In Vitro Evaluation of the Probiotic Potential of Bacteriocin Producer *Lactobacillus sakei* 1

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ABSTRACT

*Lactobacillus sakei* 1 is a food isolate that produces a heat-stable antimicrobial peptide (sakacin 1, a class Ila bacteriocin) inhibitory to the opportunistic pathogen *Listeria monocytogenes*. Bacterial isolates with antimicrobial activity may be useful for food biopreservation and also for developing probiotics. To evaluate the probiotic potential of *L. sakei* 1, it was tested for (i) in vitro gastric resistance (with synthetic gastric juice adjusted to pH 2.0, 2.5, or 3.0); (ii) survival and bacteriocin production in the presence of bile salts and commercial prebiotics (inulin and oligofructose); (iii) adhesion to Caco-2 cells; and (iv) effect on the adhesion of *L. monocytogenes* to Caco-2 cells and invasion of these cells by the organism. The results showed that *L. sakei* 1 survival in gastric environment varied according to pH, with the maximum survival achieved at pH 3.0, despite a 4-log reduction of the population after 3 h. Regarding the bile salt tolerance and influence of prebiotics, it was observed that *L. sakei* 1 survival rates were similar (*P > 0.05*) for all de Man Rogosa Sharpe (MRS) broth formulations when tests were done after 4 h of incubation. However, after incubation for 24 h, the survival of *L. sakei* 1 in MRS broth was reduced by 1.8 log (*P < 0.001*), when glucose was replaced by either inulin or oligofructose (without Oxgall). *L. sakei* 1 was unable to deconjugate bile salts, and there was a significant decrease (1.4 log) of the *L. sakei* 1 population in regular MRS broth plus Oxgall (*P < 0.05*). In spite of this, tolerance levels of *L. sakei* 1 to bile salts were similar in regular MRS broth and in MRS broth with oligofructose. Lower bacteriocin production was observed in MRS broth when inulin (3,200 AU/ml) or oligofructose (2,400 AU/ml) was used instead of glucose (6,400 AU/ml). *L. sakei* 1 adhered to Caco-2 cells, and its cell-free pH-neutralized supernatant containing sakacin 1 led to a significant reduction of in vitro listerial invasion of human intestinal Caco-2 cells.

The development of products containing starter cultures with probiotic potential is a very promising approach in the field of functional foods. *Lactobacillus sakei* is a lactic acid bacterium (LAB) originally isolated from rice wine and is used as starter culture for meat fermentation, with potential to be applied in food biopreservation (3, 29, 41). There is also interest in the application of *L. sakei* as a probiotic with potential for health benefit. Recently, Woo et al. (44) studied the effect of the intake of *L. sakei* KCTC 10755BP by children with atopic eczema-dermatitis syndrome and showed a positive correlation between ingestion of LAB and alleviation of the clinical severity of atopic eczema-dermatitis syndrome, as well as decreased chemokine levels (44).

The ability to resist low pH, digestive enzymes, and bile salts (32, 36, 40) enables probiotic organisms to reach the site of action in the intestine and to survive in the gastrointestinal tract (GIT). The World Health Organization (14) recommends the selection of probiotic cultures by evaluation of (i) gastrointestinal resistance, (ii) capability to hydrolyze bile salts, (iii) adhesion to intestinal epithelial cells, (iv) inhibition of pathogens’ adhesion, and (v) antimicrobial action against pathogenic bacteria. The last property is important for the protection of GIT from microbial infection, and it has been the focus of many studies (27, 31, 35).

Recently, Santos et al. (34) reported that *Lactobacillus delbrueckii* UFV-H2b20 protected germ-free mice against *Listeria monocytogenes* infection by favoring effector responses and preventing immunopathological consequenc-
eses. Listeriosis is of special concern for food safety because it is transmitted mainly by foods and may cause high fatality rates among neonates, the elderly, and immunocompromised persons. The GIT health may also be improved by the consumption of prebiotics, such as inulin and oligofructose. Prebiotics are defined as nondigestible soluble carbohydrates with ability to selectively stimulate the activity, survival, or even the colonization of gut by beneficial microorganisms in humans and animals (16, 33). *Lactobacillus* and *Bifidobacterium* are the main genera of colonic microorganisms stimulated by prebiotic compounds (10, 37).

The purpose of the present study was to evaluate the probiotic potential of *L. sakei* 1, an isolate from fresh pork
sauce that produces a bacteriocin of 4.4 kDa (sakacin 1), with antilisterial activity and potential to inhibit early stages of biofilm formation by L. monocytogenes on an abiotic surface (1, 10–12, 26, 43). Experiments were conducted with L. sakei 1 to evaluate in vitro gastric survival and the influence of commercial prebiotics on bile salt tolerance and bacteriocin production. The adhesion ability of L. sakei 1 to Caco-2 cells was evaluated, and the effect of its bacteriocin on adhesion and invasion of Caco-2 cells by L. monocytogenes was also studied.

**MATERIALS AND METHODS**

**Bacterial strains and culture media.** Bacteriocin producer L. sakei 1 was previously isolated from Brazilian fresh pork sausage and produces sakacin 1 (10, 12). L. monocytogenes ATCC 19115 was used as indicator strain in agar antagonism tests (19).

Working cultures were prepared (1%, vol/vol) in de Man Rogosa Sharpe broth (MRS; Oxoid, Basingstoke, UK) for LAB and in brain heart infusion (BHI) broth (Oxoid) for L. monocytogenes and incubated at 25 and 37°C, respectively, for 24 h. Stock cultures were kept at −80°C in appropriate liquid media containing 20% (vol/vol) glycerol (Synth, Diadema, Brazil). Prebiotics inulin (Orafti-HP, BENEO-Orafti, Tienen, Belgium) and oligofructose (P95, BENEO-Orafti) were used at 20 mg ml⁻¹ (Table 1). In selected experiments, glucose from MRS broth was replaced by inulin (MRS plus inulin) or oligofructose (MRS plus oligofructose).

**In vitro survival of L. sakei 1 in gastric juice.** Synthetic gastric juice was prepared by addition of 3.0 g liter⁻¹ pepsin (Sigma, St. Louis, MO) in sodium chloride solution (0.85%, wt/vol) and adjusted to pH 2.0, 2.5, or 3.0 with 0.1 N HCl (6). Populations of L. sakei 1 in overnight cultures of MRS broth were enumerated by surface plating (0.1 ml) of appropriate dilutions on MRS agar before challenge and incubated at 37°C for 48 h. The remaining broth (ca. 30 ml) was centrifuged (6,000 × g, 20 min, 5°C; Sorvall Legend Mach 1.6R, Wilmingston, DE), washed twice with phosphate-buffered saline (PBS, pH 7.4), and the pellet was suspended in 3.0 ml of the same buffer. One milliliter of the suspension was centrifuged (12,000 × g, 5 min, 5°C) and L. sakei 1 cells were resuspended in 10 ml of the synthetic gastric juice. The suspension was kept at 37°C, and populations were enumerated at 0, 1, 2, and 3 h by surface plating on MRS agar followed by incubation at 37°C for 48 h. The results were expressed as log CFU per milliliter (6, 42).

**Bile salt hydrolysis activity.** Overnight culture of L. sakei 1 in MRS broth was spotted (10 μl) on the surface of MRS agar supplemented with 0.5% (wt/vol) taurodeoxycholic acid (Sigma) plus 0.37 g of CaCl₂ per liter (Vetc, Rio de Janeiro, Brazil) and incubated at 37°C in anaerobic jars (Anaerogen, Oxoid). Bile salt hydrolysis activity was scored by measuring precipitation zones around colonies as per the method of Franz et al. (15).

**Effect of commercial prebiotics on tolerance of L. sakei 1 to bile salts.** Overnight culture of L. sakei 1 in MRS broth (1 ml) was inoculated into 9 ml of MRS broth containing prebiotics inulin or oligofructose at 20 mg ml⁻¹. Both these cultures also contained bile salts at 3 mg ml⁻¹ (Oxgall, Sigma). Regular MRS broth (20 mg ml⁻¹ glucose) containing bile salts was used as control in these experiments. Samples of each broth (0.1 ml) were withdrawn after 4 and 24 h, and viable cells were enumerated by surface plating on MRS agar following incubation at 37°C for 48 h (22, 38).

**Bacteriocin production by L. sakei 1 in broths containing prebiotics.** Bacteriocin was quantified by critical dilution assay (28) after overnight growth of L. sakei 1 in MRS broths containing 20 mg of prebiotics (inulin or oligofructose) per ml and regular MRS broth (control). Briefly, the cell-free pH-neutralized supernatants of L. sakei 1 (CFNS-S1) from each broth formulation were serially diluted with PBS (10 mM, pH 7.0). Each dilution (10 μl) was spotted onto BHI agar plates previously overlaid with 7 ml of soft BHI agar inoculated with ca. 10⁵ to 10⁶ CFU of L. monocytogenes per ml. Plates were incubated at 37°C for 24 h, and results were expressed in arbitrary units per milliliter (AU/ml), defined as the reciprocal of the highest dilution that presented an inhibition zone.

**Caco-2 cell culture.** Human intestinal epithelial Caco-2 cells (ATCC HBT37) were obtained from the Rio de Janeiro Cell Bank (UFRJ, Rio de Janeiro, Brazil) and routinely cultivated in 75-cm² tissue culture flasks (TPP, Trasadingen, Switzerland) at 37°C under 5% CO₂, humidified atmosphere (Forma Scientific, Marietta, OH), in complete RPMI medium, composed of RPMI 1640 medium (Gibco, Gaithersburg, MD) supplemented with 2.4 g of HEPES per liter (Inviitrogen, Carlsbad, CA), 2.0 g of sodium bicarbonate per liter, 100 g of heat-inactivated fetal bovine serum per liter (Invitrogen), 100 U of streptomycin per ml, and 100 μg of penicillin per ml (Sigma). The medium was replaced every 2 days until a confluent monolayer was achieved. The Caco-2 cell culture was trypsinized, transferred (10⁴ cells per well) to 24-well tissue culture plates (TPP) containing 12-mm-diameter glass coverslips (30), and incubated under 5% CO₂ in complete RPMI medium for 24 h. The medium was removed, adhered Caco-2 cells were washed twice with PBS, and 1 ml of RPMI incomplete (without antibiotics) was added (17, 30).

**L. sakei 1 adhesion to Caco-2 cells.** Cultures of Caco-2 cells were prepared as previously described, inoculated with 100 μl of an active culture of L. sakei 1 (ca. 10⁶ bacteria per well), and incubated at 37°C for 2 h under 5% CO₂. The coverslips were gently washed three times with PBS (pH 7.4) to remove nonadherent bacterial cells, fixed with methanol, stained with May-Grunwald-Giemsa (Laborclin, Pinhais, Brazil), and observed under oil immersion lens with a magnification of ×1,000 (Axiolab-Zeiss, Stuttgart, Germany).

**Preparation of CFNS-S1.** An overnight culture of L. sakei 1 was inoculated in MRS broth (1%, vol/vol) and incubated at 37°C for 24 h. The broth culture was centrifuged at 4°C at 6,720 × g for 25 min (Sorvall RC Plus, Du Pont, Wilmington, DE), the pH of supernatant was adjusted to pH 7.0, and it was filter sterilized.

**TABLE 1. Description of the commercial prebiotics used in this study**

<table>
<thead>
<tr>
<th>Prebiotic</th>
<th>Source</th>
<th>Commercial name</th>
<th>Composition³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inulin</td>
<td>Extracted from chicory root</td>
<td>Beneo HPX–Orafti</td>
<td>Inulin (99.5%)</td>
</tr>
<tr>
<td>Oligofructose</td>
<td>Partial enzymatic hydrolysis of inulin</td>
<td>Beneo P95–Orafti</td>
<td>Oligofructose (93.2%)</td>
</tr>
</tbody>
</table>

³ Data from the manufacturer, BENEO-Orafti Active Food Ingredients, Tienen, Belgium.
through 0.22-μm-pore-size membranes (Millipore, São Paulo, Brazil). The titer of sakacin 1 was determined by the critical dilution assay (26, 28) using L. monocytogenes as the indicator microorganism. The CFNS-S1 containing the semipurified sakacin 1 was diluted in incomplete RPMI at final concentrations of 0, 10, 25, 50, 75, and 90% (vol/vol) and immediately used in adhesion and invasion assays.

Adhesion of L. monocytogenes to Caco-2 cells in the presence of CFNS-S1. Caco-2 cells were cultivated up to semiconfluent growth in 24-well tissue culture plates with no coverslips. Overnight culture of L. monocytogenes (1 ml) grown in BHI broth at 37°C was diluted to 10^2 CFU/ml in RPMI incomplete medium, added to the wells to obtain the initial multiplicity of infection of 100:1, and incubated for 30 min at 37°C under 5% CO₂. Cells were washed twice with PBS buffer to eliminate nonadherent bacteria, trypsinized, and centrifuged at 10,000 × g for 5 min. The pellet was suspended in 1 ml of PBS, serial dilutions were prepared, and each dilution was surface plated on tryptic soy agar supplemented with 6 g of yeast extract per liter (TSAYE; Oxoid) and incubated for 24 h at 37°C. The percentage of adhesion was defined as the log of the number of adherent bacterial cells (log CFU) divided by the log of the total number of bacteria inoculated, multiplied by 100 (2, 31).

To evaluate the effect of the bacteriocin sakacin 1 in L. monocytogenes adhesion to Caco-2 cells, different dilutions of CFNS-S1 (0, 10, 25, 50, 75, and 90%) were used in the wells of the tissue culture plate, either at the same time as the addition of L. monocytogenes or 30 min before or 30 min after inoculation. All adhesion assays were performed independently at least three times, in triplicate.

Influence of CFNS-S1 on L. monocytogenes invasion of Caco-2 cells. L. monocytogenes was inoculated in Caco-2 cell cultures as described in the previous section and kept for 60 min at 37°C in 5% CO₂ atmosphere. To kill noninternalized bacteria, cells were washed twice with PBS and incubated in 250 μl of incomplete RPMI medium containing 250 μg of gentamicin per ml for 60 min at 37°C in 5% CO₂ atmosphere. Cells were washed with PBS, and Caco-2 cells were lysed with 0.1% (vol/vol) Triton X-100 (Sigma). Cell lysates were serially diluted in PBS buffer and plated onto TSAYE agar (0.1 ml) to enumerate intracellular bacteria. To evaluate the possible protective role of sakacin 1 against L. monocytogenes invasion, these assays were repeated using incomplete RPMI medium containing CFNS-S1 at 0, 10, 25, 50, 75, and 90% dilutions, concomitant to addition of L. monocytogenes.

The invasion efficiency (percentage) was calculated as the log of the number of intracellular bacterial cells (log CFU) divided by the log of the total number of bacteria inoculated, multiplied by 100 (4, 31). Results are presented as averages of three independent assays done in triplicate.

**RESULTS AND DISCUSSION**

**L. sakei** 1 survival under conditions simulating gastric environment. It is desirable that microorganisms intended to be used as probiotic cultures be able to survive under the adverse conditions of the GIT (13, 14, 32). Although discrepancies can be observed between in vitro and in vivo assays (42), in vitro tests still constitute an important tool for screening probiotic strains.

Results of tests from the present study on bile salt hydrolysis showed no precipitation halos in agar plates containing taurodeoxycholic acid, which indicated L. sakei 1 did not deconjugate bile salts (data not shown). However, Vinderola and Reinheimer (42) reported that it is possible that probiotic strains present good growth rate in media containing bile salts, despite being unable to produce bile salt hydrolases.

In Figure 1 it is shown that the L. sakei 1 population was below the detection limit after 1 h of exposure to synthetic gastric juice at pH values of 2.0 and 2.5. However, at pH 3.0, L. sakei 1 presented a slower death rate and it remained viable for up to 3 h, despite a 4-log reduction of the population. In a study by Charteris et al. (6), it was observed that most strains of Lactobacillus spp. and Bifidobacterium spp. evaluated died after exposure to synthetic gastric juice (pH 2.0, 0.3% pepsin, 0.5% sodium chloride). In contrast, it has been demonstrated that LAB isolated from diverse niches tolerated gastrointestinal conditions well in the presence of foods due to a buffering effect, reinforcing the hypothesis that the food matrix may improve tolerance of probiotic bacteria to gastrointestinal conditions (5, 23, 24). Vinderola and Reinheimer (42) highlighted that gastric tolerance is a strain-specific effect, reinforcing the hypothesis that the food matrix may improve tolerance of probiotic bacteria to gastrointestinal conditions (5, 23, 24).
characteristic and affirmed that low gastric resistance of LAB should not exclude a strain from probiotic classification if it exhibits other properties of interest.

Effect of commercial prebiotics on bacteriocin production by *L. sakei* 1. In this research, a high production of sakacin 1 was observed in standard MRS medium (6,400 AU/ml), while with the prebiotics inulin and oligofructose the titers of bacteriocin were only 3,200 and 2,400 AU/ml, respectively. Our results are in accordance with those reported by Vamanu and Vamanu (39), who analyzed bacteriocin production by *Lactobacillus paracasei* CMGB16 using the prebiotics inulin, raffinose, and lactulose and found that inulin did not increase bacteriocin production (24-h growth).

On the other hand, in the literature there are several reports on the improvement of bacteriocin production when LAB were grown in the presence of raffinose, lactulose, fructooligosaccharides, and trehalose (7, 39). According to Leroy and De Vuyst (21), the conditions for production of bacteriocins are not easily predicted because the regulation of the synthesis of these antimicrobial peptides depends on complex parameters.

Effect of commercial prebiotics on tolerance of *L. sakei* 1 to bile salts. At 4 h, there were no significant differences in *L. sakei* 1 viable counts (*P* > 0.05) for any MRS formulations tested (Fig. 2A). However, after 24 h, the viability of *L. sakei* 1 in MRS broth was 1.8 log lower (*P* < 0.001) when glucose was replaced by either inulin or oligofructose (without Oxgall). A statistically significant decrease (1.4 log) of the *L. sakei* 1 population in regular MRS broth plus Oxgall (Fig. 2B) after 24 h was also observed. The replacement of glucose as the main carbohydrate source by inulin or oligofructose did not improve (*P* < 0.05) tolerance of *L. sakei* 1 to bile salts.
(Fig. 2B). However, the survival of L. sakei 1 was statistically similar when broths containing bile salts were formulated with either glucose or oligofructose. This may represent an advantage for the development of symbiotic food formulations, since it has been demonstrated that some prebiotics may protect probiotic strains during passage through GIT models (25), besides the intrinsic prebiotic effect of selective stimulation of beneficial bacteria from the gut residential microbiota.

Inhibition of L. monocytogenes adhesion to and invasion of Caco-2 cells. The microphotography presented in Figure 3 shows L. sakei 1 adhered to Caco-2 cells, an important property for a transient colonization of GIT, for stimulation of the immune system, and for antagonistic activity against other enteropathogens (9, 20, 45). Haller et al. (18) showed the adhesion of several food-fermenting bacterial strains to Caco-2 cells and observed that soluble factors from spent culture supernatants of lactobacilli cultures promoted adhesion to the eukaryotic cells.

Culture supernatant of L. sakei 1 (CFNS-S1) influenced L. monocytogenes adhesion to Caco-2 cells (Fig. 4). Application of 90% CFNS-S1 (1,440 AU/ml) for 30 min before or during inoculation of L. monocytogenes led to significant reduction (P < 0.05) of L. monocytogenes adhesion to Caco-2 cells (Fig. 4A and 4B). However, addition (Fig. 4C) of the CFNS-S1 after 30 min of incubation did not affect L. monocytogenes adhesion to Caco-2 cells. Similar results were reported by Coconnier et al. (8), who found that pretreatment of Salmonella enterica serovar Typhimurium SL1344 cells with spent culture supernatant of Lactobacillus acidophilus strain LB-SCS inhibited the infection and decreased the number of intracellular viable cells in cultured human intestinal Caco-2 cells. The protective effect was not observed when LB-SCS was added after Salmonella infection to Caco-2 cells, indicating that its mechanism of action involved interaction with the pathogen (8).

![FIGURE 3. Photomicrography showing adhesion of L. sakei 1 in Caco-2 epithelial cells at 2 h, stained with May-Grunwald-Giemsa. The image was obtained with an oil immersion lens (magnification, ×1,000). Bar, 5 μm.](http://meridian.allenpress.com/jfp/article-pdf/75/6/1083/1683422/0362-028x_jfp-11-523.pdf)

![FIGURE 4. Adhesion (in percentages) of L. monocytogenes in Caco-2 cells when incubated with different concentrations of the cell-free neutralized supernatant of L. sakei 1 containing sakacin L. (A) CFNS-S1 added 30 min before L. monocytogenes; (B) CFNS-S1 added concomitantly during incubation; (C) CFNS-S1 added 30 min after addition of L. monocytogenes. Results are means of triplicates ± standard deviations. An asterisk (*) indicates statistically significant difference from control.](http://meridian.allenpress.com/jfp/article-pdf/75/6/1083/1683422/0362-028x_jfp-11-523.pdf)

Our results of L. monocytogenes invasion to Caco-2 cells (37.91% ± 3.74%) were comparable to the results of Moroni et al. (31), who used L. monocytogenes LSD348 in a similar in vitro model (44.65%). The present results (Fig. 5) also revealed that L. monocytogenes was unable to invade Caco-2 cells when 90 and 75% CFNS-S1 were added to the cultures (P < 0.05). The addition of 50 and 25% CFNS-S1 also resulted in reduced invasion, while 10% CFNS-S1 did not modify invasion of L. monocytogenes compared to the control without CFNS-S1.

Botes et al. (2) reported that cell-free neutralized supernatants of Lactobacillus plantarum 423 (plantaricin 423) and Enterococcus mundtii ST4SA (peptide ST4SA) did not prevent adhesion of L. monocytogenes to Caco-2 cells but did inhibit invasion of L. monocytogenes strain...
Scott A into Caco-2 cells, corroborating results from our present study.

L. sakei 1 survived in vitro gastric conditions at pH 3, and it was able to survive in the presence of bile salts. Moreover, it was demonstrated that the use of L. sakei 1 plus oligofructose may be of interest to obtain a symbiotic effect, since the tolerance levels of L. sakei 1 to bile salts were similar in the presence of glucose and of oligofructose. The CFNS-S1 presented a protective effect for intestinal Caco-2 cells against invasion of L. monocytogenes, thus suggesting that bacteriocins may influence the dynamic of GIT colonization by pathogens.

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REFERENCES

In vitro evaluation of gastrointestinal survival of *Lactobacillus amylovorus* DSM 16698 alone and combined with galactooligosaccharides, milk and/or *Bifidobacterium animalis* subsp. *lactis* Bb-12. *Int. J. Food Microbiol.* 149:152–158.


