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Isolation and functional characterization of a biosurfactant produced by *Lactobacillus paracasei*

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ABSTRACT

In this study, the crude biosurfactant produced by a *Lactobacillus paracasei* strain isolated in a Portuguese dairy industry was characterized. The minimum surface tension (41.8 mN/m) and the critical micelle concentration (2.5 mg/ml) obtained were found to be similar to the values previously reported for biosurfactants isolated from other lactobacilli. The biosurfactant was found to be stable to pH changes over a range from 6 to 10, being more effective at pH 7, and showed no loss of surface activity after incubation at 60 °C for 120 h. Although the biosurfactant chemical composition has not been determined yet, a fraction was isolated through acidic precipitation, which exhibited higher surface activity as compared with the crude biosurfactant. Furthermore, this isolated biosurfactant showed antimicrobial and anti-adhesive activities against several pathogenic microorganisms. In addition, *L. paracasei* exhibited a strong autoaggregating phenotype, which was maintained after washing and resuspending the cells in PBS, meaning that this attribute must be related to cell surface components and not to excreted factors. The autoaggregation ability exhibited by this strain, together with the antimicrobial and anti-adhesive properties observed for this biosurfactant opens the possibility for its use as an effective probiotic strain.

1. Introduction

Biosurfactants are amphiphilic compounds produced by microorganisms with pronounced surface and emulsifying activities [1,2]. Microbial surfactants comprise a diverse group of surface-active molecules which are categorized by their chemical composition and microbial origin. They include glycolipids, lipopeptides, polysaccharide–protein complexes, protein-like substances, lipopolysaccharides, phospholipids, fatty acids and neutral lipids [1]. Diverse properties and physiological functions in the producer organisms are expected for different groups of biosurfactants, including enhancing the solubility of hydrophobic/water-insoluble compounds (which facilitates their uptake into the cell), heavy metal binding, bacterial pathogenesis, cell adhesion and aggregation, quorum sensing and biofilm formation [3,4]. Furthermore, some of these molecules have been described as antimicrobial and anti-adhesive agents [4,5].

Bacteria are the main group of surfactant-producing microorganisms, although they are also produced by some yeasts and filamentous fungi. These compounds can be synthesized by microorganisms growing on water-immiscible hydrocarbons as well as on water-soluble compounds such as glucose, sucrose, glycerol or ethanol, and can be excreted or remain attached to the cell wall [6]. Diversity existing among the biosurfactant producing microorganisms suggests that their production represents an important survival strategy and appears to have evolved in an independent yet parallel fashion [7].

The most commonly isolated biosurfactants are glycolipids and lipopeptides. They include rhamnolipids released by *Pseudomonas aeruginosa* [8], sophorolipids from *Candida* species [9], as well as surfactin and iturin produced by *Bacillus subtilis* strains [10]. The production yields of these biosurfactants are relatively high (2–10 g/l) and they reduce the surface tension of water to values bellow 30 mN/m. On the other hand, biosurfactants produced by lactobacilli are less effective, lowering the surface tension of water to values around 36–40 mN/m, and they are produced in lower amounts (20–100 mg/l) [11–18].

Information on the chemical structure of biosurfactants produced by probiotic microorganisms is limited. Biosurfactants produced by different lactobacilli have been characterized as complex biological mixtures that inhibit the adhesion of pathogens to both biomaterial and cell surfaces, but their composition has not been extensively studied and only a few have been partially characterized [11–13,17–21]. Better knowledge of biosurfactant's composition is required to fully understand their active components and be able to modify them in order to improve their properties.

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The ability to form multi-cellular aggregates has been reported for a number of bacterial species belonging to quite different groups. This phenomenon is described either as autoaggregation (involving bacteria from the same strain) or as co-aggregation (where different bacterial species are involved) [22]. In the last years, there has been an increasing recognition of the role of lactobacilli in the maintenance of the homeostasis within dynamic ecosystems such as the gastrointestinal and urogenital tracts, and in the prevention of colonization and infection caused by pathogenic microorganisms [23]. Some lactobacilli play a protective role by producing compounds such as hydrogen peroxide, lactic acid, bacteriocins and biosurfactants, which inhibit the growth of potential pathogens [24]. Furthermore, lactobacilli interfere with pathogens by competitive exclusion from receptors present on the surface of the epithelial cells and by co-aggregation with them, which contributes to create a barrier that prevents colonization by pathogens [25,26]. Consequently, the ability to adhere to epithelial cells and mucosal surfaces has been suggested to be an important property of many bacterial strains used as probiotics [27]. Previous studies indicate that autoaggregation of probiotic strains is necessary for adherence to epithelial cells, and this increases their colonization in environments with short residence times, such as the oral cavity and the urogenital tract [24,26,28,29].

The aim of the present study was to isolate and characterize the main functional properties of the crude biosurfactant produced by *Lactobacillus paracasei*. Characterization included the determination of minimum surface tension, critical micelle concentration and stability to different factors such as pH and temperature. The antimicrobial and anti-adhesive activities of this biosurfactant were assayed against several microorganisms. Furthermore, the autoaggregation ability of this strain was also studied.

2. Materials and methods

2.1. Strains and culture conditions

A *Lactobacillus* strain isolated in a Portuguese dairy industry, *L. paracasei*, was used for biosurfactant production. This strain was previously found to be a biosurfactant-producing strain (*unpublished work*).

The strain was stored at −80 °C in conventional synthetic MRS-Lac broth [medium introduced by DeMan et al. [30] for cultivation of *Lactobacillus* species where glucose was replaced by lactose] containing 15% (v/v) glycerol solution until it was used [30]. From a frozen stock, bacterium was streaked on MRS agar plates and incubated overnight at the optimum growing temperature (37 °C) for further culturing. The agar plates were stored at 4 °C, no longer than 2 weeks.

For antimicrobial and anti-adhesive assays, the following strains kindly provided by the Faculty of Pharmacy, University of Porto (Portugal) were used: *Escherichia coli*, *P. aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae* and *Streptococcus pyogenes*. All the strains were cultured in Trypticase Soy Broth (TSB) (OXOID, Basingstoke, England) at 37 °C.

2.2. Biosurfactant production and isolation

For crude biosurfactant production by *L. paracasei* in flasks, 600 ml of culture broth were inoculated with 6 ml of an overnight subculture and incubated for 72 h at 37 °C and 120 rpm. The growth media used for the production of biosurfactant was MRS-Lac medium (standard medium where glucose was replaced by lactose). After 72 h, cells were harvested by centrifugation (10 000 × *g*, 5 min, 10 °C), washed twice in demineralized water, and resuspended in 100 ml of phosphate-buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0). The bacteria were left at room temperature for 2 h with gentle stirring for biosurfactant release, as previously described [17,18]. Subsequently, the bacteria were removed by centrifugation and the remaining supernatant liquid was filtered through a 0.22 μm pore-size filter (Millipore, Bedford, USA). The supernatant was dialyzed against demineralized water at 4 °C in a Cellu-Sep© membrane (molecular weight cut-off 6000–8000 Da, Membrane Filtration Products, Seguin, USA) and freeze-dried. Once dried, the biosurfactant was stored at −20 °C for further studies. In order to confirm the biosurfactant production, the surface tension was routinely measured using the Ring method as previously described [31].

2.3. Biosurfactant acidic precipitation

The freeze-dried crude biosurfactant isolated from *L. paracasei* as previously described was subjected to acidic precipitation according to Van Hoogmoed et al. [21]. Briefly, the biosurfactant was resuspended in PBS (pH 7.0) to a concentration of 10 mg/ml, and subsequently the pH was adjusted to 2.0 by adding 1 M HCl. The acidified sample was kept at 4 °C for 2 h and the precipitate was collected by centrifugation (10 000 × *g*, 15 min, 4 °C) and washed twice with acidic water (pH 2.0). Afterwards the precipitate was dissolved in distilled water by adjusting the pH to 7.0 with 1 M NaOH, dialyzed against demineralized water at 4 °C in a Cellu-Sep© membrane (molecular weight cut-off 6000–8000 Da) and freeze-dried. The remaining supernatant was adjusted to pH 7.0 with 1 M NaOH, dialyzed (as previously described) and freeze-dried. Once dried, both products were stored at −20 °C for further studies.

2.4. Surface–activity determination

Surface tension measurements of culture broth and PBS extracts samples were performed according to the Ring method described elsewhere [31]. A KRUSS K6 Tensiometer equipped with a 1.9 cm De Noüy platinum ring was used. To increase the accuracy of the surface tension measurements, an average of triplicates was determined. All the measurements were performed at room temperature (20 °C).

2.5. Critical micelle concentration (cmc)

Critical micelle concentration is the concentration of an amphiphilic component in solution at which the formation of micelles is initiated. It is important for several biosurfactant applications to establish their *cmc*, as above this concentration no further effect is expected in the surface activity. The *cmc* was determined by plotting the surface tension as a function of the logarithm of biosurfactant concentration and was found at the point of intersection between the two lines that best fit through the pre- and post-*cmc* data. Concentrations ranging from 0.001 to 50 mg/ml of the crude biosurfactant isolated from *L. paracasei* were prepared in PBS (pH 7.0) and the surface tension of each sample was determined by the Ring method at room temperature (20 °C) as described above. All measurements were done in triplicate.

2.6. Biosurfactant stability

The applicability of biosurfactants can be conditioned by their stability to pH changes; thus the stability of the isolated crude biosurfactant was determined by measuring the surface tension of several samples prepared from the freeze-dried biosurfactant dissolved at a concentration of 50 mg/ml at different pH values (4.0–10.0). The surface tension of each sample was determined by the Ring method at room temperature (20 °C) as described above. Measurements were done in triplicate.

Furthermore, we evaluated the stability of the crude biosurfactant at different temperatures. For that purpose, several samples of the freeze-dried biosurfactant dissolved at a concentration of 50 mg/ml in PBS (pH 7.0) were prepared and incubated at different temperatures (25 °C, 37 °C and 60 °C) for 120 h. Surface tension of each sample was determined as described above and all measurements were performed in triplicate.

2.7. Antimicrobial assays

The antimicrobial activity of the isolated crude biosurfactant against several microbial strains was determined by the microdilution method in 96-well flat-bottomed plastic tissue culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany). Briefly, 125 μl of sterile double strength medium (TSB) were placed into the first column of the 96-well microplate, and 125 μl of sterile single strength growth medium in the remaining wells. Subsequently, 125 μl of biosurfactant solution in PBS (50 mg/ml) were added to the first column of the microplate and mixed with the medium; this results in a biosurfactant concentration of 25 mg/ml; serially, 125 μl were transferred to the subsequent wells, discarding 125 μl of the mixture in the tenth column, so that the final volume for each well was 125 μl. This process results in two-fold serial dilutions of the biosurfactant in the first 10 columns (25–0.048 mg/ml). Columns 11 and 12 do not contain biosurfactant and serve as negative and growth controls, respectively. All the wells (except for the 11th column) were inoculated with 2.5 μl of a pre-culture growth overnight in TSB at 37 °C diluted to a final concentration of 1×10^8 cfu/ml. Microplates were covered and incubated for 48 h at 37 °C. After 48 h of incubation, the optical density at 600 nm was determined for each well. The growth percentages at different biosurfactant concentrations for each microorganism were calculated as:

$$\%\text{Growth}_c = \left(\frac{\text{OD}_c}{\text{OD}_0}\right) \times 100$$

where OD_c represents the optical density of the well with a biosurfactant concentration c and OD_0 is the optical density of the control well (without biosurfactant). Triplicate assays were performed at all the biosurfactant concentrations for each strain.

2.8. Anti-adhesion assays

The anti-adhesive activity of the isolated crude biosurfactant against several microbial strains (the same microorganisms that were used in the antimicrobial assay) was determined according to the procedure described by Heinemann et al. [19]. Briefly, the wells of a sterile 96-well flat-bottomed plastic tissue culture plate (Greiner Bio-One) were filled with 200 μl of a crude biosurfactant solution in PBS. Several biosurfactant concentrations were tested ranging from 3 to 25 mg/ml. The plate was incubated for 18 h at 4 °C and subsequently washed twice with PBS. Control wells contained PBS buffer only. An aliquot of 200 μl of a washed bacterial suspension (3×10^8 cfu/ml in PBS) was added and incubated in the wells for 4 h at 4 °C. Unattached microorganisms were removed by washing the wells three times with PBS. The adherent microorganisms were fixed with 200 μl of 99% methanol per well, and after 15 min the plates were emptied and left to dry. Then the plates were stained for 5 min with 200 μl of 2% crystal violet used for Gram staining per well. Excess stain was rinsed out by placing the plate under running tap water. Subsequently, the plates were air dried, the dye bound to the adherent microorganisms was resolubilized with 200 μl of 33% (v/v) glacial acetic acid per well and the optical density readings of each well were measured at 595 nm. The microbial inhibition percentages at different biosurfactant concentrations for each microorganism were calculated as:

$$\%\text{Microbial inhibition}_c = \left[1 - \left(\frac{\text{OD}_c}{\text{OD}_0}\right)\right] \times 100$$

where OD_c represents the optical density of the well with a biosurfactant concentration c and OD_0 is the optical density of the control well. Triplicate assays were performed at all the biosurfactant concentrations for each strain. The microtiter-plate anti-adhesion assay permits the estimation of the crude biosurfactant concentrations that are effective in inhibiting adhesion of the studied microorganisms.

2.9. Autoaggregation assays

Autoaggregation assays were performed according to Del Re et al. [25]. *L. paracasei* was grown in flasks with 100 ml MRS-Lac broth (conventional MRS broth with glucose replaced by lactose) for 48 h at 37 °C and 120 rpm. Cells were harvested by centrifugation (10 000 × *g*, 10 min, 10 °C), washed twice in demineralized water, and resuspended in their own culture supernatant fluid or in phosphate-buffered saline (PBS, pH 7.0) to a concentration of 10^8 cfu/ml. Cell suspensions (4 ml) were prepared in tubes and mixed by vortexing for 10 s. Autoaggregation was determined during 5 h at room temperature (20 °C); every hour, 0.1 ml of the upper suspension was transferred (without disturbing the microbial suspension) to another tube with 3.9 ml of PBS, and after mixing, the optical density was measured at 600 nm. The autoaggregation coefficient (AC) was calculated at different times according to Kos et al. [28] as:

$$\text{AC}_t = \left[1 - \left(\frac{\text{OD}_t}{\text{OD}_i}\right)\right] \times 100$$

where OD_t represents the optical density of the microbial suspension at 600 nm at time t (0.5, 1, 2, 3, 4 or 5 h) and OD_i is the optical density at $t = 0$.

3. Results

3.1. Critical micelle concentration and minimum surface tension

In order to establish the critical micelle concentration of the crude biosurfactant isolated from *L. paracasei*, the relationship between biosurfactant concentration and surface tension was determined. The freeze-dried biosurfactant was dissolved in PBS (pH 7.0) at different concentrations ranging from 0.001 to 50 mg/ml. As can be seen in Fig. 1A, a progressive decrease in surface tension is observed with the increase of biosurfactant concentration; for biosurfactant concentrations higher than 2.5 mg/ml, the surface tension becomes stable, and there is not any significant reduction even at the highest concentrations tested, therefore that value can be considered as the *cmc* for this biosurfactant. Therefore, a minimum surface tension value of 41.8 mN/m was obtained for a biosurfactant concentration of 50 mg/ml. The same *cmc* value was obtained by the semi-logarithmic representation of surface tension *versus* biosurfactant concentration (Fig. 1B).

3.2. Stability to pH and temperature

The applicability of biosurfactants in several fields depends on their stability at different temperatures and pH values. In order to study the pH effect on biosurfactant stability, the surface tension of several biosurfactant samples prepared with a concentration of 50 mg/ml at different pH values (4–10) was determined. As can be seen in Fig. 2, the minimum surface tension value was obtained at pH 7 (41.8 mN/m). The surface activity of the crude biosurfactant remained relatively stable to pH changes between pH 6 and

Table 1
Surface tension values (mN/m) of the crude biosurfactant isolated from *L. paracasei* dissolved in PBS (pH 7.0) with a concentration of 50 mg/ml. Measurements were done at room temperature (20 °C) after incubation at the different temperatures for different time intervals. The reference surface tension value was 72.0 mN/m. Results are expressed as means ± standard deviations of values from triplicate experiments.

	Surface tension (mN/m)					
	0 h	24 h	48 h	72 h	96 h	120 h
25 °C	41.9 ± 0.2	41.8 ± 0.5	42.0 ± 0.4	41.9 ± 0.1	42.1 ± 0.3	42.1 ± 0.2
37 °C	41.9 ± 0.2	42.1 ± 0.1	42.0 ± 0.2	41.8 ± 0.3	42.0 ± 0.4	41.9 ± 0.5
60 °C	41.9 ± 0.2	43.2 ± 0.4	43.0 ± 0.3	43.3 ± 0.1	43.5 ± 0.3	43.4 ± 0.2

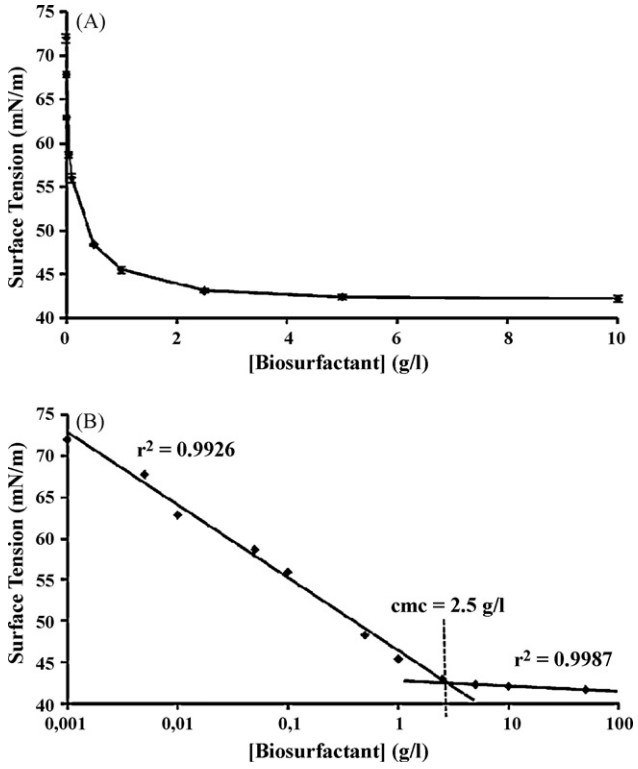

Fig. 1. Effect of the biosurfactant concentration on the PBS surface tension. (A) Surface tension (mN/m) of the crude biosurfactant obtained from *L. paracasei* dissolved in PBS (pH 7.0) at different concentrations at room temperature (20 °C). (B) Surface tension *versus* logarithm of biosurfactant concentration. The *cmc* was determined from the intersection of regression lines that better describe the two parts of the curve, below and above *cmc*. The reference surface tension value was 72 mN/m. Results represent the average of three independent measurements and error bars represent standard deviations of the mean values.

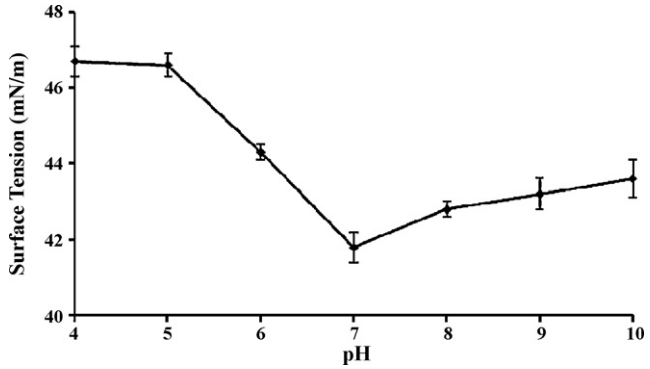

Fig. 2. Effect of pH on the surface tension of the crude biosurfactant isolated from *L. paracasei*. Samples were prepared with a concentration of 50 mg/ml at different pH values and measurements were done at room temperature (20 °C). The reference surface tension value was 72 mN/m. Results represent the average of three independent measurements and error bars represent standard deviations of the mean values.

10, showing higher stability at alkaline than acidic conditions. At pH 10, the surface tension value is only two units higher than at pH 7, whereas at pH 6 it is three units higher. In addition, for pH values lower than 6, the samples become turbid, due to partial precipitation of the biosurfactant.

As can be seen in Table 1, biosurfactant activity remains unaltered after incubation for 120 h at 25 or 37 °C, and even incubation at 60 °C for 120 h does not result in a significant loss of activity. Therefore, it can be concluded that this biosurfactant maintains its surface properties unaffected in the range of temperatures between 25 and 60 °C for incubation times of at least 120 h.

3.3. Partial purification of the crude biosurfactant

The acid-precipitated fraction obtained from the crude biosurfactant, as well as the freeze-dried supernatant, were assayed for surface activity at different concentrations and compared with the crude biosurfactant. The acid precipitated fraction constituted approximately 20% of the total dry-weight of the crude biosurfactant and was found to be more surface active than the crude mixture (Table 2). Furthermore, the freeze-dried product obtained from the supernatant left after the acidic precipitation showed poor surface activity, suggesting that the acid precipitated fraction contains the greater part of the surface active compounds existent in the crude biosurfactant.

3.4. Antimicrobial activity

The isolated crude biosurfactant showed antimicrobial activity against all the bacterial strains tested (Fig. 3). A total growth inhibition was observed for *E. coli*, *S. agalactiae* and *S. pyogenes* with a biosurfactant concentration of 25 mg/ml. For the other microorganisms assayed, although a complete inhibition was not observed, high growth reductions were obtained (from 83.5 to 96.7%) at the same biosurfactant concentration.

3.5. Anti-adhesive activity

The isolated crude biosurfactant was found to possess anti-adhesive activity against all the microorganisms assayed (Fig. 4). The highest anti-adhesive percentages were obtained for *S. aureus*

Table 2
Surface tension values (mN/m) obtained for the acid precipitated fraction, the freeze-dried supernatant and the crude biosurfactant isolated from *L. paracasei*, dissolved in PBS (pH 7.0) at different concentrations. Measurements were done at room temperature (20 °C). The reference surface tension (PBS) was 72.0 mN/m. Results represent the means ± standard deviations of three independent experiments.

Fraction	Concentration (mg/ml)			
	0.01	0.1	1.0	10.0
Crude biosurfactant	62.9 ± 0.4	56.0 ± 0.6	45.5 ± 0.2	42.1 ± 0.4
Acid precipitated fraction	57.6 ± 0.5	51.1 ± 0.4	40.8 ± 0.6	39.5 ± 0.3
Freeze-dried supernatant	71.9 ± 0.6	65.9 ± 0.5	62.2 ± 0.4	61.7 ± 0.6

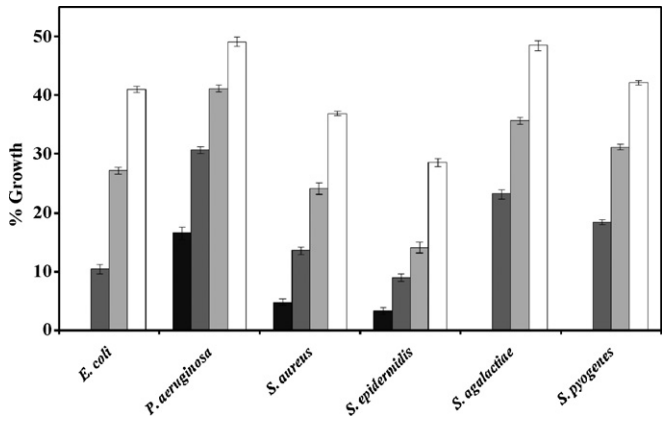

Fig. 3. Growth percentages obtained with the crude biosurfactant isolated from *Lactobacillus paracasei* at different concentrations (25 mg/ml [■], 12.5 mg/ml [▓], 6.25 mg/ml [▒] and 3.12 mg/ml [□]) when compared with a control without biosurfactant. Results are averages of triplicate experiments and error bars represent the standard deviation values.

(72.0%), *S. epidermidis* (62.1%) and *S. agalactiae* (60.0%) for a biosurfactant concentration of 25 mg/ml. On the contrary, a low activity was observed for *P. aeruginosa* (16.5%) and *E. coli* (11.8%) at the same biosurfactant concentration.

3.6. Autoaggregation phenotype of L. paracasei

L. paracasei exhibits a strong autoaggregation phenotype, producing macroscopic granules clearly observable. After mixing the stationary phase culture, snow flake-like aggregates can be observed, which rapidly sediment under resting conditions. Aggregates are also visible under light microscope.

The sedimentation rate of *L. paracasei* was measured over a period of 5 h using washed cells resuspended in PBS (pH 7.0). To avoid the possibility of removing extracellular components, which may participate in autoaggregation, measurements were also done using washed cells resuspended in their own culture supernatant fluid. The autoaggregation percentages obtained at different times were similar in both cases (51.1 and 49.4%, respectively, after 2 h), and confirmed the autoaggregating ability of this strain (Fig. 5). These results also suggest that autoaggregation must be related to cell surface components and not to excreted factors, because it is not lost after repeated washing (three times) of the cells with demineralized water and suspending them in PBS.

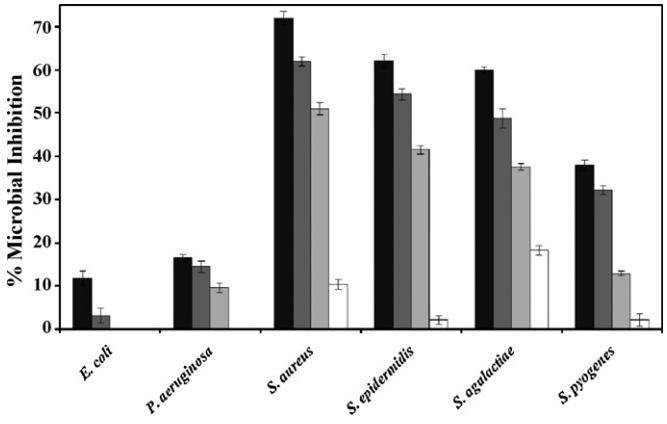

Fig. 4. Microbial inhibition percentages obtained from the anti-adhesion assays with the crude biosurfactant isolated from *Lactobacillus paracasei* at different concentrations (25 mg/ml [■], 12.5 mg/ml [▓], 6.25 mg/ml [▒] and 3.12 mg/ml [□]). Results are averages of triplicate experiments and error bars represent the standard deviation values.

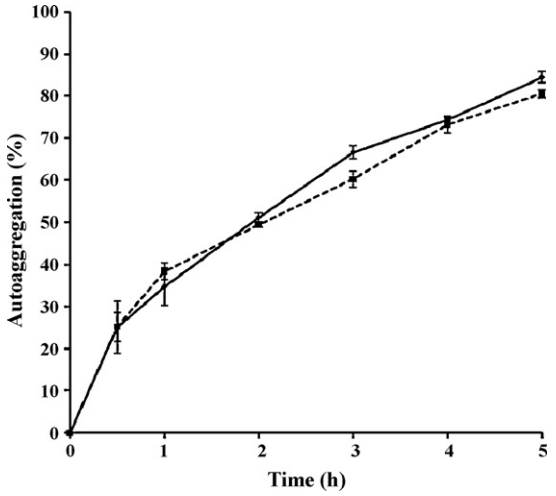

Fig. 5. Comparison of the autoaggregation ability of *L. paracasei* cells resuspended in PBS (pH 7.0) after grown on MRS-Lac broth (----), or resuspended in their own culture supernatant fluid (—-). Error bars represent standard deviations of the mean values of results from three independent experiments.

4. Discussion

The critical micelle concentration of the crude biosurfactant isolated from *L. paracasei* was established at 2.5 mg/ml. Additionally, the minimum surface tension value obtained for a biosurfactant concentration of 50 mg/ml (in PBS at pH 7.0) was 41.8 mN/m. The effectiveness of a surfactant is determined by its ability to reduce the surface and interfacial tension; for instance, a good surfactant can reduce the surface tension of water from 73.2 to 35.0 mN/m, and the interfacial tension between water and hexadecane from 40.0 to 1.0 mN/m [32]. Table 3 compiles the surface activities and *cmcs* of SDS and several biosurfactants isolated from different lactobacilli strains. Those compounds can reduce the surface tension of water to values around 36–39 mN/m and their *cmcs* range from 1.0 to 20.0 mg/ml. Therefore, our results are in agreement with those obtained for biosurfactants isolated from other lactobacilli.

The applicability of biosurfactants in several fields depends on their stability at different temperatures and pH values. In this case, the surface activity of the isolated crude biosurfactant was found to be quite stable over a pH range from 6 to 10, being more effective at pH 7. Additionally, it was observed a loss of activity at acidic and basic conditions, being the surface activity more negatively affected by acidic pH values. The higher instability of biosurfactants isolated from other lactobacilli to acidic conditions has been described by other authors and related to the presence of negative charged groups at the polar ends of the molecules, which are protonated under those conditions [33]. In that sense, the characterization of the intracellular biosurfactants produced by some lactobacilli has

Table 3
Minimal surface tensions and critical micelle concentrations (*cmcs*) obtained for various surface active compounds (sodium dodecylsulphate and biosurfactants isolated from different lactobacilli strains).

Surfactant	Surface tension (mN/m)	cmc (mg/ml)	Reference
Synthetic surfactants			
Sodium dodecylsulphate	37.0	1.8	[32]
Biosurfactants from			
Lactobacillus fermentum RC-14	39.0	1.0	[11]
Lactobacillus fermentum B54	39.0	2.5	[11]
Streptococcus thermophilus B	37.0	2.0	[13]
Streptococcus thermophilus A	36.0	20.0	[17]
Lactococcus lactis 53	36.0	14.0	[18]

revealed the presence of proteic fractions probably associated to phosphate groups [11,12,17,18,20], which could be also negatively affected by denaturalization at acidic pH. In accordance with that, in our case, for pH values lower than 6, precipitation of some of the biosurfactant components was observed contributing to the increase measured in the surface tension. Regarding the stability at different temperatures, the biosurfactant remained stable after incubation for 120 h to temperatures from 25 to 60 °C, with practically no loss of activity. In that sense, Desai and Banat [34] observed that heat treatment (autoclaving at 120 °C for 15 min) on some biosurfactants caused no appreciable changes in their surface and emulsifying activities.

Although several studies on biosurfactants produced by lactobacilli have been performed, little is known about their chemical structure, due to their complexity. For instance, the crude biosurfactant released by *S. thermophilus* B was reported to be a mixture of various components, including polysaccharides and glycolipids, but not proteins or phospholipids [13]. A glycolipid-like fraction obtained from that biosurfactant resulted extremely surface-active, reducing the surface tension of water to 35 mN/m at a concentration of 0.5 mg/ml [13]. Also, biosurfactants released by *Streptococcus mitis* BA and *S. mitis* BMS contained extremely low levels of proteins, and the main components were glycolipids [21]. An acid precipitated fraction isolated from the *S. mitis* BMS biosurfactant and identified as a rhamnolipid-like compound reduced the surface tension of water to 35 mN/m at a concentration of 1 mg/ml, while the crude biosurfactant reduced the surface tension to approximately 48 mN/m at the same concentration, and the freeze-dried supernatant (the supernatant left after acid precipitation) hardly decreased the surface tension of water in the same concentration range [21].

In our case, although the crude biosurfactant isolated from *L. paracasei* has not been chemically characterized yet, results suggest that it consists of a mixture of several compounds. A fraction was purified by acidic-precipitation, which represents approximately 20% of the dry weight of the crude biosurfactant and exhibits higher surface activity, suggesting that it must contain the greater part of the surface active compounds existent in the mixture, a result similar to that obtained by Van Hoogmoed et al. [21] with a biosurfactant isolated from *S. mitis* BMS.

Biosurfactants isolated from several lactobacilli were characterized as multi-component mixtures, consisting of protein and polysaccharides, probably containing phosphate groups [11,12,17,18]. One fraction isolated from the biosurfactant obtained in the stationary growth phase of *L. fermentum* B54 showed the same surface activity as the crude biosurfactant (39 mN/m), even though with a lower *cmc* (0.5 mg/ml), and was richer in protein, containing less polysaccharides and phosphate groups than the crude biosurfactant [11]. Also, a fraction with higher surface activity was partially purified from biosurfactants produced by *L. lactis* 53 (with a similar protein content but less polysaccharide and phosphate contents as compared to the crude biosurfactant) and *S. thermophilus* A (with no protein content) [17,18]. Howard et al. [20] identified a number of collagen-binding proteins in the crude biosurfactants obtained from different *Lactobacillus* strains, being one of those proteins present in the biosurfactant isolated from *L. fermentum* RC-14 and responsible for the anti-adhesive effect against *Enterococcus faecalis in vitro* [19].

Several biosurfactants which exhibit antimicrobial activity against various microorganisms have been previously described. They include surfactin and iturin produced by *B. subtilis* strains [10], rhamnolipids from *Pseudomonas* species [35,36], mannosylerythritol lipids from *Candida antarctica* [37] and biosurfactants produced by some fungi [38]. However, there are few reports about the antimicrobial activity of biosurfactants isolated from lactobacilli; only biosurfactants obtained from *S. thermophilus* A and *L. lactis* 53 showed significant antimicrobial activity against several bacterial and yeast strains isolated from explanted voice prostheses [17,18]. In addition to the antimicrobial properties, the biosurfactant isolated in the present study exhibited a considerable anti-adhesive activity against most of the microorganisms tested. Involvement of biosurfactants in microbial adhesion and desorption has been widely described. The prior adsorption of biosurfactants to solid surfaces might constitute an effective strategy to reduce microbial adhesion and preventing colonization by pathogenic microorganisms, not only in the biomedical field, but also in other areas, like the food industry [2,39–41]. The anti-adhesive activity observed with this biosurfactant against several pathogenic microorganisms, such as *S. aureus* (a common cause of community and hospital acquired infections), *S. epidermidis* and *S. agalactiae*, are very promising for further studies and applications aiming to reduce/prevent microbial colonization on different materials.

Table 4
Autoaggregation percentages calculated for different lactobacilli strains after 1 h (a) or 2 h (b).

Strain	%Autoaggregation	Reference
Bifidobacterium longum B2	≈50(b)	[25]
Bifidobacterium longum B7	≈80(b)	[25]
Lactobacillus acidophilus M92	45(b)	[28]
Lactobacillus kefir 8347	29(a)	[45]
Lactobacillus kefir 8345	58(a)	[45]
L. paracasei	51(b)	This study

The ability to form multicellular aggregates has been reported for a number of bacterial species belonging to quite different groups, including lactobacilli. The cellular aggregation between microorganisms of the same strain (autoaggregation) or between genetically different strains (co-aggregation) is of considerable relevance in several ecological niches and is important to maintain the microbial populations on different mucosal surfaces [22,29]. Autoaggregation of lactobacilli appears to be necessary for their adhesion to epithelial cells and mucosal surfaces, and this attribute is a desirable property of probiotic bacteria [27,28,42]. The autoaggregation ability is one of the proposed mechanisms to explain the protective role displayed by lactobacilli in the human vagina. This property, together with the adhesion to epithelial cells, facilitates the formation of biofilms by lactobacilli on the vaginal epithelia, preventing the access to pathogens [43]. In most cases, bacterial adhesion to epithelial cells and autoaggregation are mediated by the same factors [25,28,43]. In the same way, co-aggregation between lactobacilli and pathogenic microorganisms contributes to create a barrier that prevents their adhesion to the epithelia and subsequent access to the tissues, constituting an important host defense mechanism against infections in the urogenital and gastrointestinal tracts [25,43,44].

The results obtained for *L. paracasei* suggest that the autoaggregation ability must be related to cell surface components and not to excreted factors, because it is not lost after washing the cells and suspending them in PBS. Furthermore, the autoaggregation coefficients obtained for *L. paracasei* in this study, both in PBS or in the culture broth supernatant, are similar to those achieved by other authors for other probiotic lactobacilli (Table 4).

5. Conclusions

In this study, the crude biosurfactant produced by a *L. paracasei* strain was characterized. The minimum surface tension and the critical micelle concentration were similar to the values previously reported for biosurfactants isolated from other lactobacilli, and it showed stability to pH changes and temperature. A fraction was isolated through acidic precipitation, which exhibited higher surface activity when compared with the crude biosurfactant. Fur-

thermore, in view of the autoaggregating phenotype exhibited by *L. paracasei*, it would be interesting to evaluate its ability to co-aggregate with other microorganisms (particularly pathogenic microorganisms) and to adhere to epithelial cells. The mechanisms employed by the healthy microflora to interfere with the adhesion of invading pathogens include competitive exclusion and displacement, production of antibacterial compounds (such as lactic acid, hydrogen peroxide, bacteriocins, and bacteriocin-like substances), co-aggregation and release of biosurfactants [23,46]. *L. paracasei* produced a biosurfactant which antimicrobial and anti-adhesive activities against several pathogens have been demonstrated, and the ability to co-aggregate with those pathogens and to adhere to epithelial cells would enhance the probiotic properties and applications of this strain. The results obtained suggest the possible use of this biosurfactant as an alternative antimicrobial agent in the medical field for applications against microorganisms responsible for diseases and infections in the urinary, vaginal and gastrointestinal tracts, as well as in the skin, making it a suitable alternative to conventional antibiotics.

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