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Research paper

Liposomes containing biosurfactants isolated from *Lactobacillus gasseri* exert antibiofilm activity against methicillin resistant *Staphylococcus aureus* strains

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ABSTRACT

Staphylococcus aureus is the major causative agent of skin and soft tissue infections, whose prevention and treatment have become more difficult due to the emergence of antibiotic-resistant strains. In this regard, the development of an effective treatment represents a challenge that can be overcome by delivering new antibiofilm agents with appropriate nanocarriers. In this study, a biosurfactant (BS) isolated from *Lactobacillus gasseri* BC9 and subsequently loaded in liposomes (LP), was evaluated for its ability to prevent the development and to eradicate the biofilm of different methicillin resistant *S. aureus* (MRSA) strains. BS from *L. gasseri* BC9 was not cytotoxic and was able to prevent formation and to eradicate the biofilm of different MRSA strains. BS loaded liposomes (BS-LP) presented a mean diameter (lower than 200 nm) suitable for topical administration and a low polydispersity index (lower than 0.2) that were maintained over time for up 28 days. Notably, BS-LP showed higher ability than free BS to inhibit *S. aureus* biofilm formation and eradication. BS-LP were loaded in lyophilized matrices able to quickly dissolve (dissolution time lower than 5 s) upon contact with exudate, thus allowing vesicle reconstitution. In conclusion, in this work, we demonstrated the antibiofilm activity of *Lactobacillus*-derived BS and BS-LP against clinically relevant MRSA strains. Furthermore, the affordable production of lyophilized matrices containing BS-LP for local prevention of cutaneous infections was established.

1. Introduction

Staphylococcus aureus is one of the major human pathogens causing a wide range of diseases including skin infections, bacteraemia and infective endocarditis [1]. *S. aureus* strains are featured by genetic diversity [2] and possess the ability to form biofilm and to acquire antibiotic resistance [3,4]. These characteristics have contributed to an increase in the number of *S. aureus* infections [5]. In particular, methicillin resistant *S. aureus* (MRSA) strains usually possess multiple antibiotic resistance [6] with the consequence of reducing the number of applicable therapies [7]. In addition, the development of some *Staphylococcus* resistance mechanisms appears to be intrinsic to biofilm growth mode [8,9].

Currently, the increase of MRSA in community-acquired skin and soft tissue infections [10] poses a significant health burden [11], and novel strategies are especially needed for the prevention of chronic infections due to biofilm formation. Among the different strategies [12,13], the use of novel and natural antibiofilm agents delivered by appropriate nanocarriers represents a promising approach for biofilm related-infection treatment and prevention [14].

Biosurfactants (BS) are amphiphilic compounds, produced by microorganisms, which can be localized on their cell surface, or secreted extracellularly. These molecules exert critical functions in the survival of their producing microorganisms by facilitating nutrient transport, interfering in microbe-host interactions and quorum sensing mechanisms, or by acting as antimicrobial, antiadhesive and antibiofilm agents

Abbreviations: BS, biosurfactant; LP, liposomes; BS-LP, biosurfactant loaded liposomes

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[15–17]. BS are considered "green" compounds, owing to their natural origin, biodegradability and low toxicity [18] and have been widely studied for applications in food and cosmetics industries, hydrocarbon dissolution and bioremediation [16,18]. During the past decade, their antibacterial, antifungal and antiviral activities have made them appropriate candidates for the prevention and treatment of infection diseases [19–21]. In particular, BS produced by human lactobacilli represent possible antipathogenic molecules delivered by probiotic and commensal bacterial strains. The antimicrobial and antiadhesive properties of BS isolated from *Lactobacillus* spp. were previously demonstrated [22,23], but only few papers reported data concerning their direct antibiofilm activity [17,24,25].

In the last decades, different novel delivery systems have been developed for the prevention and treatment of biofilm related infections, including carriers of nanometric size. Indeed, nanocarriers such as liposomes, solid lipid nanoparticles, polymeric nanoparticles and micelles, and dendrimers are considered safe systems that can improve treatment efficacy [14]. In particular, liposomes (LP) with appropriate size and characteristics of the phospholipid bilayer penetrate the extracellular matrix of biofilms thus providing efficient delivery [14]. Furthermore, the release of the active molecules to the surface or the interior of the microorganisms can be improved by using nanocarriers whose structure resembles the cell membrane one [26]. For skin and soft tissue infections, topical formulations able to lie at the application site for suitable time periods, are generally preferred. For this reason, topical antibiofilm and antimicrobial LP suspensions have been often formulated in suitable vehicles, such as viscous solutions or hydrogels [27]. At the same time, LP suspensions can be lyophilized in the presence of appropriate excipients thus obtaining solid matrices with improved characteristics [28]. In fact, lyophilized matrices can ensure LP stability as well as prevent the release of the active molecules during the storage period. Moreover, upon contact with local exudate, they can quickly provide the formation of a more or less viscous solution containing the active LP.

In order to highlight the potential application of biologically active lactobacilli metabolites, the present study aims to investigate the antibiofilm activity of BS isolated from a *Lactobacillus gasseri* strain of vaginal origin, against some clinically isolated MRSA strains. The innovative approach of this research consisted in the association of a lactobacilli BS with a nanometric carrier (BS in liposomes; BS-LP) aiming to contrast *S. aureus* biofilm development. A further goal was the preparation of an easy-to-use final dosage form suitable for the application at the skin level, that could be envisaged in preclinical studies.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Lactobacillus gasseri BC9, previously isolated from a human vaginal swab [29], was cultured in de Man, Rogosa and Sharpe (MRS) broth supplemented with 0.05% L-cysteine, at 37 °C for 24 h in anaerobic jars containing GasPak EZ.

Staphylococcus aureus subsp. *aureus* ATCC 29213 and *S. aureus* clinical isolates (Table 1) were used as test microorganisms to evaluate the antibiofilm activities of BS produced by *L. gasseri* and BS encapsulated in vesicles. *S. aureus* strains 2–88 were isolated from positive blood cultures submitted to the Microbiology Unit of St. Orsola-Malpighi University Hospital of Bologna (Italy), for routine diagnostic procedures, from patients with suspected sepsis or bacteraemia. The bacterial identification at the species level was obtained by means of a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), using a Bruker Microflex MALDI-TOF MS instrument (Bruker Daltonics) [30]. Antimicrobial susceptibility testing (AST) was performed by broth microdilution method using the MicroScan WalkAway plus System (Beckman Coulter srl, Milan, Italy) and EUCAST criteria were used for the interpretation of results and for the definition of antimicrobial susceptibility category. Strains 2, 7, 45, 83, and 86 were classified as MRSA and were characterized by different resistance pattern, whereas strain 88 was resistant to gentamicin only (Table 1). All *S. aureus* strains were grown in Nutrient broth (NB) or Nutrient agar plates (NA), aerobically for 24 h at 37 °C.

The culture media and GasPak EZ were supplied by Becton Dickinson and Company (Sparks, MD).

2.2. Biosurfactant production and isolation

Cell-bound BS were isolated from *L. gasseri* BC9 following the protocol already published [31]. Briefly, 40 mL of an overnight culture of *L. gasseri* BC9 were inoculated in 400 mL of MRS broth and allow to grow for 24 h, and cell pellets was harvested by centrifugation (10000*g*, 10 min). Cells were washed twice in sterile water, re-suspended in 150 mL of PBS and gently stirred at room temperature for 2 h to release the cell-bound BS. Afterwards the suspension was centrifuged and the supernatant was filtered through a 0.22 µm pore size filter. For BS purification, cell-free supernatant was then subjected to dialysis against demineralized water in a Cellu-Sep© membrane (molecular weight cut-off 6000–8000 Da; Spectra/Por 2 dialysis membrane Spectrum Laboratories Inc., USA) for 24 h at room temperature, and lyophilized at 0.01 atm and −45 °C (Christ Freeze Dryer ALPHA 1–2, Milan, Italy). As reported in [31], BS produced by *L. gasseri* BC9 was composed by peptide-like molecules containing hydrocarbon chains and possessed a high surface activity together with a low critical micelle concentration.

2.3. Cytotoxicity of BS on human and murine fibroblasts

Normal human adult fibroblast (HDFa) and the murine fibroblast cell line NIH/3T3 were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were seeded at 1.5×10^4 cells/well in a 96-well culture plastic plate (Orange Scientific, Braine-l'Alleud, Belgium) and cultured in 5% CO_2 at 37 °C in RPMI 1640 medium (Labtek Eurobio, Milan, Italy), supplemented with 10% FBS (Euroclone, Milan, Italy) and 2 mM L-glutamine (Sigma-Aldrich, Milan, Italy). After 24 h of growth, cells were exposed for additional 24 or 48 h to increasing concentrations of BS (0.05–10 mg/mL) solubilized in RPMI 1640 medium. On the day of measurement, the culture medium was replaced with 0.1 mL of 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) dissolved in PBS at the concentration of 0.2 mg/mL, and samples were incubated for 2 h at 37 °C. To dissolve the blue-violet formazan salt crystals formed, 0.1 mL of isopropyl alcohol was added to each well and incubated for 20 min. The absorbance at 570 nm was measured using a multiwell plate reader (Wallac Victor2, PerkinElmer), viability was compared with that of untreated cells, used as controls.

2.4. Liposome preparation and characterization

Biosurfactant loaded liposomes (BS-LP) were prepared by the ethanol injection method [32]. Briefly, Phospholipon 90G also known as L-α-phosphatidylcholine (PC) (Phospholipon 90G from soybean lecithin, containing not less than 94% PC, was a kind gift from Lipoid GmbH, Ludwigshafen, Germany) and BS (weight ratio of 3:1) were dissolved in 5 mL of ethanol 96% (Carlo Erba, Milan, Italy). The ethanol solution was then injected into 10 mL of water under continuous stirring at 200 rpm at injection rate of 1 mL/min. Ethanol was removed by means of rotary evaporation (Buchi Rotavapor R-200, Flawil, Switzerland) under vacuum at 60 °C for 10 min with stirring at 210 rpm. Final concentrations of PC and BS were 7.5 mg/mL e 2.5 mg/mL, respectively. Liposomes without BS were prepared as control formulation (control-LP). All formulations were stored at 4–8 °C.

The prepared LP were characterized in terms of size, polydispersity, zeta potential and stability over time. BS-LP and control-LP size

Table 1
S. aureus clinical isolates, origin of sepsis and antibiotic resistance pattern.

S. aureus strain	Origin of sepsis	Antimicrobial resistance pattern: class (agent tested)
2	Cutaneous (and respiratory) infection	Beta-Lactams (oxacillin and cefoxitin), Macrolides (erythromycin), Lincosamides (clindamycin), Fluoroquinolones (levofloxacin), Aminoglycosides (gentamicin), Ansamycins (rifampicin)
7	Primary bacteremia	Beta-Lactams (oxacillin and cefoxitin), Fluoroquinolones (levofloxacin), Ansamycins (rifampicin)
45	Unknown	Beta-Lactams (oxacillin and cefoxitin), Macrolides (erythromycin), Lincosamides (clindamycin), Fluoroquinolones (levofloxacin)
83	Central venous catheter infection	Beta-Lactams (oxacillin and cefoxitin), Fluoroquinolones (levofloxacin)
86	Unknown	Beta-Lactams (oxacillin and cefoxitin), Macrolides (erythromycin), Lincosamides (clindamycin), Tetracyclines (tetracycline), Fusidane (fusidic acid)
88	Unknown	Aminoglycosides (gentamicin)

distribution was measured by PCS (Photon-Correlation Spectroscopy) using a Brookhaven 90-PLUS instrument (Brookhaven Instruments Corp., Holtsville, NY) with He-Ne laser beam at a wavelength of 532 nm (scattering angle of 90°). Suspensions were diluted (1:1000; v/v) in ultrapure water (18.2 MΩ cm, MilliQ apparatus by Millipore, Milford, MA). Zeta potential measurements were carried out at 25 °C on a Malvern Zetasizer 3000 HS instrument (Malvern Panalytical Ltd., Malvern, UK), after the same dilution. LP size and polydispersity were evaluated immediately after the preparation and during storage (28 days at 4–8 °C).

2.5. Biofilm dispersal and inhibition of biofilm development

BS, BS-LP and control-LP were prepared by dissolving lyophilized BS or mixing LP suspensions in NB, and sterilized by filtration (0.45 μm; GVS Abluo Syringe Filter, Merck KGaA, Darmstadt, Germany). Biofilm dispersal was evaluated as previously described [17], with some modifications. Briefly, *S. aureus* strains were grown on NA plates at 37 °C, overnight. Biomass from the plates was resuspended in NB to a final optical density $(OD)_{600}$ of 0.03 and 150 μL were inoculated in a 96-well culture plate (Corning Inc., Pisa, Italy). The multi-well plates were incubated at 37 °C under shaking (100 rpm) for 24 h. Afterwards, the liquid culture was removed, leaving only *S. aureus* biofilm in the well, and 150 μL of BS, BS-LP or control-LP suspensions were added to each well. Control wells were supplied with NB only. Plates were further incubated at 37 °C with shaking (100 rpm) for 24 h. *S. aureus* biofilm quantification was performed using crystal violet assay. Specifically, biofilms were washed twice with 180 μL of Phosphate Buffer Saline pH 7.4 (PBS) (2.38 g/L Na_2HPO_4; 0.19 g/L KH_2PO_4; 8 g/L NaCl), fixed with 180 μL of 100% ethanol for 5 min and stained with 180 μL of 0.41% crystal violet (w/v) in 12% ethanol for 5 min. Samples were then washed 3 times with 200 μL of sterile ultrapure water to remove excess stain. The *S. aureus* biofilm quantification was performed through the addition of 200 μL of 100% ethanol to each well and reading OD_{595} using an EnSpire Multimode Plate Reader (PerkinElmer Inc., Waltham, MA). *S. aureus* biofilm dispersal was expressed in percentage relative to the control wells (without BS or LP), based on the average of three biological replicates, following the Eq. (1).

Inhibition of *S. aureus* biofilm development was evaluated following a protocol similar to biofilm dispersal assay. Briefly, *S. aureus* biomass grown overnight on NA plates was directly re-suspended in NB containing BS, BS-LP or control-LP to reach a final OD_{600} of 0.03. 150 μL of each suspension were inoculated in a 96-well culture plate. Control wells contained *S. aureus* suspensions in NB only, without the supply of BS or LP formulations. The multi-well plates were incubated at 37 °C with shaking (100 rpm) for 24 h, and biofilm quantification was performed by crystal violet assay, as previously described. Inhibition of *S. aureus* biofilm development was expressed in percentage relative to the control wells, based on the average of three biological replicates, following Eq. (1).

$$\text{Biofilm Dispersal/Inhibition of Biofilm Development (\%)} = [1 - (\text{mean } OD_{595\ \text{sample}} / \text{mean } OD_{595\ \text{control}})] \times 100 \quad (1)$$

2.6. Preparation and characterization of lyophilized matrices

In order to obtain fast dissolving matrices able to restore the vesicles immediately after application, LP prepared in the presence of suitable excipients were lyophilized in blister dies. Gelatin (Type B Gelatin from bovine skin, Sigma-Aldrich, Milan, Italy) was selected as matrix forming polymer and glycerol (Glycerol 98.8%, Sigma-Aldrich, Milan, Italy) as lyoprotectant. For the preparation of the matrices, LP were obtained by injecting the ethanol solution of Phospholipon 90G and BS (5 mL) into water (10 mL) containing gelatin (1%, w/w) or gelatin (1%, w/w) and glycerol (0.125%, w/w). After the removal of ethanol by rotary evaporation, PC and BS concentrations were the same as in Section 2.4. Aliquots (500 μL) of the prepared suspensions were placed in the dies (13 mm diameter) of a plastic blister (Farmalabor Tech., Barletta-Andria-Trani, Italy), subsequently frozen overnight at −20 °C, lyophilized at 0.01 atm and −45 °C and stored at room temperature.

The matrices were weighted and characterized in terms of thickness and diameter by means of an Electronic Digital Caliper (1367 E 2900, Shanghai ShangErBo Import & Export Co., Shanghai, China). Moreover, the impact of gelatin and glycerol on the dissolution rate of the matrices, as well as on the reconstitution of LP, were evaluated. The time for complete dissolution was detected by visual observation, after immersion of the matrices in 5 mL of Simulated Wound Fluid pH 7.5 (SWF) (bovine serum albumin 2.0 g/L; calcium chloride 2.20 g/L; sodium chloride 23.4 g/L; 2-amino-2-(hydroxymethyl)-1,3-propanediol 6.06 g/L) [33]. Furthermore, size and polydispersity index (PDI) of the LP were measured before lyophilization and after reconstitution in water with a gentle mixing by a horizontal shaker (Certomat H, B-Braun spa, Milan, Italy).

2.7. Statistical analysis

All the results are reported as mean ± standard deviation (SD), calculated from 3 independent experiments, each with 3 replicates, except for dissolution results that were calculated from 5 independent experiments. Statistical analysis was performed using *t*-test. One-way Anova test was used for MMT results. Differences were considered significant for $P < 0.05$.

3. Results and discussion

3.1. Safety assessment of BS

Considering the possible application for the prevention of skin infections, the safety of BS was assessed on HDFa and NIH/3T3 cell lines. Cell survival after 24 or 48 h of treatment with different concentrations of BS was evaluated by MTT assay. Fig. 1 shows that viability of human (A) and murine (B) fibroblasts was not affected by BS up to 10 mg/mL,

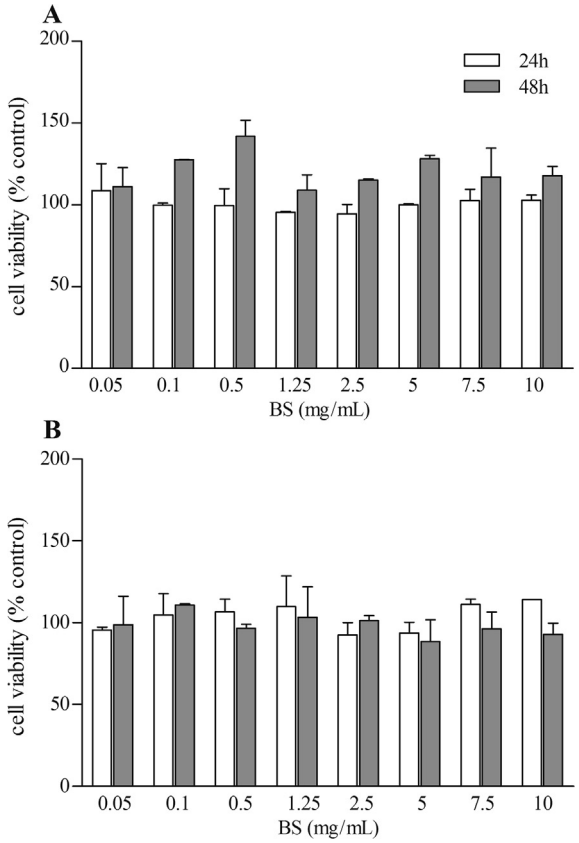

Fig. 1. MTT assay of (A) HDFa and (B) NIH/3T3 cells after incubation with BS for 24 h and 48 h. The MTT values were normalized to control. The Y-axis represents the percentage of cell viability whereas the X-axis represents the treatment. Data is reported as mean ± standard deviation of 3 repeats and statistical significance was determined by one-way ANOVA.

thus indicating that BS is not cytotoxic.

3.2. Antibiofilm activity of Lactobacillus BS against S. aureus strains

Previous studies with BS isolated from *Lactobacillus* spp. have demonstrated their antiadhesive properties against *Candida* [23], however there are few reports about the direct antibiofilm activity of *Lactobacillus* spp. BS towards pathogenic bacteria [17,24,25]. The effect of BS produced by *L. gasseri* BC9 was evaluated on five clinically isolated MRSA strains (strains 2, 7, 45, 83, 86), a gentamicin-resistant clinical isolate (strain 88) and a sensitive reference strain (ATCC 29213). Notably, isolates 2, 7, 45, 86 are resistant to more than three antimicrobial classes, being therefore classified as multi drug-resistant (MDR) *S. aureus* strains (Table 1). BS were tested at the final concentrations of 1.25, 2.5 and 5 mg/mL. According to the Critical Micellar Concentration (CMC), previously calculated for this BS (2 mg/mL) [31], we chose to evaluate the antibiofilm effect of a concentration immediately above the CMC (2.5 mg/ml) and concentrations equal to half (1.25 mg/mL) and double (5 mg/mL) this value. Preformed biofilm dispersal and biofilm development inhibition were investigated (Fig. 2). BS produced by *L. gasseri* BC9 were able to interfere with preformed *S. aureus* biofilms, causing significant biofilm dispersal for all the strains tested. BS were particularly active on biofilms formed by strains 2, 83, 86, and 88, showing dispersal percentage above 30%, at a concentration as low as 2.5 mg/mL. Notably, BS were overall more effective in eradicating biofilms of MRSA as compared to the reference strain ATCC 29213 (Fig. 2A). BS were also able to inhibit *S. aureus* biofilm development, reducing biofilm formation for all the tested strains, and reaching 30% of inhibition for strains 2, 88 and ATCC 29213 at a concentration of 2.5 mg/mL (Fig. 2B). The antibiofilm results are comparable to biofilm reduction described by Morais et al. using crude BS of *L. gasseri* P_{65} towards *E. coli* and *S. saprophyticus* [25]. Overall, the antibiofilm activity of BS was not dose-dependent, although the best profile was obtained with the BS concentration of 2.5 mg/mL. Different levels of biofilm dispersal and formation inhibition were achieved depending on the *S. aureus* strain under analysis, this feature being possibly related to the great genetic variability of *S. aureus*. Indeed, the global population of *S. aureus* is divided in various lineages, which differ in hundreds of genes, that are present/absent, and have variant regions or SNP differences [2].

3.3. Characterization of liposomes containing BS

The ethanol injection method is a simple and reproducible technique for scaling-up LP production [34]. The immediate dilution of the ethanol in the aqueous phase provides the formation of bilayer planar fragments that tend to acquire a quasi-spherical structure upon stirring and/or ultrasonication [35]. Table 2 reports control-LP and BS-LP characteristics. All the LP presented a diameter lower than 200 nm, that is considered suitable for potential antibiofilm application [26]. Immediately after the preparation, BS-LP showed higher mean diameter with respect to control-LP, probably due the entrapment of BS. Moreover, all the prepared formulations showed a good PDI (0.156 ± 0.029 for BS-LP and 0.199 ± 0.036 for control-LP), thus confirming that the injection technique is a suitable method to obtain homogeneously dispersed vesicles. As reported by several papers [36], vesicles prepared with the switterionic PC generally exhibited a negative zeta potential. The presence of BS in the vesicles did not provide statistically significant difference in the zeta potential with respect to the control-LP (Table 2). Zeta potential values are near or above the theoretically appointed 30 mV limit required for stability [37]. The size and PDI of control-LP and BS-LP were also detected during storage at 4–8 °C for 28 days (Fig. 3). For both types of LP, size and PDI measured immediately after preparation were not significantly different with respect to those detected during 28 days of storage at 4–8 °C.

3.4. Antibiofilm activity of liposomes containing BS against S. aureus strains

The antibiofilm activity of BS-LP was evaluated by means of biofilm dispersal and biofilm formation inhibition assays. BS-LP were used at the final BS concentration of 2.5 mg/mL because, at this concentration, *L. gasseri* BC9 BS were previously described to exert a maximal overall effect on *S. aureus* biofilm (Fig. 4). The biofilm dispersal assay revealed that BS-LP induced significant biofilm dispersal on all the strains tested. Notably, the formulation of BS into LP enhanced their eradication activity, as demonstrated by higher dispersal effect of BS-LP as compared to BS alone. BS-LP were particularly effective on biofilms formed by strains 2, 45, 83, 86, and 88, generally showing dispersal percentage above 50%, with a maximum dispersal effect of 92.4% on MRSA strain 45 (Fig. 4A). BS-LP were also able to reduce the biofilm formation of all the tested strains (except for MRSA strain 2), revealing even higher performance as compared to BS alone (Fig. 4B). In this regard, MRSA strain 2 was the most sensitive to BS; this could justify the inability of the liposome formulation to further improve the antibiofilm activity towards MRSA strain 2. In general, LP containing BS displayed a significant increase of antibiofilm properties as compared to crude BS, demonstrating that phospholipid vesicles can improve the activity of BS. Moreover, LP without BS (control-LP) did not possess significant biofilm dispersal or biofilm formation inhibition activities against *S. aureus* (Fig. 4), indicating that the carrier itself did not contribute to the observed antibiofilm activity. Liposomes are supposed not only to support BS stabilization, but also to mediate BS delivery to *S. aureus* biofilm and BS interaction with cell surface [26].

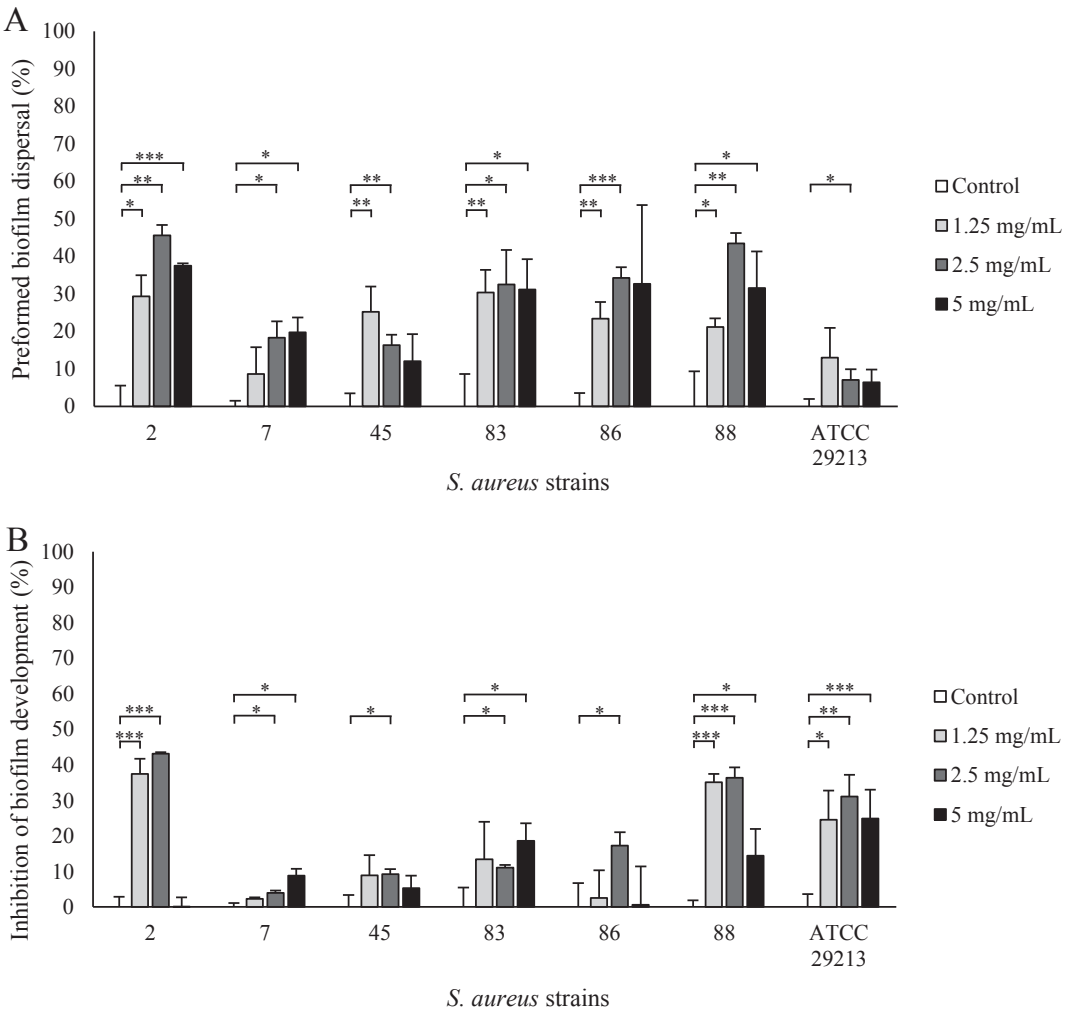

Fig. 2. Antibiofilm activity of *L. gasseri* BC9 biosurfactant (BS) towards *S. aureus* strains. (A) Dispersal of preformed *S. aureus* biofilm; (B) Inhibition of the development of *S. aureus* biofilm. Biofilms were untreated (white bar) or treated with BS at the final concentrations of 1.25 mg/mL (light grey bar), 2.5 mg/mL (dark grey bar) and 5 mg/mL (black bar). Data are expressed as mean value ± standard deviation (%). Statistical significance was evaluated using the student's *t*-test against the control (untreated). *** ($P < 0.001$); ** ($P < 0.01$); * ($P < 0.05$).

Table 2
Liposome characteristics.

Liposome	Size (nm)	PDI	Zeta potential (mV)
BS-LP	149.2 ± 6.6	0.156 ± 0.029	− 36.14 ± 2.12
control-LP	114.2 ± 10.5	0.199 ± 0.036	− 38.41 ± 1.11

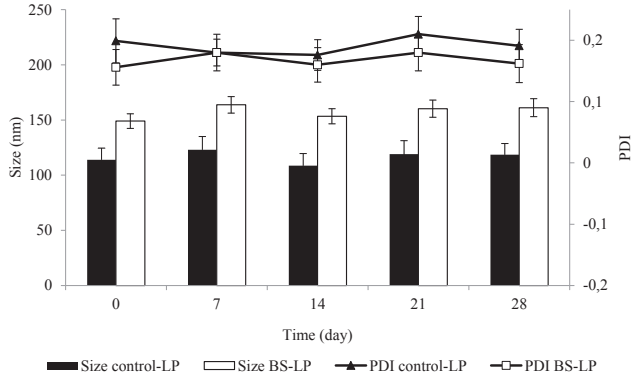

Fig. 3. Variation of LP size and polydispersity index (PDI) during 28 days of storage at 4–8 °C.

3.5. Formulation of lyophilized matrices containing BS loaded liposomes

Gelatin matrices were used as solid carriers, able to quickly release liposomes on the skin. Lyophilized matrices were cylindrical shaped and presented a homogeneous and smooth surface; they were easy to remove from blisters, with no or minimum residue and with good handling characteristics. The results of thickness, size and weight (Table 3) also demonstrated high uniformity with minimum intra-batch variability. Table 3 also reports the time for complete dissolution after matrix immersion in 5 mL of SWF. As can be seen, both matrices containing BS-LP and control-LP dissolved in the SWF within 6 s.

To check the integrity of liposomes before freeze-drying, size and PDI were measured. Size of BS-LP and control-LP were 138.1 ± 9.7 nm and 120.2 ± 4.6 nm, respectively (not significantly different with respect to LP prepared without gelatin, reported in Table 2). Moreover, PDI values (0.169 ± 0.045 for BS-LP and 0.271 ± 0.057 for control-LP) confirmed that LP were homogeneously dispersed.

After matrix dissolution in purified distilled water, LP were released and monitored again for their size and PDI. A slight size increase was observed for both formulations (225.3 ± 17.2 nm for BS-LP and 171.6 ± 8.1 nm for control-LP), despite the homogeneity of the samples as demonstrated by PDI values (0.275 ± 0.078 for BS-LP and 0.220 ± 0.018 for control-LP). Considering that several studies reported that liposomes with size below 500 nm can efficiently delivery

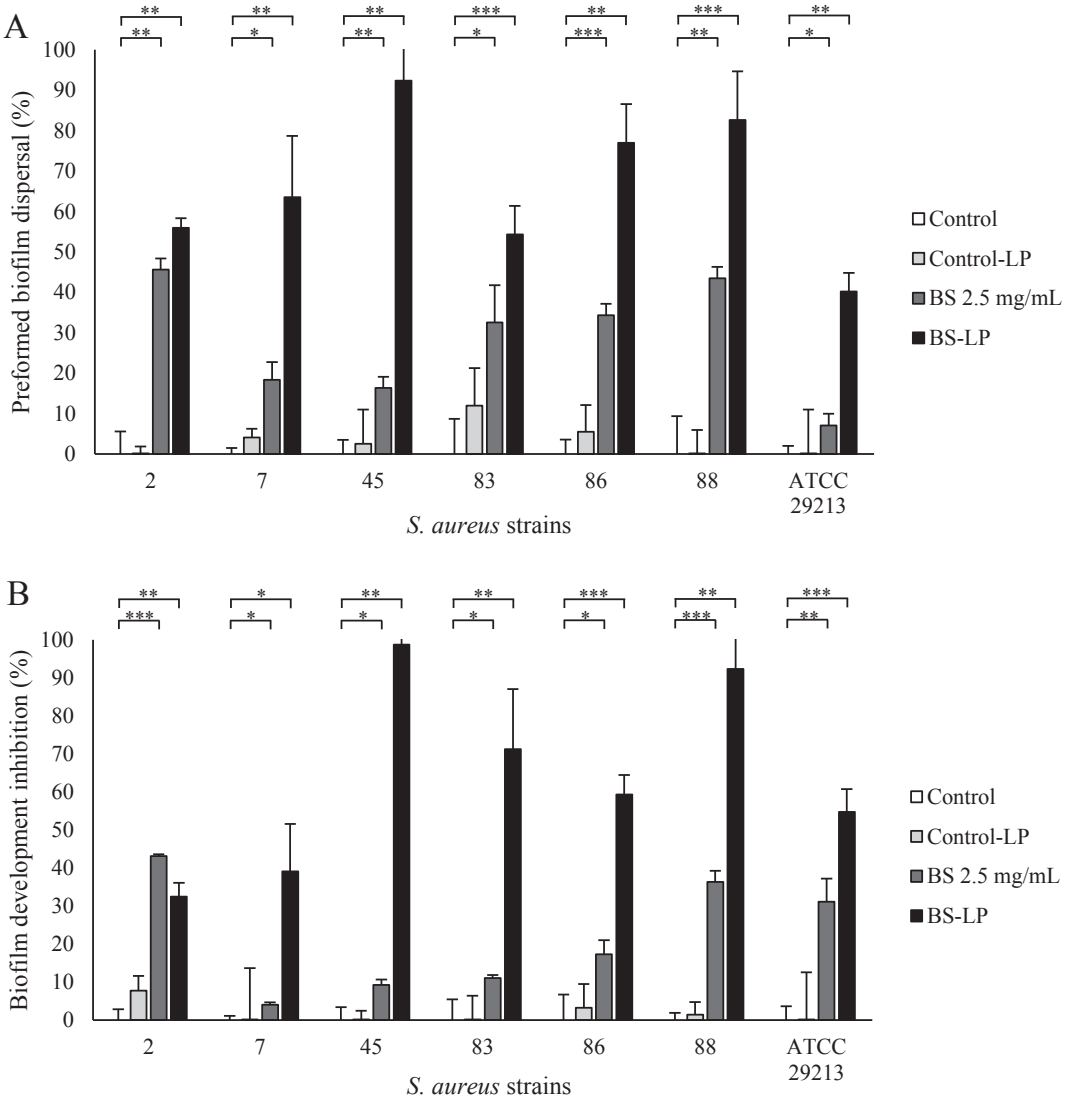

Fig. 4. Antibiofilm activity of *L. gasseri* BC9 biosurfactant (BS) included in liposomes (BS-LP) and control-LP towards *S. aureus* strains. (A) Dispersal of preformed *S. aureus* biofilm; (B) Inhibition of the development of *S. aureus* biofilm. Biofilms were untreated (white bar) or treated with control-LP (light grey bar), BS (dark grey bar) and BS-LP (black bar). BS was tested at the final concentration of 2.5 mg/ml. The same concentration of BS was used in BS-LP. Control-LP were prepared without BS. Data are expressed as mean value ± standard deviation (%). Statistical significance was evaluated using the student's *t*-test against the control (untreated): *** ($P < 0.001$); ** ($P < 0.01$); * ($P < 0.05$).

active molecules to biofilms [26], the observed increase of BS-LP size did not represent a limiting factor for BS delivery. These preliminary results confirmed that LP were efficiently incorporated into gelatin matrices without affecting their properties.

4. Conclusions

In this study, we demonstrated that a *Lactobacillus*-derived BS, delivered in topical LP carriers, is able to counteract clinically relevant MRSA biofilms, which are of high relevance due to their relation with recurrent skin and soft tissue infections. BS is not cytotoxic and possess great antibiofilm properties that can be significantly increased by the encapsulation in LP. As the incorporation into lyophilized gelatin matrices does not affect BS-LP properties, the possibility to vehicle this novel antibiofilm agent through a solid dosage form, with improved characteristics in terms of stability and application at the treatment site, represents a further strength of our work. Overall, this study highlights the potential of using gelatin based matrices containing BS-LP as a new option to prevent MRSA biofilm infections.

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Table 3
Properties of lyophilized matrices.

Matrix	Thickness (mm)	Diameter (mm)	Weight (mg)	Dissolution time in SWF (sec)
BS-LP	3.34 ± 0.18	13.21 ± 0.19	11.34 ± 0.93	4.50 ± 0.71
control-LP	3.38 ± 0.36	13.31 ± 0.16	9.60 ± 0.10	5.75 ± 0.54

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Competing Interests

None.

Ethical Approval

Not required.

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