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ORIGINAL ARTICLE

Effect of human defensins on lactobacilli and liposomes

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Abstract

Aims: To study the effect of human β-defensins (HBD-1 and HBD-2) on lactobacilli membranes as well as on liposomes prepared from purified bacterial lipids.

Methods and Results: *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 331 and *Lact. delbrueckii* subsp. *lactis* CIDCA 133 were grown in Man, Rogosa, Sharpe broth for 16 h at 37°C. After being washed, micro-organisms were treated with 0·1–10 μg ml^{-1} of HBD-1 and HBD-2 (30 min, 37°C). Bacterial damage was determined by flow cytometry after propidium iodide staining. In parallel experiments, release of carboxyfluorescein from liposomes prepared from bacterial lipids was determined fluorometrically (excitation 485/20 nm, emission 528/20 nm) in the presence of HBD-1, HBD-2 or Nisin. Exposure of lactobacilli to HBD-2 resulted in a significant membrane permeabilization being *Lact. delbrueckii* subsp. *bulgaricus* CIDCA 331 the most susceptible strain. Liposomes prepared with lipids from strain CIDCA 133 were destabilized neither by HBD-1 nor by HBD-2, whereas liposomes derived from strain CIDCA 331 were susceptible to HBD-2 but not to HBD-1. Effect of defensins was strongly inhibited in the presence of NaCl, and the activity increased in water.

Conclusions: Results reported in the presented work indicate that lipid composition of bacterial membranes lead to a different interaction with cationic peptides such as defensins.

Significance and Impact of the study: The results represent an advance in the understanding of the differential effect of HBDs on micro-organisms. Differences in susceptibility to anti-microbial peptides could modify the fate of micro-organisms after the interaction with host's cells.

Introduction

Defensins are one of the most common types of anti-microbial peptides (AMPs; Menendez and Brett Finlay 2007). They are widespread in nature and have activity against a broad range of pathogens. They have direct anti-microbial effects and constitute key components of the immune response (Doss *et al.* 2010). Moreover, the imbalance between the production of defensins and the composition of the intestinal microbiota leads to the development and maintenance of inflammatory bowel disease (Gersemann *et al.* 2012). Defensins from vertebrates are small cationic molecules that have three intramolecular disulfide bonds involving six conserved cysteines. On the basis of cysteine pairing and length of peptide fragments between cysteines, two main groups of defensins can be defined: α and β. In humans, six α and four β-defensins have been characterized in some detail (Menendez and Brett Finlay 2007; Wong *et al.* 2007). Alpha defensins are highly concentrated in the granules of neutrophils (PMN) and Paneth cells, and β-defensins are secreted by epithelial cells lining mucosal surfaces

(Wu and Hazlett 2011). According to their location, human β-defensins (HBDs) provide a first line of defence against potentially pathogenic microbes (Huttner and Bevins 1999).

Similar to other AMPs, the principal function of β-defensins is to control bacteria or fungi growth by affecting their surrounding membrane. Positive charges of defensins lead to interactions with negatively charged cell components (e.g. membrane phospholipids), thus increasing membrane permeability that in turns results in cell death (Wu and Hazlett 2011).

HBD-1 and HBD-2 present high activity against gram-negative bacteria and low activity against gram positive such us *Staphylococcus aureus* (Harder *et al.* 2001). The expression of both defensins is different; HBD-1 is a constitutive AMP, whereas HBD-2 is inducible by factors triggering inflammation (Harder *et al.* 2001).

Lactic acid bacteria have demonstrated an interesting probiotic potential. The ability to resist the passage through the gastrointestinal tract and to exert their beneficial properties *in vivo* are essential properties for a strain intended to be included in probiotic preparations (Klaenhammer and Kullen 1999). At a mucosal level, defensins play an important role as protective molecules. Differences in the interaction of micro-organisms with defensins could be relevant for the fate of those micro-organisms, thus shaping the effect of probiotics on the host.

We have demonstrated that two related lactic acid bacteria, *Lactobacillus delbrueckii* subsp. *lactis* CIDCA 133 and *Lact. delbrueckii* subsp. *bulgaricus* CIDCA 331 have a different susceptibility to HBD-1 and HBD-2 and enterocyte-secreted factors (Hugo *et al.* 2010). Interestingly, strain CIDCA 133 is able to resist the inhibitory effect of both defensins, whereas strain CIDCA 331 is strongly inhibited (Hugo *et al.* 2010). This behaviour suggests a differential interaction between defensins and lactobacilli in plasma membrane domains, and this effect is relevant when strains are to be selected to be applied in functional foods.

In the present study, we aimed to gain further insight into the effect of HBD-1 and HBD-2 on lactobacilli whole cells as well as on liposomes prepared from their lipids. This approach will allow for a better understanding of the mechanisms involved in the differential susceptibility of these two strains of lactobacilli to HBD-1 and HBD-2.

Material and methods

Bacterial strains and growth conditions

Lactobacillus delbrueckii subsp. *bulgaricus* CIDCA 331 and *Lact. delbrueckii* subsp. *lactis* CIDCA 133 belong to the CIDCA culture collection (Gomez-Zavaglia *et al.* 2000). Stock cultures were stored at −80°C, and micro-organisms were re-activated twice in de Man, Rogosa, Sharpe broth (Biokar Diagnostics, Beauvais, France) at 37°C for 16 h. Bacteria were harvested by centrifugation at 10 000 ***g*** for 10 min and washed twice with phosphate-buffered saline (PBS) [K_2HPO_4 0·144 g l^{-1}; NaCl 9·00 g l^{-1}; Na_2HPO_4 g l^{-1}].

Peptide treatment

Washed micro-organisms were diluted 1/100 in water, 50 mmol l^{-1} PBS or 50 mmol l^{-1} *N*-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid (Hepes) buffer, pH 7·5 to obtain an OD (550 nm) of 0·06 ($1{\cdot}10^6$ CFU ml^{-1}). Aliquots of 100 μl were incubated in glucose 90 mmol l^{-1} at 37°C for 10 min and then, treated with concentrations of HBD-1 and HBD-2 ranging from 0·1 to 10 μg ml^{-1} (Sigma–Aldrich Co, St Louis, MO, USA) for 30 min at 37°C. Nisin (Sigma–Aldrich Co) at 3 μg ml^{-1} was used as a positive control because it is a well-known AMP (Winkowski *et al.* 1996).

Flow cytometry

Bacteria incubated with defensins and controls without treatment were incubated with the membrane-impermeant DNA-binding probe propidium iodide (PI), that only gain access to the intracellular domain when membranes are damaged (Wouters *et al.* 2001; Tymczyszyn *et al.* 2012). Stock solutions of PI (Molecular Probes, Leiden, the Netherlands) were prepared in distilled water to a final concentration of 10 mg ml^{-1} and stored in the dark at 4°C. PI was added to a final concentration of 0·5 mg ml^{-1}. For flow cytometry analysis, the concentration of micro-organisms was adjusted to approximately 10^5 CFU ml^{-1}. Samples were incubated with the probe for 5 min at room temperature, and the ratio of PI (+) bacteria was assessed by flow cytometry in a FL2-H (PI) *vs* FSC-H scatter plot (FACSCalibur, CellQuest software; Becton Dickinson, Mountain View, CA, USA). For each sample, 10 000 events were analysed. Nontreated bacteria and bacteria heated for 3 min at 80°C were used as controls.

Lipid extraction

Lipids were extracted according to the modified Bligh and Dyer method (Marinetti 1993). Briefly, cell pellets were suspended in chloroform-methanol-water (1 : 2 : 0·8 by volume; 4·75 ml per g of cells) for 12 h at 4°C and then centrifuged at 8000 ***g***, 10 min at 10°C. The supernatant was collected and a second extraction was

performed on the pellet. Both supernatants were mixed and chloroform-water (1 : 1) was added (12·5 ml per g of cell culture). The final mixture was centrifuged at 8000 ***g*** for 20 min. The chloroform phase was collected and dried under vacuum (Rotavapor® RE 120; Büchi, Flawil, Switzerland). Lipids were dissolved in chloroform and stored at −20°C for up to 2 weeks.

Preparation of 5 (6)-carboxyfluorescein (CF)-loaded liposomes

Large unilamellar vesicles were prepared according to Winkowski *et al.* (1996). Briefly, lipids in chloroform were dried under a stream of nitrogen. The dried phospholipids were hydrated to a final concentration of 7 mg ml^{-1} in 50 mmol l^{-1} Hepes buffer and 50 mmol l^{-1} 5 (6)-carboxyfluorescein (CF), pH 7·5. The mixture was vortexed 10 min at regular intervals for 1 h at room temperature. Nonentrapped CF was removed by two centrifugations for 10 min at 5600 ***g***. Afterwards, liposomes were washed with 50 mmol l^{-1} Hepes buffer, pH 7·5 (Hollmann *et al.* 2007). In these conditions, CF in liposomes is self-quenched, and the increase in fluorescence after treatments can be ascribed to the release of CF from liposomes (Chen and Knutson 1988).

CF release assays

Release of CF was determined fluorometrically in polyvinyl chloride microtiter plates. CF-loaded liposomes were diluted in 100 ml of 50 mmol l^{-1} Hepes buffer (pH 7·5) with or without 100 mmol l^{-1} NaCl. On the basis of the dry mass of lipid extract used for the preparation of liposomes, the final lipid concentration was approximately 20 μg ml^{-1}.

The peptide-induced leakage of CF was monitored for 20 min at room temperature by determining the increase in fluorescence intensity on a Synergy HT fluorescence microplate reader (Bio-Tek Instruments, Winooski, VT, USA) with excitation and emission wavelengths at 485/20 and 528/20 nm, respectively. CF release was initiated by addition of 0 to 4 μg ml^{-1} of HBD-1, HBD-2 or nisin (control of pore-forming peptide) to 100 μl of CF-loaded liposomes in buffer. Reaction progress was expressed as the percentage of CF release relative to the total fluorescence determined by the addition of 1 μl of 10% (v/v) Triton X-100 at the end of each experiment.

The peptide-induced leakage was calculated by the following equation:

$$\%\text{Efflux} = 100^{*}(F - F_0)/(F_{100} - F_0), \qquad (1)$$

where F is the fluorescence intensity induced by the peptide, F_0 is the fluorescence of intact vesicles and F_{100} represents the intensity after Triton X-100 treatment (Hadjicharalambous *et al.* 2008).

Statistics

Determinations were done in duplicate from three independent cultures of bacteria. The relative differences were reproducible independently of the cultures used. Analysis of variance (ANOVA) was carried out using the statistical program STATISTIX 8 Software (Analytical Software, Tallahassee, FL, USA). Means were compared by Tukey's test.

Results

Flow cytometric analysis of membrane permeabilization

Propidium iodide uptake was used to assess the effect of AMPs on *Lact. delbrueckii* subsp. *bulgaricus* CIDCA 331 and *Lact. delbrueckii* subsp. *lactis* CIDCA 133. Figure 1 shows representative plots obtained from the flow cytometry analysis. A region (R1) containing bacteria was evident in FSC-H *vs* SSC-H plots (Fig. 1a). For untreated control cells, only 4·3% of PI (+) cells was observed (Fig. 1b). These values increased to 82·3 and 60·3% after 30 min of nisin or HBD-2 treatments, respectively (Fig. 1c,d). Similar results were obtained for strain CIDCA 133 (data not shown).

The percentage of PI (+) bacteria after defensin treatment is shown in Table 1. When cell suspensions were prepared in PBS buffer no effect was observed (Table 1). Even at high defensin concentrations (8 μg ml^{-1}), no differences in the percentage of PI (+) cells between samples and controls were found (data not shown).

In contrast, when bacteria were suspended in water (in absence of NaCl), a strong activity of HBD-2 was observed on CIDCA 331 and low effect on CIDCA 133 (Table 1). It is worth noting the different behaviour of the strains under study after treatment with HBD-1 and HBD-2 in water: HBD-1 does not have effect, whereas HBD-2 leads to 27·7 and 65·5 of PI (+) cells for strains CIDCA 133 and CIDCA 331, respectively (Table 1).

Considering the different susceptibility of the strains under study to HBD-2, the effect of this peptide was evaluated at different concentrations in the absence of NaCl (Hepes buffer, Fig. 2). Exposure of lactobacilli to HBD-2 resulted in a significant membrane permeabilization as compared with control cells. Figure 2 shows a clear difference in the percentage of PI (+) cells between the strains under study. In strain CIDCA 331, the percentage of PI (+) cells increased in a dose-dependent manner. Noteworthy, percentages of PI (+) cells are 2·6 to 3·5-folds higher for strain CIDCA 331 than for strain CIDCA 133 at 4 and 8 μg ml^{-1} of HBD-2, respectively.

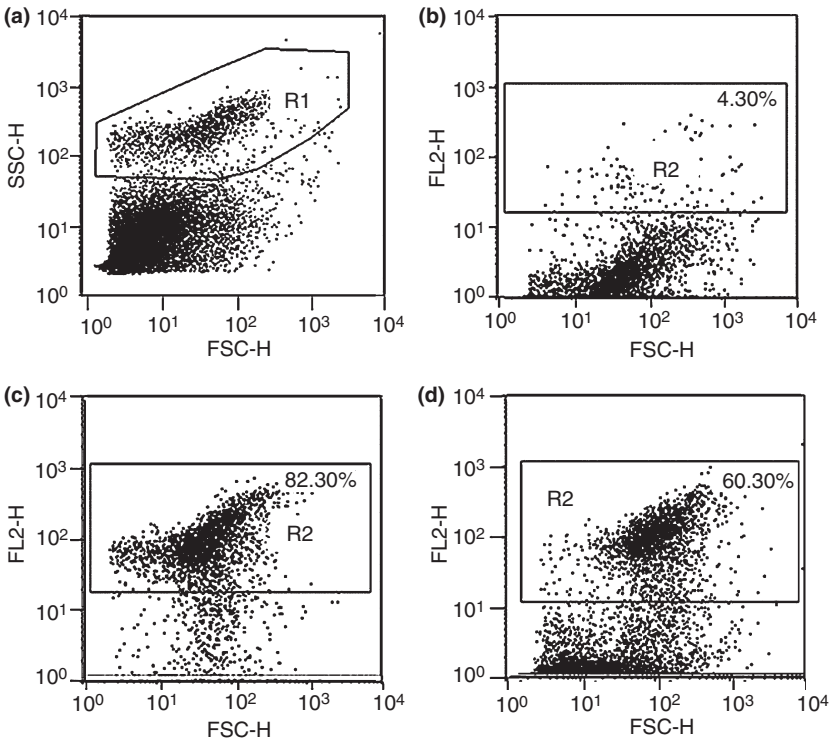

Figure 1 Flow cytometry analysis of *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 331. (a) FSC-H *vs* SSC-H plot showing the gate of bacteria (R1). (b–d) Percentage of propidium iodide (PI) positive cells. (b) control cells, (c), nisin-treated cells, (d) HBD-2-treated cells. R2 represents FL2-positive bacteria. Gated bacteria (R1) were analysed for PI uptake (FL2-H; R2). Bacteria were suspended in Hepes buffer and treated for 30 min.

Table 1 Percentage of PI (+) cells after treatment of whole bacteria with defensins for 30 min

	Strain			
	Lact. lactis CIDCA 133		*Lact. bulgaricus* CIDCA 331	
	PBS	Water	PBS	Water
Control	2·79	8·70	1·53	8·50
HBD-1	1·65	8·60	2·01	13·23
HBD-2	3·02	27·70*	2·83	65·47*

PBS, phosphate-buffered saline; PI, propidium iodide.
Values were obtained by flow cytometry. Defensin concentration: 3 μg ml^{-1}. SD = ±2·48%.
*Indicate significant differences with regard to the corresponding controls ($P < 0{\cdot}05$).

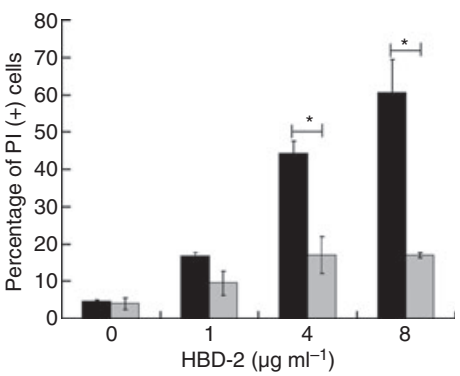

Figure 2 Percentage of propidium iodide (PI) (+) bacteria after treatment with HBD-2 in 50 mmol l^{-1} Hepes buffer. *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 331 (black bars), *Lact. delbrueckii* subsp. *lactis* CIDCA 133 (grey bars). Values were obtained by flow cytometry. *Significant differences ($P < 0{\cdot}05$).

CF release assays

Taking into account that membrane permeabilization could be due to the insertion of defensins in the lipid bilayer, the effect of the bacterial lipid composition on the interaction with both defensins was investigated. For this purpose, CF-containing liposomes prepared from bacterial lipids were exposed to different concentrations of HBD-1, HBD-2 or nisin, and the kinetics of fluorophore efflux were analysed. The peptide–liposomes interaction leads to the release of CF, thus giving rise to an increase of the fluorescence because of dequenching.

Figure 3(a,b) depicts kinetics of CF efflux after the addition of peptides. Nisin (1 μg ml^{-1}), a well-characterized pore-forming peptide, leads to the leakage of approximately 60% CF from liposomes of both strains. The addition of HBD-1 did not produce effect on liposomes from both strains (Fig. 3a,b). However, HBD-2

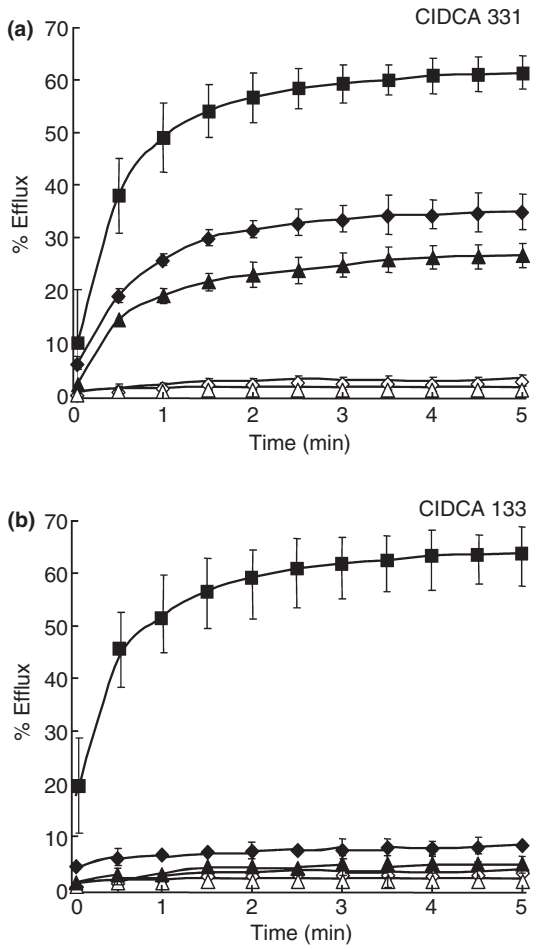

Figure 3 Kinetics of 5 (6)-carboxyfluorescein (CF) release from liposomes after addition of anti-microbial peptides. Lipids were from *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 331 (a) and *Lact. delbrueckii* subsp. *lactis* CIDCA 133 (b). Nisin 1 μg ml^{-1} (■), HBD-1 1 μg ml^{-1} (△) and 2 μg ml^{-1} (◊), HBD-2 1 μg ml^{-1} (▲) and 2 μg ml^{-1} (♦). % Efflux was calculated as indicated in Materials and methods. The % Efflux after 5 min remained unchanged until the end of the experiment.

has a different effect on the lipids from both strains (Fig. 3a,b).

Concentrations of 1 and 2 μg ml^{-1} HBD-2 produced a release of 26 and 35% CF, respectively (Fig. 3a). In contrast, in lipid vesicles of strain CIDCA 133 exposed to HBD-2, no significant difference respect to the control was observed (Fig. 3b). These results obtained with lipid vesicles are consistent with those obtained for whole bacteria by flow cytometry, shown in Table 1.

The decrease in the activity of HBD-2 in the presence of NaCl (also shown in Table 1) was also analysed on liposomes. Figure 4 depicts the release of CF from liposomes derived from lipids of strain CIDCA 331 after a 20 min treatment with HBD-2. The presence of NaCl leads to a six-fold decrease in the probe efflux, indicating a strong inhibition on the activity of HBD-2. Even when high concentrations of HBD-2 increased the liposomes leakage (12 and 19% efflux at 4 and 6 μg ml^{-1}, respectively), the percentages of CF efflux were lower than those obtained in the absence of NaCl at concentrations of 1 μg ml^{-1}.

Discussion

The interaction between human defensins and membranes has been studied in several bacterial species and also in liposomes prepared from synthetic lipids (Fujii *et al.* 1993; Hadjicharalambous *et al.* 2008). The effect of defensins on both commensal and pathogenic bacteria could be relevant for the composition and function of intestinal microbiota. Nuding *et al.* (2009) reported the effect of HBD-1, HBD-2, HBD-3 and alpha defensin 5 on membrane depolarization in several species of micro-organisms belonging to the intestinal microbiota, including lactobacilli and bifidobacteria.

The main mechanism described for the action of defensins on micro-organisms is the disruption of cytoplasmic membrane permeability with further loss of viability. This effect involves the interaction between positively charged peptides and negatively charged lipids (Fujii *et al.* 1993).

We previously reported a differential susceptibility to human defensins of the lactobacilli studied in the present work (Hugo *et al.* 2010).

Strains CIDCA 331 and CIDCA 133 have a similar lipid composition, including phosphatydylglycerol, cardiolipin, three different glycolipids and a neutral lipid (Gomez-Zavaglia *et al.* 2000). However, the ratios of both

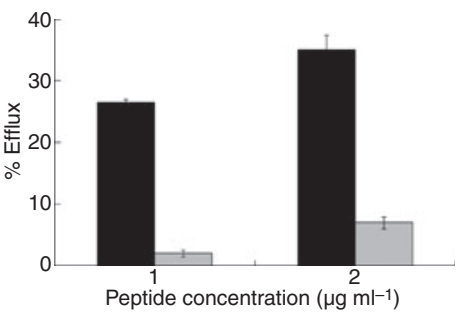

Figure 4 Release of 5 (6)-carboxyfluorescein (CF) from liposomes. Lipids were extracted from *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 331. Values were measured 20 min after the addition of 1 or 2 μg ml^{-1} HBD-2. Liposomes were suspended in 50 mmol l^{-1} Hepes buffer, pH 7·5 with (grey bars) or without (black bars) NaCl 100 mmol l^{-1}. % Efflux was calculated as indicated in Materials and methods.

glycolipids/phospholipids (GLY/PL) and saturated/unsaturated fatty acids are different for both strains (Gomez-Zavaglia *et al.* 2000).

Results of the present work indicate that strain CIDCA 331 is more susceptible to HBD-2 than strain CIDCA 133. As already reported, the effect of HBD-2 was highly inhibited by the presence of NaCl (Singh *et al.* 1998). The salt sensitivity of HBDs has been described for HBD-1, HBD-2 and HBD-4 but not for HBD-3 (Scudiero *et al.* 2010). The anti-microbial effect of HBD-3 in the presence of NaCl has been explained at high charge (+11) respect to (+4) and (+6) for HBD-1 and HBD-2, respectively (Scudiero *et al.* 2010; Jung *et al.* 2011).

In agreement with results obtained in whole micro-organisms, HBD-2 induced a higher CF release in liposomes of strain CIDCA 331 than in liposomes of strain CIDCA 133. Given that efflux was reduced in the presence of NaCl at physiological concentrations, electrostatic forces seem to play a crucial role in the lipid –peptide interaction. The higher sensibility of strain CIDCA 331 indicates a stronger interaction of HBD-2 with lipids. This could be related to the lower GLY/PL ratio in this strain as compared with strain CIDCA 133. Similar results were reported by Hadjicharalambous *et al.* (2008). They found that cryptdin-4 (a murine α-defensin present in Paneth cells) leads to the efflux of CF in liposomes with 70% of negative charge [phosphatidylethanolamine (PE):phosphatidylglycerol (PG) with a ratio equal to 3 : 7], but not when the PE/PG ratio is inverted (7 : 3; 30% of negative charges). In addition, slight changes in the amino acid sequence can also modify the lipid/peptide interaction, thus re-inforcing the crucial role of electrostatic interactions in the effect of AMPs (Hadjicharalambous *et al.* 2008).

Our results show that HBD-1 does not produce any effect on the percentage of PI (+) cells and does not induce the CF release from liposomes of both strains at the concentrations assayed. These findings can be related with the low positive charge of HBD-1. Indeed, net positive charges are +4 and +6 for HBD-1 and HBD-2, respectively (Scudiero *et al.* 2010; Jung *et al.* 2011). The low surface charge of HBD-1 is probably not high enough to allow for the interaction with bacterial membranes in the experimental conditions of the present study. It is worth noting that other authors reported that HBD-1 is 10-fold less efficient than HBD-2 as bactericidal agent (Singh *et al.* 1999). The different patterns of expression of HBD-1 and HBD-2 may explain their different roles in mucosa protection. Indeed, whereas HBD-1 is constitutively expressed, HBD-2 is an inducible peptide that might reach higher concentrations in the gastrointestinal tract (Harder *et al.* 2001).

In addition, Schroeder *et al.* proposed that free cysteines in the carboxy terminus are important for the bactericidal effect. Therefore, the lower activity of HBD-1 may be ascribed to the absence of reducing conditions (Schroeder *et al.* 2011).

Findings reported in the presented work suggest that differences in lipid composition of bacterial membranes could lead to different interaction with cationic peptides such as defensins. Interestingly, the GLY/PL ratio in strain CIDCA 133 is higher than in strain CIDCA 331 (Gomez-Zavaglia *et al.* 2000), thus reducing the negative charges available to interact with HBD-2. This fact may stabilize liposomes from strain CIDCA 133 in the presence of HBD-2. The relevance of surface charges for the biological effect of AMPs has been demonstrated (Winkowski *et al.* 1996; Hale and Hancock 2007; Jung *et al.* 2011). However, other factors besides electrical charges (e.g. peptide folding) could also play a role in the effect of AMPs (Schroeder *et al.* 2011).

It must be mentioned that no differential effects were found with nisin. The percentages of CF release were in agreement with those reported by Winkowski *et al.* (1996), and they were similar for both strains under study (Fig. 3). Nisin is a well-characterized pore-forming AMP, which in addition inhibits peptidoglycan synthesis by interacting with lipid II (Brötz *et al.* 1998).

Our results constitute a further insight into the understanding of the effect of human defensins on potentially probiotic microorganisms. Although more studies are needed to unravel the consequences of our findings, we can hypothesize that differences in susceptibility to central players of the innate immunity (i.e. defensins) could modify the fate of micro-organisms after interaction with the host, thus leading to different immune responses.

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