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## Antimicrobial and anti-adhesive activities of cell-bound biosurfactant from *Lactobacillus agilis* CCUG31450

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In this work, biosurfactant production by different *Lactobacillus* strains was studied using the conventional MRS medium for lactic acid bacteria. *Lactobacillus agilis* CCUG31450 produced a cell-bound biosurfactant that reduced the surface tension of water to 42.5 mN m<sup>-1</sup>, and exhibited a high emulsifying activity ( $E_{24} = 60\%$ ). The amount of biosurfactant produced was 84 mg l<sup>-1</sup>, with a cmc of 7.5 mg ml<sup>-1</sup>. A preliminary chemical characterization by Fourier transform infrared spectroscopy indicated that the biosurfactant is a glycoprotein. Using cheese whey as an alternative culture medium, biosurfactant production was increased up to 960 mg l<sup>-1</sup>. The biosurfactant exhibited a considerable anti-adhesive activity against *Staphylococcus aureus*, as well as antimicrobial activity against *S. aureus*, *Streptococcus agalactiae* and *Pseudomonas aeruginosa*. This study represents the first description of a biosurfactant produced by a *L. agilis* strain. The results obtained open future prospects for the application of this biosurfactant to reduce or inhibit the adhesion of pathogenic microorganisms (such as *S. aureus*) in several biomedical applications.

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### 1. Introduction

Biosurfactants are a structurally diverse group of surface-active compounds synthesized by microorganisms. They include, among others, glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids and neutral lipids. Due to their amphipathic nature, biosurfactants accumulate at the interface between fluid phases with different polarities, reducing the surface and interfacial tensions, and forming stable emulsions between aqueous and oil phases.<sup>1</sup> Although the function attributable to biosurfactants in the producer organisms is not completely understood, it is generally accepted that these molecules enhance the solubility of water-insoluble compounds, facilitating their uptake into the cell, and participate in processes such as cell adhesion and aggregation, quorum sensing, biofilm formation and defense against other microorganisms.<sup>2</sup>

Many biosurfactants have been reported to possess a similar or better performance when compared with synthetic surfactants, which in addition to their lower toxicity, higher biodegradability and effectiveness at extreme temperatures, salinities and pH values, make them a green alternative to their chemical counterparts in different applications, including agriculture, food, cosmetics or petroleum industries, as well as in bioremediation.<sup>3–5</sup> Furthermore, biosurfactants can affect

microorganisms' adhesion to different surfaces,<sup>6,7</sup> exhibit anti-bacterial, antifungal or antiviral activity<sup>8–11</sup> and, in some cases, anti-tumor activity, interfering with cancer progression processes.<sup>1,12</sup> Altogether these properties contribute to the biosurfactants potential as alternatives to conventional therapeutic agents in many biomedical applications.

Lactic acid bacteria (LAB) represent a significant part of the natural microbiota in the human gastrointestinal and genitourinary tracts, playing an important role in the maintenance of homeostasis within those ecosystems and contributing to prevent pathogen colonization. These bacteria are considered probiotic agents (live microorganisms which, when administered in adequate amounts, confer a health benefit to the host) that inhibit the growth of pathogens, producing antibacterial compounds (*e.g.* lactic acid, hydrogen peroxide, bacteriocins, and bacteriocin-like substances) and releasing biosurfactants.<sup>13</sup>

Biosurfactants produced by LAB are less studied than other biosurfactants like lipopeptides or rhamnolipids (produced by *Bacillus* and *Pseudomonas* species, respectively) for several reasons; namely they are less effective, reducing the surface tension to values around 36–45 mN m<sup>-1</sup>; their critical micelle concentrations (cmc) are relatively high (from 1.0 to 20.0 mg ml<sup>-1</sup>); they are produced in lower amounts (20–100 mg l<sup>-1</sup>); and they are associated to the cell wall, thus an extraction process is required for their recovery.<sup>10,11,14</sup> However, the main advantage of these biosurfactants is that LAB are generally regarded as safe (GRAS), and most of them are already used in many food manufacturing and industrial processes. Therefore, LAB can be viewed as a promising source of biosurfactants.

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In this work, the biosurfactant production by different *Lactobacillus* strains was evaluated. The cell-bound biosurfactant produced by *Lactobacillus agilis* CCUG31450 was characterized. The critical micelle concentration and the effect of several environmental parameters on surface and emulsifying activities were determined. Furthermore, a preliminary chemical characterization was performed. Alternative culture media were tested for the biosurfactant production by this isolate. And the antimicrobial and anti-adhesive activities against different pathogenic microorganisms were studied.

## 2. Results and discussion

### 2.1. Screening of biosurfactant-producing strains

The production of cell-bound and excreted biosurfactants by the different *Lactobacillus* strains was studied using MRS medium. None of the isolates could reduce the surface tension of the culture medium, meaning that these strains are unable to produce extracellular biosurfactants. However, all the isolates were found to produce cell-bound biosurfactants, as surface tension reductions (between 15.5 and 27.9 mN m<sup>-1</sup>) after the extraction procedure at the end of the fermentation process, were observed (Table 1). Taking into account the minimum decrease in surface tension (8 mN m<sup>-1</sup>) generally accepted to consider a microorganism as a biosurfactant-producer,<sup>15</sup> it can be concluded that all the studied lactobacilli produced biosurfactants. The highest surface tension reduction (27.9 mN m<sup>-1</sup>) was obtained with the isolate *L. agilis* CCUG31450, which also showed the highest emulsifying activity (20.0%) and the highest biosurfactant production (84.4 mg l<sup>-1</sup>). Surface tension reductions after extraction of cell-bound biosurfactants between 12 and 33 mN m<sup>-1</sup> have been reported for different *Lactobacillus* species,<sup>6,9,16–20</sup> which is in good agreement with the results obtained with the strains herein studied. Although LAB produce mainly cell-bound biosurfactants, in some cases cell-bound and extracellular biosurfactants are produced simultaneously; however, in those cases, extracellular biosurfactants are usually produced in lower amounts.<sup>17–21</sup> On the contrary, in the last years, a few *Lactobacillus* and *Lactococcus* species have been reported to exclusively produce extracellular biosurfactants.<sup>22–25</sup>

The amounts of cell-bound biosurfactants produced by other LAB vary between 20 and 100 mg l<sup>-1</sup>.<sup>10,11,19–21,26</sup> In the case of

lactobacilli producing exclusively extracellular biosurfactants, the amounts reported are usually higher (1–5 g l<sup>-1</sup>).<sup>22,24,25</sup> Therefore, the amounts of cell-bound biosurfactant produced by *L. agilis* CCUG31450 are in accordance with the previously reported data for other LAB. In view of the results obtained, *L. agilis* CCUG31450 was selected to perform the following studies, as it showed the best results regarding biosurfactant production.

### 2.2. Critical micelle concentration (cmc)

The cmc of the cell-bound biosurfactant produced by *L. agilis* CCUG31450 in MRS medium was determined by measuring the surface tension of the freeze-dried biosurfactant dissolved at different concentrations in PBS buffer, as described elsewhere<sup>10</sup> (Fig. 1). The cmc was found to be 7.5 mg ml<sup>-1</sup>, with a minimum surface tension value of 42.5 mN m<sup>-1</sup>, and a maximum emulsifying index of 60%.

The cmc and the surface tension values herein obtained for the cell-bound biosurfactant produced by *L. agilis* CCUG31450 are in good agreement with the values reported in the literature for biosurfactants produced by other LAB, although lactobacilli

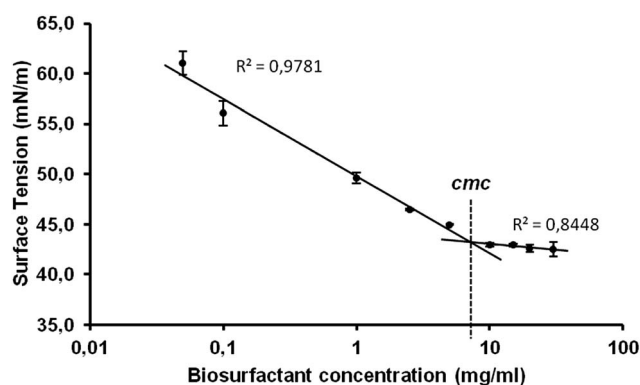


Fig. 1 Surface tension values (mN m<sup>-1</sup>) versus logarithm of biosurfactant concentration (mg ml<sup>-1</sup>) obtained with the cell-bound biosurfactant produced by *L. agilis* CCUG31450 in MRS medium. The cmc was determined from the intersection of the regression lines that better describe the two parts of the curve, below and above the cmc. Results represent the average of two independent experiments ± standard deviation.

**Table 1** Screening of biosurfactant production by lactobacilli. Surface tension values (mN m<sup>-1</sup>), emulsifying indexes ( $E_{24}$ , %), biomass concentration (OD<sub>600 nm</sub>) and cell-bound biosurfactant concentration (mg l<sup>-1</sup>) obtained with the different lactobacilli strains grown in MRS medium at 37 °C and 100 rpm. The surface tension values and the emulsifying indexes were measured in the PBS extracts after the recovery of the cell-bound biosurfactants. The surface tension of PBS was 71.9 ± 0.1 mN m<sup>-1</sup>. Results represent the average of three independent experiments ± standard deviation

Microorganism	<i>t</i> (h)	ST (mN m <sup>-1</sup> )	OD <sub>600 nm</sub>	$E_{24}$ (%)	[Biosurfactant] (mg l <sup>-1</sup> )
<i>L. agilis</i> CCUG31450	96	44.0 ± 0.4	1.042 ± 0.156	20.0 ± 2.0	84.4 ± 1.6
<i>L. animalis</i> ATCC35046	120	46.6 ± 1.4	1.635 ± 0.108	16.0 ± 1.2	27.6 ± 5.6
<i>L. buchneri</i> ATCC4005	72	56.0 ± 2.1	1.876 ± 0.243	0.0	18.6 ± 3.7
<i>L. hamsteri</i> ATCC43851	96	52.9 ± 1.1	4.065 ± 0.134	0.0	24.2 ± 1.6
<i>L. intestinalis</i> ATCC49335	72	56.4 ± 0.9	1.289 ± 0.201	0.0	19.0 ± 4.2

strains that produce exclusively extracellular biosurfactants can exhibit lower cmc values (Table 2).

The biosurfactant produced by *L. agilis* CCUG31450 exhibited an emulsifying index of 60% against *n*-hexadecane at concentrations equal or higher than the cmc. This emulsifying index is higher than the results reported for biosurfactants produced by other LAB. Emulsifying indexes between 40 and 49% were found for biosurfactants produced by *Lactobacillus pentosus* CECT4023 against gasoline, kerosene and octane;<sup>3,27</sup> and biosurfactants produced by *Lactobacillus plantarum* CFR2194 exhibited emulsifying indexes against a variety of water-immiscible substrates between 13.6 and 38.2%.<sup>9</sup>

### 2.3. Stability studies

The effect of several environmental parameters on the activity of the cell-bound biosurfactant produced by *L. agilis* CCUG31450 was studied at a concentration of 7.5 mg ml<sup>-1</sup> (the cmc for this biosurfactant).

Regarding the effect of pH, the minimum surface tension was obtained at pH 7. For pH values lower than 7, an increase in the surface tension values was observed (from 42.8 mN m<sup>-1</sup> at pH 7 to 45.0 mN m<sup>-1</sup> at pH 6). As the pH decreases, the surface tension continued increasing, up to 47.0 mN m<sup>-1</sup> at pH 2. However, the emulsifying activity was less affected by low pH values (60.0% at pH 7 and 57.0% at pH 3). Under alkaline conditions, from pH 7 to 9, only a slight increase in the surface tension was found, and the emulsifying activity remained constant. However, for pH values higher than 9, a rapid increase in the surface tension values was detected, as well as a reduction in the emulsifying activity, which disappeared at pH values higher than 11. The most favorable conditions regarding surface and emulsifying activity were obtained between pH 7 and 9 (Fig. 2A).

The instability of biosurfactants produced by some LAB when exposed to acidic conditions, as well as a higher stability at alkaline conditions, has been previously reported by other researchers.<sup>10,11,14,27</sup> The characterization of some of those

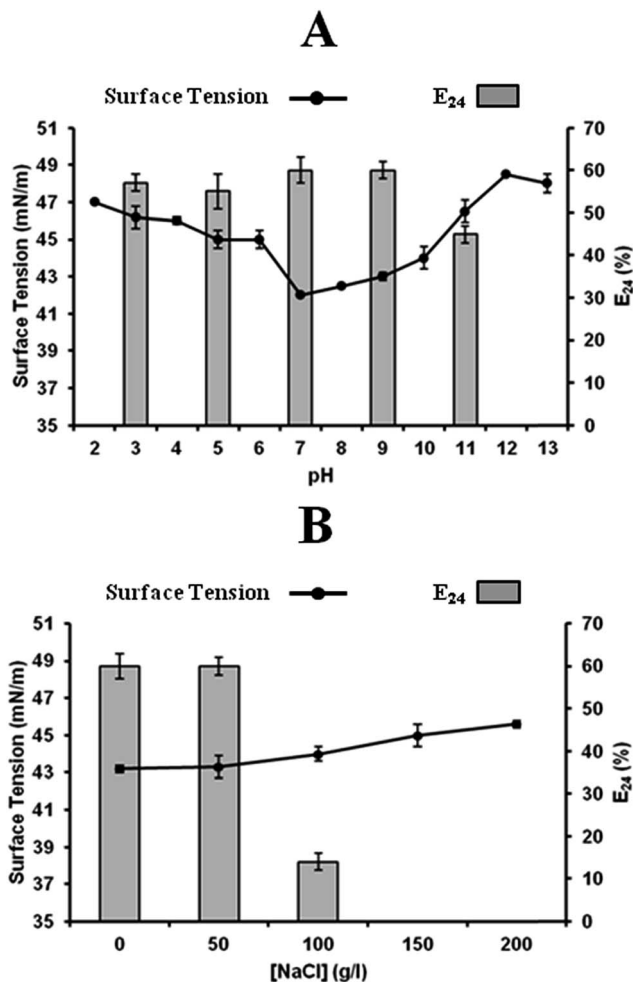


Fig. 2 Effect of pH (A) and salinity (B) on the surface tension and the emulsifying activity of the cell-bound biosurfactant produced by *L. agilis* CCUG31450 dissolved in demineralized water at a concentration of 7.5 mg ml<sup>-1</sup>. Results represent the average of three independent experiments  $\pm$  standard deviation.

Table 2 Critical micelle concentrations (mg ml<sup>-1</sup>) and surface tension values (mN m<sup>-1</sup>) reported for biosurfactants produced by different LAB

	Surface tension (mN m <sup>-1</sup> )	cmc (mg ml <sup>-1</sup> )	Reference
<b>Cell-bound biosurfactants</b>			
<i>Lactobacillus plantarum</i> CFR2194	44.3	6.0	9
<i>Lactococcus lactis</i> 53	36.0	14.0	10
<i>Streptococcus thermophilus</i> A	36.0	20.0	11
<i>Lactobacillus paracasei</i> A20	41.8	2.5	14
<i>Lactobacillus fermenti</i> 126	45.1	9.0	16
<i>Lactobacillus rhamnosus</i> CCM1825	43.6	6.0	16
<i>Lactobacillus acidophilus</i> RC14	39.0	1.0	20
<i>Lactobacillus agilis</i> CCUG31450	42.5	7.5	This study
<b>Extracellular biosurfactants</b>			
<i>Lactobacillus</i> sp. CV8LAC	45.4	0.1	22
<i>Lactococcus lactis</i>	40.5	3.5	24

biosurfactants revealed the presence of protein fractions probably associated to phosphate groups, which can be negatively affected by the denaturation at acidic pHs.<sup>10,11</sup> Also, negatively charged groups at the polar ends of the molecules, which are protonated at low pH values, contribute to the biosurfactants instability in those conditions.<sup>14</sup>

Regarding the effect of salinity, both surface tension and emulsifying activity remained almost constant for NaCl concentrations up to 50 mg ml<sup>-1</sup> (Fig. 2B). In this case, the emulsifying activity was more affected at high NaCl concentrations than the surface tension. A surface tension value of 45.6 mN m<sup>-1</sup> was obtained with the highest NaCl concentration tested (200 mg ml<sup>-1</sup>), whereas NaCl concentrations higher than 50 mg ml<sup>-1</sup> led to an abrupt loss of emulsifying activity.

Furthermore, the biosurfactant was found to be very stable when exposed to high temperatures, since the surface tension value (42.8 mN m<sup>-1</sup>) and the emulsifying index (60%) remained constant after incubation at 121 °C for 20 minutes. On the contrary, Gudiña *et al.*<sup>14</sup> reported some loss of activity for the

biosurfactant produced by *Lactobacillus paracasei* ssp. *paracasei* A20 when it was incubated at 60 °C for 120 hours; and Portilla-Rivera *et al.*<sup>27</sup> reported that biosurfactants produced by *L. pentosus* CECT4023 were only stable in the range of temperatures between 10 and 40 °C.

#### 2.4. Fourier transform infrared spectroscopy (FTIR)

The molecular characterization of the cell-bound biosurfactant produced by *L. agilis* CCUG31450 in MRS medium was performed using Fourier Transform Infrared spectroscopy (FTIR), which is a useful technique to identify different functional groups in mixtures of unknown composition. This technique has been widely used to obtain a preliminary characterization of complex biosurfactant mixtures produced by different lactobacilli. The most relevant peaks obtained (Fig. 3) are described below.

The broad peak at 3250 cm<sup>-1</sup> indicates the presence of OH and NH groups, characteristic of proteins. The big and well defined peaks at 1643 cm<sup>-1</sup> and 1535 cm<sup>-1</sup> correspond to C=O stretching (AMI protein band) and N-H bending (AMII protein band), respectively. These groups suggest the presence of proteins in the sample. The small peak at 2925 cm<sup>-1</sup>, corresponding to C-H stretching and the very small peak at 1460 cm<sup>-1</sup>, corresponding to CH (scissor), indicate the presence of bonds occurring in aliphatic chains. At 1060 cm<sup>-1</sup>, a large, broad and well defined peak corresponding to the PII polysaccharide band typical of bond vibrations in the C-O-C group was observed. Moreover, a small but well defined peak was observed at 1394 cm<sup>-1</sup> (1000–1300 cm<sup>-1</sup>), corresponding to C-O stretching in sugars.

The spectrum obtained for the cell-bound biosurfactant produced by *L. agilis* CCUG31450 suggests that it must be a glycoprotein, and it is closely related to those obtained by other authors for biosurfactants produced by *L. pentosus* CECT4023, *Lactococcus lactis* 53, *L. paracasei* ssp. *paracasei* A20, *L. plantarum* CFR2194 or *Lactobacillus fermentum* ATCC9338.<sup>3,9,10,28</sup>

The existing information on the chemical structure of biosurfactants produced by lactobacilli is very limited, mainly due to their complexity. Some of them (*i.e.* *Lactobacillus fermenti* 126, *Lactobacillus rhamnosus* CCM1825, *Lactobacillus acidophilus* DSM20079, *L. lactis* 53, *Streptococcus thermophilus* A) have been partially characterized as multi-component mixtures, consisting of protein and polysaccharides associated to phosphate groups.<sup>10,11,16,17,20,21,29</sup> In other cases, they have been found to be glycolipids (*Streptococcus mitis* BA and *S. mitis* BMS)<sup>30</sup> or a mixture of polysaccharides and glycolipids (*S. thermophilus* B).<sup>26</sup>

#### 2.5. Alternative culture media

In order to reduce the production costs, cheese whey was evaluated as an alternative substrate for biosurfactant production by *L. agilis* CCUG31450. The best results regarding biosurfactant production were obtained with a medium containing cheese whey at a concentration of 100 g l<sup>-1</sup>. This medium contained, per liter, 34.9 ± 0.8 g of carbohydrates and 8.0 ± 0.9 g of protein. The cultures were performed at 37 °C and 100 rpm for 120 hours. Similarly to the results obtained with MRS medium, the surface tension of the culture medium supernatants remained constant along the fermentation, thus no extracellular biosurfactants were produced. However, the production of cell-bound biosurfactants was confirmed since the surface tension of the PBS after the extraction of cell-bound biosurfactants at the end of the fermentation was reduced up to 49.3 mN m<sup>-1</sup>. The amount of crude biosurfactant recovered was 959 ± 9.9 mg l<sup>-1</sup>, which is about 11 times the amount of biosurfactant produced in MRS medium. However, this biosurfactant exhibited a higher cmc value (13 mg ml<sup>-1</sup>) when compared with the one produced in MRS medium.

Cheese whey, containing high levels of lactose, protein, organic acids and vitamins, has been described as a good substrate for biosurfactant production by LAB. Rodrigues *et al.*<sup>11</sup> reported the production of 1400 mg of biosurfactant per liter by *L. pentosus* CECT4023 using cheese whey as culture medium. Moreover, *L. lactis* 53 and *S. thermophilus* A produced higher

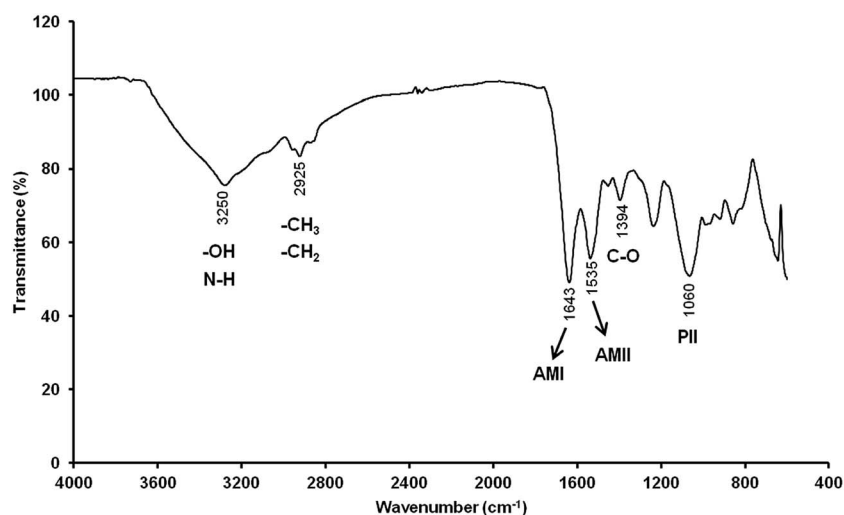


Fig. 3 FTIR spectrum obtained with the cell-bound biosurfactant produced by *L. agilis* CCUG31450 in MRS medium.

amounts of biosurfactant using cheese whey supplemented with peptone or yeast extract when compared with the corresponding synthetic media.<sup>31</sup> The results herein obtained confirm that cheese whey can be used as an alternative culture medium for biosurfactant production by *L. agilis* CCUG31450. The amount of biosurfactant produced with this medium is considerably higher when compared with the conventional MRS medium, thus it can contribute to reduce the production costs of this biosurfactant and increase its competitiveness in the market.

## 2.6. Anti-adhesive activity

The anti-adhesive activity of the cell-bound biosurfactant produced by *L. agilis* CCUG31450 was evaluated. The biosurfactant exhibited a considerable anti-adhesive activity against *S. aureus*, even at the lowest concentration tested (1 mg ml<sup>-1</sup>), which was higher than the reported for biosurfactants produced by other LAB at the same concentrations (Table 3). However, no anti-adhesive activity could be observed against the other microorganisms studied.

Studies performed with biosurfactants produced by different LAB demonstrated their ability to reduce or inhibit the adhesion of pathogenic microorganisms (including Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi) to glass, polystyrene, silicone rubber, surgical implants, voice prostheses, metallic surfaces and epithelial cell surfaces.<sup>6,7,9,16,17,20,22,26,28–30,32–36</sup> Consequently, biosurfactant treatments can provide an optional way for controlling biofilm development and inhibit the adhesion of pathogens, reducing the use of synthetic drugs and chemicals.

The anti-adhesive activity exhibited by the cell-bound biosurfactant produced by *L. agilis* CCUG31450 against *S. aureus* (a common cause of community and hospital acquired infections) opens future prospects regarding its possible application to

reduce the adhesion of *S. aureus* on different surfaces and prevent biofilm formation and microbial colonization on different materials, such as medical insertional devices.

Usually the effect of biosurfactants on the adhesion of undesirable microorganisms is attributed to modifications in bacterial–surface interactions, like changes in the surface tension and bacterial cell-wall charge, or changes in the surface properties, which affect the adhesion and detachment of microorganisms. However, in most cases the precise mechanisms of such activity have not been fully explained, and more complex mechanisms can be involved.

Heinemann and co-workers<sup>33</sup> isolated a collagen-binding protein from the crude biosurfactant produced by *L. fermentum* RC-14 which is involved in the adhesion of lactobacilli to the epithelium. At the same time, that protein inhibited the adhesion of uropathogenic bacteria probably by competition with the pathogens for binding to host sites. Likewise, that protein was proposed as the responsible for inhibiting the adhesion of *S. aureus* to biosurfactant-treated surgical implants *in vivo* probably due to the competition with extracellular matrix-binding proteins of *S. aureus* for binding to host sites.<sup>32</sup> Similar collagen-binding proteins were identified in biosurfactants produced by *L. rhamnosus* and *Lactobacillus casei* strains<sup>37</sup> and it is supposed that these proteins are involved in the biosurfactants anti-adhesive activity.

Biosurfactants released by *L. fermentum* ATCC9338 and *L. acidophilus* DSM20079 reduced the adhesion and biofilm formation of *Streptococcus mutans*, one of the main organisms responsible for dental caries, due to a complex process that alters the expression of genes in the target microorganism.<sup>28,29</sup> Therefore, the exact mechanisms involved in the protective effect of biosurfactants still remain to be clarified and a better knowledge of their composition is required to completely understand their activities.

## 2.7. Antimicrobial activity

The cell-bound biosurfactant produced by *L. agilis* CCUG31450 exhibited antimicrobial activity at the highest concentration tested (5 mg ml<sup>-1</sup>) against *S. aureus* (20.0 ± 8.1%), *P. aeruginosa* (13.5 ± 0.7%) and *S. agalactiae* (10.7 ± 2.7%). However, no antimicrobial activity was observed against *E. coli* and *C. albicans*.

Usually, the beneficial effect of biosurfactants produced by LAB is due to their anti-adhesive activity. However, some few studies on their antimicrobial activity have been reported. Purified fractions of cell-bound biosurfactants produced by *S. thermophilus* A and *L. lactis* 53 exhibited significant antimicrobial activity against *Staphylococcus epidermidis*, *Streptococcus salivarius*, *S. aureus*, *Candida tropicalis* and *C. albicans* at concentrations higher than 2.5 mg ml<sup>-1</sup>.<sup>10,11</sup> The crude biosurfactant produced by *L. paracasei* ssp. *paracasei* A20 exhibited growth inhibition percentages (at a concentration of 3 mg ml<sup>-1</sup>) against *S. aureus*, *P. aeruginosa* and *S. agalactiae* of 63.1 ± 0.4, 50.9 ± 0.8 and 51.5 ± 0.8%, respectively.<sup>8</sup> Also, the crude biosurfactant produced by *L. plantarum* CFR2194 completely inhibited the growth of *E. coli*, *S. aureus* and *Yersinia*

**Table 3** Anti-adhesive activity of cell-bound biosurfactants produced by *Lactobacillus agilis* CCUG31450 against *Staphylococcus aureus* (results represent the average of triplicate experiments ± standard deviation) compared with the results obtained with biosurfactants produced by different LAB

	Biosurfactant concentration (mg ml <sup>-1</sup> )	Anti-adhesive activity (%)	Reference
<i>L. agilis</i> CCUG31450	10.0	64.6 ± 0.7	This study
	5.0	62.7 ± 1.2	
	2.5	56.3 ± 0.6	
	1.0	50.3 ± 3.2	
<i>L. paracasei</i> ssp. <i>paracasei</i> A20	12.5	62.0 ± 1.1	8
	6.2	51.1 ± 1.4	
	3.1	10.3 ± 1.2	
<i>L. plantarum</i> CFR 2194	4.0	25.9 ± 0.1	9
	10.0	37	
<i>L. lactis</i> 53	10.0	37	10
	5.0	34	
	2.5	13	
<i>S. thermophilus</i> A	10.0	42 ± 0	11
	5.0	22 ± 1	
	2.5	11 ± 1	

*enterocolitica* at a concentration of 25 mg ml<sup>-1</sup>.<sup>9</sup> The extracellular biosurfactant produced by a *L. lactis* strain partially inhibited the growth of *E. coli* and *S. aureus* strains as determined by agar well diffusion assays, although the biosurfactant concentration used in these assays was not provided.<sup>24</sup>

## 3. Experimental

### 3.1. Strains and standard culture conditions

The following strains were screened for biosurfactant production: *Lactobacillus animalis* ATCC35046, *Lactobacillus buchneri* ATCC4005, *Lactobacillus hamsteri* ATCC43851 and *Lactobacillus intestinalis* ATCC49335, obtained from the American Type Culture Collection (USA); *Lactobacillus agilis* CCUG31450, obtained from the Culture Collection of University of Gothenburg (Sweden). All strains were grown in DeMan, Rogosa and Sharpe (MRS) medium (OXOID, England) at 37 °C and aerobic conditions for different time intervals.

For the antimicrobial and anti-adhesive assays, the following strains, kindly provided by the Faculty of Pharmacy, University of Porto (Portugal), were used: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Candida albicans*. These strains were grown overnight in Luria-Bertani (LB) medium at 37 °C and aerobic conditions. The composition of LB medium was (g l<sup>-1</sup>): NaCl 10; tryptone 10; yeast extract 5; pH 7.0.

All strains were stored at -80 °C in the appropriate medium supplemented with glycerol (20% (v/v)) until use. Whenever required, frozen stocks were streaked on agar plates and incubated overnight at 37 °C for further culturing.

### 3.2. Screening of biosurfactant-producing strains

Biosurfactant production by the lactobacilli strains was conducted in 500 ml flasks containing 200 ml of MRS medium. Each flask was inoculated with 2 ml of a pre-culture grown overnight at 37 °C and 100 rpm. Pre-cultures were prepared by transferring a single colony from an agar plate into 10 ml of MRS medium. The cultures were incubated at the same conditions as the corresponding pre-cultures.

In order to evaluate bacterial growth and excreted biosurfactant production, samples (4 ml) were taken at different time points during the fermentation. Biomass concentration was determined by measuring the optical density at 600 nm. After measuring the cell growth, the samples were centrifuged (9000 rpm, 20 min) and the cell-free supernatants were used to measure the surface tension as described below.

Biosurfactants can be excreted to the culture medium (extracellular biosurfactants) or remain attached to the cell wall (cell-bound biosurfactants). LAB have been reported to mainly produce cell-bound biosurfactants. The surface tension measured in the cell-free supernatants gives an indication of the extracellular biosurfactants produced; while the production of cell-bound biosurfactants is determined at the end of the fermentation after their recovery (as described below) and further measurement of the surface tension values. All the cultures were maintained until the maximum biosurfactant

production was achieved (*i.e.* until the surface tension values remained constant).

### 3.3. Cell-bound biosurfactants recovery

At the end of the fermentation, the cells were harvested by centrifugation (9000 rpm, 20 min), washed twice with the same volume of demineralized water, and resuspended in phosphate-buffered saline (PBS: 10 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> and 150 mM NaCl, with pH adjusted to 7.0), as described by Gudiña *et al.*<sup>14</sup> A volume of 15 ml of PBS buffer was used per 100 ml of culture. The cell-suspension was left at room temperature (20 °C) for 2 h with gentle stirring for cell-bound biosurfactants release. Subsequently, the cells were removed by centrifugation (9000 rpm, 20 min) and the remaining supernatant liquid was filtered through a 0.2 µm pore-size filter (Whatman, GE Healthcare, UK). The solution containing the cell-bound biosurfactants was dialyzed against demineralized water at 4 °C in a Cellu-Sep® membrane (molecular weight cut-off 6000–8000 Dalton; Membrane Filtration Products, Inc., USA) for 48 h and further freeze-dried. The biosurfactants were weighed and stored at -20 °C for subsequent studies. To confirm the biosurfactant production, the surface tension was routinely measured (as described below) during the extraction process.

### 3.4. Surface-activity determination

The surface tension of the culture broth supernatants, biosurfactant solutions and the PBS extracts was measured using the Ring method as described elsewhere.<sup>14</sup> A KRÜSS K6 Tensiometer (KRÜSS GmbH, Germany) equipped with a 1.9 cm De Noüy platinum ring was used. In order 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).

### 3.5. Emulsifying activity determination

Emulsifying activity was determined by the addition of 2 ml of *n*-hexadecane to the same volume of biosurfactant solutions or PBS extracts in glass test tubes, as described elsewhere.<sup>4,5</sup> The tubes were mixed with vortex at high speed for 2 min and subsequently incubated at 25 °C for 24 h. The emulsification indexes ( $E_{24}$ , %) were calculated as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm). All the emulsification indexes were performed in triplicate.

### 3.6. Critical micelle concentration (cmc)

Critical micelle concentration (cmc) is defined as the concentration of an amphiphilic compound in solution at which the formation of micelles is initiated. Cell-bound biosurfactants produced by *L. agilis* CCUG31450 were dissolved in PBS buffer at different concentrations (from 0.05 to 50 mg ml<sup>-1</sup>), and the surface tension of each sample was measured as described above. The cmc was determined by plotting the surface tension as a function of the logarithm of biosurfactant concentration, and it was found at the point of intersection between the two

lines that best fit through the pre- and post-cmc data, as described elsewhere.<sup>14</sup> All the measurements were done in triplicate.

### 3.7. Stability studies

The applicability of biosurfactants can be conditioned by their stability to different environmental parameters. Therefore, the effect of pH, temperature and salinity on the activity of the biosurfactant produced by *L. agilis* CCUG31450 in MRS medium was determined. Stability studies were performed using the cell-bound freeze-dried biosurfactant dissolved in demineralized water at a concentration equivalent to the cmc. In order to assess the effect of salinity on biosurfactant activity, the biosurfactant solutions were supplemented with different NaCl concentrations (from 10 to 200 g l<sup>-1</sup>). The surface tension and the emulsifying activity were measured as described above and compared with the corresponding values without addition of NaCl. The stability of the biosurfactant to high temperatures was also determined. For that purpose, the biosurfactant solutions were incubated at 121 °C for 20 min and allowed to cool to room temperature. Surface tension and emulsification indexes were measured and compared to the corresponding values before the heat treatment. The pH stability was studied by adjusting the biosurfactant solutions to different pH values (2–13) using HCl or NaOH solutions, and measuring the surface tension and the emulsification indexes as described above. All the experiments were carried out in triplicate.

### 3.8. Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy in the attenuated total reflection mode (FTIR-ATR) was used to study the molecular composition of the cell-bound biosurfactant produced by *L. agilis* CCUG31450 in MRS medium. The spectra were recorded on a JASCO FTIR-4100 Spectrometer and were obtained from 64 scans with a resolution of 8 cm<sup>-1</sup> in the range of 600–4000 cm<sup>-1</sup>.

### 3.9. Alternative culture media

Biosurfactant production by *L. agilis* CCUG31450 was studied using cheese whey powder (supplied by a local dairy industry, Quinta dos Ingleses, S.A.) as an alternative substrate to replace the synthetic medium MRS. Cheese whey media (CWM) were prepared by dissolving cheese whey in demineralized water at different concentrations (between 50 and 150 g l<sup>-1</sup>). The solutions were sterilized (121 °C for 15 minutes) and the precipitates (*i.e.* proteinaceous material) were removed by centrifugation (9000 rpm, 40 min) and discharged. The supernatants obtained were adjusted to pH 6.2 and used as culture media. The CWM were analyzed for total carbohydrates and protein contents using the phenol-sulfuric and Lowry methods, respectively.<sup>38,39</sup>

### 3.10. Anti-adhesive assays

The anti-adhesive activity of the cell-bound biosurfactant produced by *L. agilis* CCUG31450 against *E. coli*, *P. aeruginosa*, *S. aureus*, *S. agalactiae* and *C. albicans* was determined as

described by Gudiña *et al.*<sup>8</sup> Briefly, the wells of a sterile 96-well flat-bottomed plastic tissue culture plate (Orange Scientific, Belgium) were filled with 200 µl of biosurfactant solutions prepared in demineralized water at different concentrations (between 1 and 10 mg ml<sup>-1</sup>). The plates were incubated for 18 h at 4 °C and subsequently washed twice with PBS buffer. An aliquot of 200 µl of a washed bacterial suspension in PBS adjusted to an optical density (600 nm) of 0.6 was added to each well and incubated for 24 h at 4 °C. Unattached microorganisms were removed by washing the wells twice 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. After that, the plates were stained for 5 min with 200 µl of 33% crystal violet (used for Gram staining) per well. Excess stain was rinsed out by washing the wells three times with PBS. Subsequently, the plates were air dried, the dye bound to the adherent microorganisms was re-solubilized with 200 µl of 33% (v/v) glacial acetic acid per well, and the optical density of each well was measured at 595 nm. The microbial inhibition percentages at different biosurfactant concentrations for each microorganism were calculated as:

$$\% \text{ microbial inhibition}_c = [1 - (\text{OD}_c/\text{OD}_0)] \times 100,$$

where OD<sub>c</sub> represents the optical density of the well with a biosurfactant concentration *c*, and OD<sub>0</sub> is the optical density of the control well (without biosurfactant). Triplicate assays were performed for each microorganism and biosurfactant concentration.

### 3.11. Antimicrobial assays

The antimicrobial activity of the cell-bound biosurfactant produced by *L. agilis* CCUG31450 against the same microorganisms used in the anti-adhesive assays was determined in 96-well plastic tissue culture plates (Orange Scientific, Belgium) at different concentrations, as described by Gudiña *et al.*<sup>8</sup> Briefly, the freeze-dried biosurfactant was dissolved in LB medium at a concentration of 5 mg ml<sup>-1</sup>, and sterilized by filtration through a 0.2 µm pore-size filter (Whatman, GE Healthcare, UK). Subsequently, 250 µl of LB medium containing the biosurfactant were placed into the first column of the 96-well microplate, and 125 µl of LB medium in the remaining wells. After that, 125 µl from the first column were transferred to the second column and mixed. Serially, 125 µl were transferred to the subsequent wells, discarding 125 µl of the mixture in the tenth column. This process results in two-fold serial dilutions of the biosurfactant in the first 10 columns. Columns 11 and 12 did not contain biosurfactant and served as growth and negative controls, respectively. All the wells (except for the 12<sup>th</sup> column) were inoculated with 5 µl of a pre-culture of the corresponding microorganism grown overnight in LB medium at 37 °C and diluted to an optical density (600 nm) of 0.3. The microplates were covered and incubated for 24 h at 37 °C. After 24 h of incubation, the optical density of each well (at 600 nm) was measured. The growth inhibition percentages at different biosurfactant concentrations for each microorganism were calculated as:

$$\% \text{ growth inhibition}_c = [1 - (\text{OD}_c/\text{OD}_0)] \times 100,$$

where  $\text{OD}_c$  represents the optical density of the well with a biosurfactant concentration  $c$ , and  $\text{OD}_0$  is the optical density of the control well (without biosurfactant). Triplicate assays were performed for each microorganism and biosurfactant concentration.

## 4. Conclusion

In this work, the biosurfactant production by *L. agilis* CCUG31450 was reported for the first time. A preliminary chemical characterization by FTIR indicated that this biosurfactant is a glycoprotein. The biosurfactant exhibited higher anti-adhesive activity against *S. aureus* when compared with biosurfactants produced by other lactobacilli. It also showed antimicrobial activity against *S. aureus*, *S. agalactiae* and *P. aeruginosa*, which is an unusual property among biosurfactants produced by LAB. Furthermore, the use of cheese whey as an alternative culture medium increased the biosurfactant production about 11 times as compared with the conventional MRS medium, which can contribute to the economic sustainability of the production process. The antimicrobial and anti-adhesive activities unveiled by this biosurfactant against *S. aureus* suggest its possible use in several biomedical applications.

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