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ORIGINAL ARTICLE

Lactobacillus delbrueckii subsp *lactis* (strain CIDCA 133) resists the antimicrobial activity triggered by molecules derived from enterocyte-like Caco-2 cells

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Abstract

Aims: The aim of the present study was to assess the ability of a potentially probiotic strain to resist, *in vitro*, the effect of intestinal antimicrobial molecules.
Methods and results: Strain CIDCA 133 of *Lactobacillus delbrueckii* subsp *lactis* was studied. *Lactobacillus delbrueckii* subsp *bulgaricus* as well as other gram-positive and gram-negative bacteria were used for comparison purposes. The effect of different antimicrobial extracts was determined by diffusion assays, viable counts and growth kinetics. Human-defensins (hβD1 and hβD2) were also included in the study. Two types of cellular fractions from Caco-2 cells were tested: (i) cytosolic fractions, obtained by sonication of cultured human enterocytes and (ii) cationic fraction, obtained by batch extraction of the cytosolic fraction with a weak cation exchange resin. In addition, the effect of Caco-2-secreted factors was studied. Strain CIDCA 133 was neither inhibited by Caco-2 secreted, cytosolic nor cationic fractions. Of note, human-defensins were inactive against strain CIDCA 133. In contrast, a related lactobacilli: *Lactobacilli delbrueckii* subsp *bulgaricus* (strain CIDCA 331) and other species of gram-positive or gram-negative bacteria were strongly inhibited.
Conclusions: Strain CIDCA 133 is able to survive and grow in the presence of enterocyte-derived antimicrobial molecules. This ability is not a general property of lactobacilli.
Significance and Impact of the Study: Results could provide a new insight into the mechanisms of the probiotic effect and encourage further studies on this field. Resistance to antimicrobial peptides can be relevant to understand the interaction of potentially probiotic strains with the host's immune system. This ability can be also relevant as a selection criterion for new probiotic strains.

Introduction

Probiotics are defined as 'live microbial food supplements that improve the health of the host' (Fuller 1989). Fermented dairy products containing probiotic bacteria are the most successful category of functional foods (Saxelin *et al.* 2005). Lactobacilli and bifidobacteria are generally accepted as suitable candidates for the formulation of probiotics for humans. Consumption of probiotics positively affects the composition of intestinal microbiota and could provide several benefits for the host including the inhibition of intestinal pathogens, immunomodulation, anticarcinogenic and antimutagenic activities, alleviation of symptoms of lactose intolerance and reduction in serum cholesterol (Hord 2008). Selection criteria of potentially probiotic strains include several recommendations as technological adaptability, accurate identification, GRAS status and capacity to survive, proliferate and

develop their metabolic activity *in vivo* (Klaenhammer and Kullen 1999). Also, acid-bile tolerance and adherence to epithelial cells are considered as relevant characteristics in a probiotic strain (Mishra and Prasad 2005).

There are multiple factors that could affect the viability of probiotic micro-organisms in the gastrointestinal tract. The intestinal mucosa is protected from the attack of harmful enteric micro-organisms by host's defences in the context of both adaptive and innate immunity. Innate immunity, the first line of defence, uses nonclonal recognition molecules and nonspecific effector molecules. Epithelial cells are the main players of innate immunity by providing a physical barrier against bacterial translocation as well as by producing several biologically active molecules. These factors, along with the resident microbiota, control pathogenic micro-organisms (Ganz 2002). The nonspecific effector molecules produced by the intestinal epithelia include, mucins, enzymes, histones and antimicrobial peptides (AMPs) such as defensins (Hecht 1999). These molecules could be either secreted to the extracellular milieu or remain in the cytoplasm and nuclei of the epithelial cells (Rose *et al.* 1998). AMPs are relatively small proteins (12–100 amino acids), positively charged, amphiphillic, and they have a broad antimicrobial spectrum (Jenssen *et al.* 2006). Antimicrobial intestinal peptides are produced by specialized cells (Paneth's cells) and also by epithelial cells (Liévin-Le Moal and Servin 2006).

Human epithelial intestinal cells produce two types of AMPs: β defensins (hβD-1 and hβD-2) and the cathelicidin hLL-37. At mucosal surfaces, AMPs can modify the balance of the colonizing microbiota (Salzman *et al.* 2007).

The aim of the present study was to assess the *in vitro* effect on potentially probiotic lactobacilli of antimicrobial factors present in cultured human enterocytes.

Materials and methods

Bacterial strains and growth conditions

Shigella flexneri, *Escherichia coli* CM1, *Bacillus cereus* M2 and enteropathogenic *Escherichia coli* (EPEC) belong to the culture collection of the Cátedra de Microbiología of the Facultad de Ciencias Exactas (Universidad Nacional de La Plata, Argentine). *Lactobacillus delbrueckii* subsp. *lactis* strain CIDCA 133 and *Lactobacillus delbrueckii* subsp *bulgaricus* strain CIDCA 331 belong to the culture collection of the Centro de Investigación y Desarrollo en Criotecnología de Alimentos. (CIDCA, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Argentine). Stock cultures were stored at −80°C, and micro-organisms were reactivated twice in liquid medium before the experiments. Gram-negative bacteria were grown in nutrient broth (Biokar Diagnostics, Beauvais, France) at 37°C for 16 h. *Bacillus cereus* was cultured in Brain Heart Infusion (BHI) broth (Biokar Diagnostics, Beauvais, France) containing 0·1% (w/v) glucose at 32°C under orbital agitation. Incubation was performed for 3 h to obtain bacteria in early stationary phase thus minimizing the ratio of sporulated *B. cereus*. Lactobacilli were grown in de Man, Rogosa, Sharp (MRS) broth (Biokar Diagnostics, Beauvais, France) in anaerobic conditions at 37°C for 16 h. Bacteria were harvested by centrifugation at 10 000 ***g*** for 10 min and suspended in phosphate buffered saline (PBS) or the appropriate growth medium.

Cell cultures

Caco-2 cells were cultured in 75-cm^2 flasks (37°C, 5% CO_2–95% air atmosphere) in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Life Technologies, Rockville, MD, USA) supplemented with 10% (v/v) of foetal calf serum (PAA; Laboratories, GmbH, Pasching, Austria), 1% nonessential amino acids (Gibco, BRL, Life Technologies) and antibiotics (12 UI ml^{-1} penicillin and 12 μg ml^{-1} streptomycin). Monolayers at postconfluence (15 days of incubation) were used throughout. Forty-four hours before the experiments, Caco-2 cells were incubated in DMEM medium without neither foetal calf serum nor antibiotics to avoid the presence of inhibitory compounds other than those produced by the epithelial cells.

Caco-2-secreted factors

After the incubation of cell monolayers with DMEM without foetal calf serum nor antibiotics, spent culture supernatant was collected, lyophilized and suspended in distilled water to obtain ten-fold concentrated extracts. Samples were stored at −20°C until use.

Caco-2 cytosolic and brush border extracts

Cytosolic fractions were obtained according to the protocol of Pinto *et al.* (1983). Briefly, cells were collected and washed thrice using cold PBS. Then, they were suspended in Tris-Manitol buffer (2 mmol l^{-1} Tris, 50 mmol l^{-1} manitol, pH 7·1). Afterwards, cell suspensions were incubated on ice and sonicated thrice for 30 s in an Ultrasonic Desintegrator, (Model 60 w; MSE Ltd., USA) The lysate was incubated with $CaCl_2$ (10 mmol l^{-1}) for 10 min at 4°C and centrifuged at 950 ***g*** for 10 min. Resulting supernatant was centrifuged at 33 500 ***g*** for 30 min at 4°C. After this step, two fractions were obtained: supernatant (cytosolic fraction) and pellet (brush border fraction). Cytosolic fraction was concentrated (ten-fold) by lyophilization as described earlier.

Batch extraction of the cationic fraction from cytosol

Batch extraction was used to concentrate the cationic fraction from cytosol (Porter *et al.* 1998). Cytosolic fraction was incubated with a weak cation exchange resin (Macro-Prep CM; Bio-Rad Laboratories, CA, USA) at a ratio of 1 : 200 (resin : sample). After being incubated overnight at 4°C with gentle stirring, the resin was separated by centrifugation (1500 ***g***, 5 min). Afterwards, three washes with sodium phosphate buffer (10 mmol l^{-1}) were performed. Adsorbed cations were eluted at 4°C by two steps of acid treatment i.e. 1 h in 10% (v/v) acetic acid and overnight in 5% (v/v) acetic acid. Supernatants collected from both extractions were pooled, concentrated by lyophilization and stored at −20°C until use.

Inhibition assays

Diffusion assays.
Antimicrobial activity of Caco-2 extracts was determined by using a diffusion assay as described by Lehrer *et al.*(1991). Briefly, solid media were obtained by adding 1% (w/v) agarose to nutrient or MRS broth. Bacterial suspensions (1×10^8 CFU ml^{-1}) were spread on the surface of solid medium by using a cotton swab. Afterwards, 10 μl of samples was added to wells (2·5 mm in diameter) performed in the agarose. Plates were incubated for 24 h at 37°C, and zone diameters were measured.

Assays in liquid media.
To assess the effect of inhibitory factors on the growth kinetics of the strains under study, Caco-2 extracts or human β-defensins (hβD1 and hβD2; Sigma-Aldrich Co., St Louis, MO, USA) were added to bacterial suspensions (1×10^5 CFU ml^{-1}) in MRS or nutrient broth. β-defensins were used at 0·5 μg ml^{-1}. The number of surviving bacteria was determined by plating appropriate dilutions on either nutrient or MRS agar.

Statistical analysis

Results were compared by means of the two-tailed Student's *t*-test using InfoStat Software (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina).

Results

Effect of Caco-2 extracts on micro-organisms

To investigate the effect on different micro-organisms of cellular fractions obtained from Caco-2 cells, diffusion assays were conducted in culture media solidified with agarose. Incubation of either the Caco-2 cytosolic fraction or the brush border fraction with different bacteria revealed an inhibitory effect. The antagonism was observed both on gram-positive and gram-negative bacteria (Fig. 1). Of note, whereas *Shigella flexneri* was strongly inhibited by both cytosolic and brush border fractions (zone diameters 14·5 ± 2·2 and 11·1 ± 1·4 mm, respectively), enteropathogenic *E. coli* (EPEC) was only inhibited by the cytosolic fraction (zone diameter 7·0 ± 1·4 mm). Interestingly, *L. delbrueckii* subsp *lactis* CIDCA 133 was not antagonized neither by the cytosolic nor the brush border extract from Caco-2 cells. In contrast, the related micro-organism *Lactobacillus delbrueckii* subsp *bulgaricus* strain CIDCA 331 was strongly inhibited by the cytosolic fraction. In addition, ten-fold concentrated spent culture supernatants from Caco-2 cells were assayed. Even though inhibition zones of 13 ± 1 mm and 14 ± 1 mm were observed for strains 331 and CM1, respectively, no inhibition on strain 133 was detected (zone diameter 2·5 ± 0·0 mm).

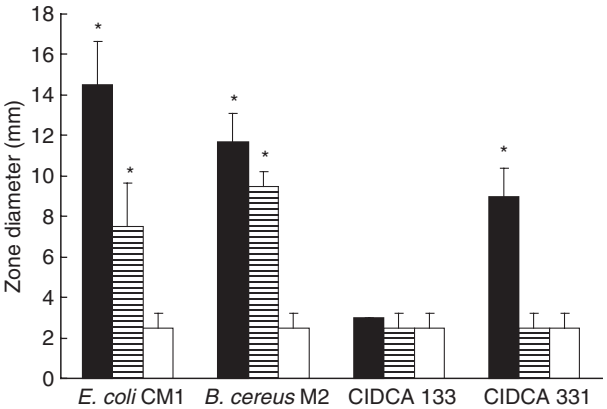

Figure 1 Effect of cytosolic and brush border fractions derived from Caco-2 cells on different bacterial strains. Antimicrobial activity was determined by diffusion assays (agarose). Micro-organisms were incubated with cytosolic fraction (black bars), brush border fraction (striped bars) or phosphate buffered saline (PBS) (white bars). Data represent the means of zone diameters ± standard deviation (n = 3). Asterisks indicate significant differences with the corresponding PBS control (P < 0·05). Internal diameter of the wells was 2·5 mm.

It is worth noting that assays performed by using agar, instead of agarose, as the solidifying agent led to smaller zone diameters (data not shown).

Characterization of the inhibitory activity

To distinguish between bacteriostatic and bactericidal activities, we performed assays in liquid medium. As shown in Fig. 2, incubation of *E. coli* CM1 with cytosolic fraction from Caco-2 cells led to a 3-log reduction in bacterial viability after 3-h incubation. This bactericidal effect was also observed for strain 331. Indeed, after 3-h

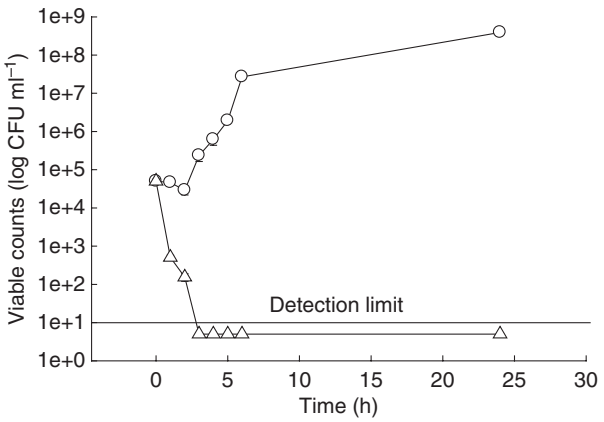

Figure 2 Killing effect of cytosolic fraction extract from Caco-2 cells against *Escherichia coli* strain CM1. Results show a representative kinetics. (△) cytosolic extract, (○) control.

Table 1 Comparison of the effect on different micro-organisms of cytosolic and cationic extracts from Caco-2 cells

Strain	Cytosolic extract	Cationic extract
Escherichia coli CM1	++	+
Bacillus cereus M2	++	+
Lactobacillus delbrueckii subsp *bulgaricus* (CIDCA 331)	+	+
Lactobacillus delbrueckii subsp *lactis* (CIDCA 133)	–	–

Results are scored according to the size of zone diameter in the diffusion assay. (+) 5–10 mm of inhibition zone; (++) 11–20 mm; (–) indicates no inhibitory effect (2·5 mm corresponding to the diameter of the well).

incubation with cytosolic extracts, viable counts of strain 331 decreased from 10^4 CFU ml^{-1} to 10^3 CFU ml^{-1}.

The findings that zone diameters in diffusion assays performed with agarose were larger than those obtained in agar-containing media, prompted us to investigate the role of cationic compounds in the inhibitory effect. To this end, we performed batch extraction of the cytosolic fraction from Caco-2 cells with a weak cation exchange resin. As given in Table 1, cationic fractions from Caco-2 cells showed similar inhibition pattern that cytosolic extracts.

Effect of human β-defensins

Even though several positively charged molecules (e.g. histones, phospholipase A2, defensins) could be present in the cationic extracts, the fact that human beta-defensins (hβDs) are the most abundant antimicrobial peptides in

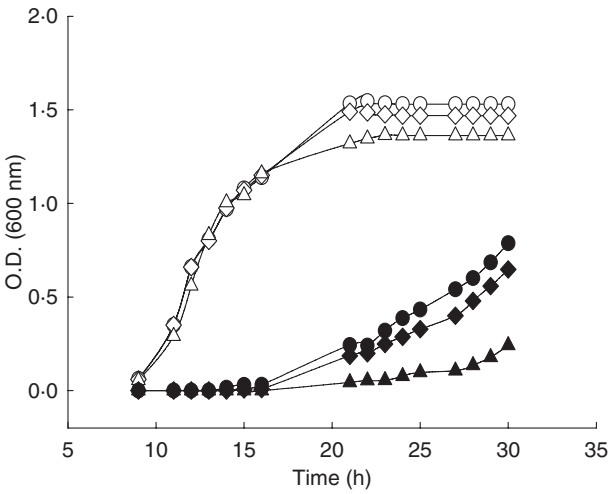

Figure 3 Growth curves of lactobacilli in the presence of human β-defensins. Symbols: (●, ○) control; (△, ▲) hβD$_1$; (◇, ♦) hβD$_2$. White symbols correspond to strain CIDCA 133, and black symbols correspond to strain CIDCA 331.

epithelial cells (Yanagi *et al.* 2007), prompted us to study the effect of purified human defensins.

Growth kinetics of lactobacilli performed in the presence of human β-defensins (hβD$_1$ and hβD$_2$) showed that whereas *Lactobacillus delbrueckii* subsp *bulgaricus* CIDCA 331 was inhibited by both defensins (mainly by hβD2), growth kinetics of *Lactobacillus delbrueckii* subsp *lactis* CIDCA 133 was not modified by defensins (Fig. 3). No effects were observed on the growth of *E. coli* CM1 in the presence of human defensins (data not shown).

Discussion

Survival of probiotic micro-organisms in the gastrointestinal tract depends on the ability to cope with several antimicrobial molecules produced by different cell populations.

Caco-2 cells constitute a widely accepted model for the study of the interaction between micro-organisms and cell populations relevant to the defence mechanisms of the intestinal tract. It is known that Caco-2 cells are able to produce a set of molecules with antibacterial activity e. g. lysozyme, defensins, phospholipases and α-antitrypsin (Bernet-Camard *et al.* 1996; Ganz 2002). In the present study, we used Caco-2-derived extracts to assess the effect of such inhibitory molecules on intestinal bacteria.

Whereas cytosolic extracts were inhibitory to most of the strains under study, strains CIDCA 133 and enteropathogenic *E. coli* (EPEC) showed a different behaviour, i.e. strain 133 was not inhibited, and EPEC was inhibited but in a lesser extent than other strains. This behaviour

could be expected for a pathogenic micro-organism (EPEC), but, surprisingly, a nonpathogenic and potentially probiotic micro-organism such as strain CIDCA 133 was also able to resist the inhibitory effect of Caco-2-derived factors. Furthermore, a different *Lactobacillus* strain was strongly inhibited by cytosolic extracts (Fig. 1). Noteworthy, concentrated spent supernatants from Caco-2 cell cultures inhibited both *E. coli* CM1 and *L. delbreuckii subsp bulgaricus* CIDCA 331, thus indicating that, in contrast to strain 133, these strains would not be active in the presence of secreted factors from enterocytes.

Defensins as well as other cationic molecules with antibacterial activity are important effectors of the innate immune system. Strain CIDCA 133 was resistant not only to the effect of the Caco-2 cationic extract but also to purified human β defensins (Table 1, Fig. 3).

The observed effect depends on the defensin/bacteria ratio. Indeed, when 10^3 CFU ml^{-1} was incubated for 24 h with different concentrations of human β-defensins, we found that strains CM1 and 133 were able to grow in the presence of 5 and 2·5 μg ml^{-1} of human beta-defensins, respectively, whereas strain 331 is able to grow at the concentrations of 0·08 μg ml^{-1} hBD (data not shown). It is worth noting that concentration of hβD2 in human faeces ranges from 0·01 to 0·06 μg ml^{-1} (Möndel *et al.* 2009).

Antimicrobial peptides (AMPs) lead to antimicrobial effect by damaging or destabilizing cytoplasmic membrane or acting at cytoplasmic level by inhibiting essential cellular process such as protein or nucleic acid synthesis (Brogden 2005). Regardless of their precise mode of action, the activities of AMPs are almost universally dependent upon interaction with the bacterial cell membrane (Hancock and Rozek 2002). Interactions between antimicrobial peptides and bacteria are determined by electrostatic attraction between the positive-charged AMPs and anionic bacterial structures such as lipids and other surface structures (Jenssen *et al.* 2006).

Bacterial pathogens have evolved mechanisms to limit the effectiveness of AMPs. These mechanisms involve the diminution of the electrostatic interaction between AMPs and bacterial surface by reducing the net negative charge of the bacterial cell envelope through the covalent modification of anionic molecules such as lipid A, teichoic acids or phospholipids (Peschel 2002).

As strain CIDCA 133 was affected neither by the cationic extract from Caco-2 cells nor by purified human β defensins, whereas strain CIDCA 331 was strongly inhibited, we could hypothesize that these strains differ in the composition of lipids or teichoic acids. In fact, chromatographic analysis revealed the different proportions of fatty acids present in cellular lipids between the two strains (Gómez Zavaglia *et al.* 2000).

The ability of probiotic micro-organisms to induce the production of antimicrobial molecules has been reported. Indeed, probiotic bacteria provoked a strong induction of hβD2 synthesis in intestinal epithelial cells Caco-2 (Schlee *et al.* 2007, 2008). Furthermore, *in vivo* studies demonstrated that oral administration of potentially probiotic *E. coli* strains increases faecal secretion of hβD2 in healthy volunteers (Möndel *et al.* 2009). It is worth noting that this probiotic strain was not resistant to the induced antimicrobial factors such as hβD2. These findings could indicate the possibility of a suicidal character of this interaction (Möndel *et al.* 2009).

Lactobacillus delbrueckii subsp *lactis* CIDCA 133 demonstrated the ability to antagonize biological effect of enterohaemorrhagic *E. coli* (EHEC) *in vitro* and to inhibit nitrate reductase activity of a nonpathogenic commensal *E. coli* strain (Hugo *et al.* 2006, 2008). In the present study, we showed the ability of this strain to resist the effects of antimicrobial factors derived from cultured human enterocytes as well as to overcome the inhibitory effect of human β defensins. These findings could be relevant to predict the competitiveness of this strain *in vivo* and to understand the interaction of selected probiotic micro-organisms with host's cells and the resulting immune response.

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