity. Since milk fat did not seem to modify the degree of *L. fermentum* adhesion to intestinal fragments (not shown), we argue that milk fat could have blocked the active site of FE enzymes. Further research is necessary to elucidate this fact. Despite this tentative inhibitory effect of milk fat, functional cheeses containing *L. fermentum* CRL1446 provided sufficient FE activity into the gut, causing a significant increase in total intestinal FE activity and enhancement of oxidative status, as demonstrated in a previous study (Abeijón Mukdsi *et al.*, 2013). Besides dairy based-products, other food matrixes, in particular vegetable-based, could be investigated in the future for the development of novel functional foods containing FE-producing LAB.

5. Conclusions

From our results, we can conclude that permeabilisation of *L. fermentum* cells by bile increases the availability of FE enzymes, thus providing meaningful FE activity in the gut, whereas the bile tolerance of cells would permit *L. fermentum* to grow and be present in high concentrations in the intestine. Moreover, adhesion of *L. fermentum* CRL1446 to intestinal epithelial cells would imply a prolonged provision and/or stimulation of FE activity at mucosa level. These *in vitro* and *ex vivo* results provide a deeper understanding of the beneficial effects of *L. fermentum* CRL1446 administration observed *in vivo*, and reinforce the use of this strain for the development of novel functional foods directed to oxidative stress-related ailments.

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References

Abeijón Mukdsi, M.C., 2009. Esterases from lactic acid bacteria in fermented foods. PhD thesis, Universidad Nacional de Tucumán, Tucumán, Argentina.

Abeijón Mukdsi, M.C., Gauffin Cano, M.P., González, S.N. and Medina, R.B., 2012. Administration of *Lactobacillus fermentum* CRL1446 increases intestinal feruloyl esterase activity in mice. Letters in Applied Microbiology 54: 18-25.

Abeijón Mukdsi, M.C., Haro, C., González, S.N. and Medina, R.B., 2013. Functional goat milk cheese with feruloyl esterase activity. Journal of Functional Foods 5: 801-809.

Abeijón Mukdsi, M.C., Medina, R.B., Katz, M.B., Pivotto, R., Gatti, P. and González, S.N., 2009. Contribution of lactic acid bacteria esterases to the release of fatty acids in miniature ewe's milk cheese models. Journal of Agricultural and Food Chemistry 57: 1036-1044.

Andreasen, M.F., Kroon, P.A., Williamson, G. and Garcia-Conesa, M.T., 2001a. Esterase activity able to hydrolyze dietary antioxidant hydroxycinnamates is distributed along the intestine of mammals. Journal of Agricultural and Food Chemistry 49: 5679-5684.

Andreasen, M.F., Kroon, P.A., Williamson, G. and Garcia-Conesa, M.T., 2001b. Intestinal release and uptake of phenolic antioxidant diferulic acids. Free Radical Biology and Medicine 31: 304-314.

Babot, J.D., Argañaraz-Martínez, E., Saavedra, L., Apella, M.C. and Perez Chaia, A., 2014. Selection of indigenous lactic acid bacteria to reinforce the intestinal microbiota of newly hatched chicken-relevance of *in vitro* and *ex vivo* methods for strains characterization. Research in Veterinary Science 97: 8-17.

Bao, Y., Zhang, Y., Zhang, Y., Liu, Y., Wang, S., Dong, X., Wang, Y. and Zhang, H., 2010. Screening of potential probiotic properties of *Lactobacillus fermentum* isolated from traditional dairy products. Food Control 21: 695-701.

Begley, M., Gahan, C.G.M. and Hill, C., 2005. The interaction between bacteria and bile. FEMS Microbiology Reviews 29: 625-651.

Bhathena, J., Kulamarva, A., Urbanska, A.M., Martoni, C. and Prakash, S., 2007. Microencapsulated bacterial cells can be used to produce the enzyme feruloyl esterase: preparation and *in-vitro* analysis. Applied Microbiology and Biotechnology 75: 1023-1039.

Bhathena, J., Martoni, C., Kulamarva, A., Urbanska, A.M., Malhotra, M. and Prakash, S., 2009. Orally delivered microencapsulated live probiotic formulation lowers serum lipids in hypercholesterolemic hamsters. Journal of Medicinal Food 12: 310-319.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Couteau, D., McCartney, A.L., Gibson, G.R., Williamson, G. and Faulds, C.B., 2001. Isolation and characterization of human colonic bacteria able to hydrolyse chlorogenic acid. Journal of Applied Microbiology 90: 873-881.

Donaghy, J., Kelly, P.F. and McKay, A.M., 1998. Detection of ferulic acid esterase production by *Bacillus* spp. and lactobacilli. Applied Microbiology and Biotechnology 50: 257-260.

Esteban-Torres, M., Landete, J.M., Reverón, I., Santamaría, L., De las Rivas, B. and Muñoz, R., 2015. A *Lactobacillus plantarum* esterase active on a broad range of phenolic esters. Applied and Environmental Microbiology 81: 3235-3242.

Faulds, C.B., 2010. What can feruloyl esterases do for us? Phytochemistry Reviews 9: 121-132.

Fazary, A.E. and Ju, Y.H., 2007. Feruloyl esterases as biotechnological tools: current and future perspectives. Acta Biochimica et Biophysica Sinica 39: 811-828.

Gobbetti, M., Smacchi, E. and Corsetti, A., 1997. Purification and characterization of a cell surface-associated esterase from *Lactobacillus fermentum* DT41. International Dairy Journal 7: 13-21.

Guglielmetti, S., De Noni, I., Caracciolo, F., Molinari, F., Parini, C. and Mora, D., 2008. Bacterial cinnamoyl esterase activity screening for the production of a novel functional food product. Applied and Environmental Microbiology 74: 1284-1288.

Lai, K.K., Lorca, G.L. and González, C.F., 2009. Biochemical properties of two cinnamoyl esterases purified from a *Lactobacillus johnsonii* strain isolated from diabetic resistant rats' (BB-DR) stool samples. Applied and Environmental Microbiology 76: 5018-5024.

Maragkoudakis, P.A., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B. and Tsakalidou, E., 2006. Probiotic potential of *Lactobacillus* strains isolated from dairy products. International Dairy Journal 16: 189-199.

Mikelsaar, M. and Zilmer, M., 2009. *Lactobacillus fermentum* ME-3 – an antimicrobial and antioxidative probiotic. Microbial Ecology in Health and Disease 21: 1-27.

Noh, D.O. and Gilliland, S.E., 1993. Influence of bile on cellular integrity and *β*-galactosidase activity of *Lactobacillus acidophilus*. Journal of Dairy Science 76: 1253-1259.

Park, Y.W., Juárez, M., Ramos, M. and Haenlein, G.F.W., 2007. Physico-chemical characteristics of goat and sheep milk. Small Ruminant Research 68: 88-113.

Plant, L.J. and Conway, P.L., 2002. Adjuvant properties and colonization potential of adhering and non-adhering *Lactobacillus* spp. following oral administration to mice. FEMS Immunology and Medical Microbiology 34: 105-111.

Ranadheera, R.D.C.S., Baines, S.K. and Adams, M.C., 2010. Importance of food in probiotic efficacy. Food Research International 43: 1-7.

Ruiz, L., Margolles, A. and Sánchez, B., 2013. Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium*. Frontiers in Microbiology 4: 396.

Srinivasan, M., Sudheer, A.R. and Menon, V.P., 2007. Ferulic acid: therapeutic potential through its antioxidant property. Journal of Clinical Biochemistry and Nutrition 40: 92-100.

Thirabunyanon, M., Boonprasom, P. and Niamsup, P., 2009. Probiotic potential of lactic acid bacteria isolated from fermented dairy milks on antiproliferation of colon cancer cells. Biotechnology Letters 31: 571-576.

Tomaro-Duchesneau, C., Saha, S., Malhotra, M., Coussa-Charley, M., Al-Salami, H., Jones, M.L., Labbé, A. and Prakash, S., 2012. *Lactobacillus fermentum* NCIMB 5221 has a greater ferulic acid production compared to other ferulic acid esterase producing lactobacilli. International Journal of Probiotics and Prebiotics 7: 23-32.

Tomaro-Duchesneau, C., Saha, S., Malhotra, M., Jones, M.L., Labbé, A., Rodes, L., Kahouli, I. and Prakash, S., 2014. Effect of orally administered *L. fermentum* NCIMB 5221 on markers of metabolic syndrome: an *in vivo* analysis using ZDF rats. Applied Microbiology and Biotechnology 98: 115-126.

Van de Guchte, M., Serror, P., Chervaux, C., Smokvina, T., Ehrlich, S.D. and Maguin, E., 2002. Stress responses in lactic acid bacteria. Antonie van Leeuwenhoek 82: 187-216.

Van Tassell, M.L. and Miller, M.J., 2011. *Lactobacillus* adhesion to mucus. Nutrients 3: 613-636.

Vitaglione, P., Napolitano, A. and Fogliano, V., 2008. Cereal dietary fibre: a natural functional ingredient to deliver phenolic compounds into the gut. Trends in Food Science and Technology 19: 451-463.

Wall, T., Båth, K., Britton, R.A., Jonsson, H., Versalovic, J. and Roos, S., 2007. The early response to acid shock in *Lactobacillus reuteri* involves the ClpL chaperone and a putative cell wall-altering esterase. Applied and Environmental Microbiology 73: 3924-3935.

Wang, X., Geng, X., Egashira, Y. and Sanada, H., 2004. Purification and characterization of a feruloyl esterase from the intestinal bacterium *Lactobacillus acidophilus.* Applied and Environmental Microbiology 70: 2367-2372.

Whitehead, K., Versalovic, J., Roos, S. and Britton, R.A., 2008. Genomic and genetic characterization of the bile stress response of probiotic *Lactobacillus reuteri* ATCC 55730. Applied and Environmental Microbiology 74: 1812-1819.

Zárate, G., Pérez Chaia, A., González, S. and Oliver, G., 2000. Viability and β-galactosidase activity of dairy propionibacteria subjected to digestion by artificial gastric and intestinal fluids. Journal of Food Protection 63: 1214-1221.

Zhao, Z. and Moghadasian, M.H., 2010. Bioavailability of hydroxycinnamates: a brief review of *in vivo* and *in vitro* studies. Phytochemistry Reviews 9: 133-145.