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Teat-apex colonizer *Bacillus* from healthy cows antagonizes mastitis-causing *Staphylococcus aureus* biofilms

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Abstract

Staphylococcus aureus is the most frequent causal agent of bovine mastitis, which is largely responsible for milk production losses worldwide. The pathogen's ability to form stable biofilms facilitates intramammary colonization and may explain disease persistence. This virulence factor is also highly influential in the development of chronic intramammary infections refractory to antimicrobial therapy, which is why novel therapies that can tackle multiple targets are necessary. Since udder microbiota have important implications in mastitis pathogenesis, they offer opportunities to develop alternative prophylactic and therapeutic strategies. Here, we observed that a *Bacillus* strain from the teat apex of lactating cows was associated to reduce colonization by *S. aureus*. The strain, identified as *Bacillus* sp. H21, was able to antagonize in-formation of mature *S. aureus* biofilms associated to intramammary infections without affecting cell viability. When exploring the metabolite responsible for this activity, we found that a widespread class of *Bacillus* exopolysaccharide, levan, eliminated the pathogenic biofilm under evaluated conditions. Moreover, levan had no cytotoxic effects on bovine cellular lines at the biologically active concentration range, which demonstrates its potential for pathogen control. Our results indicate that commensal *Bacillus* may counteract *S. aureus*-induced mastitis, and could therefore be used in novel biotechnological strategies to prevent and/or treat this disease.

Keywords

Staphylococcus aureus; bovine mastitis; biofilm; teat apex microbiota; *Bacillus;* levan

1. Introduction

Infection by *Staphylococcus aureus* is the leading cause of mastitis in dairy cattle (Campos et al., 2022). Mastitis, an intramammary inflammatory disease, drastically affects milk production and causes large economic losses (Sharun et al., 2021). It is most frequently prevented and/or treated with antibiotics and herd management programs. However, *S. aureus* has developed virulence mechanisms that protect it not only from antibiotic treatment but also from the host's innate and adaptive immune response (Zaatout et al., 2020). Biofilm formation, one of these virulence mechanisms, occurs when bacterial cells become attached to the mammary gland epithelium. Once established there, they are able to proliferate and finally accumulate in multilayers (Baselga et al., 1993). This facilitates intramammary colonization and persistent infections and may lead to chronic mastitis, which is currently difficult to eradicate (Xue et al., 2014). *S. aureus* has historically been among the most important emergent zoonotic pathogens and thus poses a threat to public health (Algammal et al., 2020). The appearance of drug-resistant clones and the dangers this may entail for food safety have created a critical need for alternative disease management strategies, which may be based on new compounds with the ability to antagonize *S. aureus*'s virulence factors (Glatthardt et al., 2020)

Intramammary infections were traditionally described as host-pathogen interactions that triggered inflammatory responses, a notion that has more recently been challenged by the finding that commensal microbiota plays a crucial role in the udder's health status (Derakhshani et al., 2018). Microbiota can benefit their host in processes that include carbohydrate metabolism, vitamin synthesis, immunomodulation, and they significantly affect host-pathogen interactions. For this reason, they might be harnessed to develop

prophylactic/therapeutic products to substitute antibiotics, both for animals and for humans (Heikkila and Saris, 2003; Iwase et al., 2010; Bouchard et al., 2015; Isaac et al., 2017; Glatthardt et al., 2020).

The commensal microbes that colonize the teat apex and the teat canal are the first line of defense against pathogens attempting to enter the udder, and thus against intramammary infections (Paulrud, 2005). Teat skin and environmental niches such as herd feces and bedding material are the most important sources of milk microbiota (Doyle et al., 2017). When culture-dependent and independent techniques were combined, the major commensal and pathogenic microorganisms in teat skin were found to be bacteria from the phyla *Firmicutes, Actinobacteria, Bacteroidetes,* and *Proteobacteria* (Woodward et al., 1987; Braem et al., 2012; Verdier-Metz et al., 2012). Among these is the genus *Bacillus*, which also resides in the bovine mammary gland (Oikonomou et al., 2014).

Bacillus species have antimicrobial, antioxidant, antibiofilm, anti-inflammatory and immunomodulatory activity, and have been used as probiotics to prevent infections (Elshaghabee et al., 2017; Piewngam and Otto, 2020). Their ability to form spores and bioactive metabolites, as well as their long shelf-life, make them potent pathogen antagonists (Barboza-Corona et al., 2009; Piewngam et al., 2018; Ngalimat et al., 2021). Rhizosphere-derived *Bacillus*, for instance, were successfully used as probiotics against *S. aureus*-experimental mastitis in murine and bovine models (Afroj et al., 2021). Besides their probiotic features and beneficial effects on health, several strains are able to grow in stable biofilms as their main lifestyle both in nature and in the laboratory, and can produce significant amounts of exopolysaccharides (EPSs) (Abid et al., 2019). Although EPSs are common structural components of the extracellular matrices of *Bacillus* biofilms, not enough is known about their functions (Marvasi et al., 2010). They may be heteropolymers or

homopolymers like glucan (Malick et al., 2017) and levan . The latter, a homopolysaccharide containing fructose, is biocompatible and biodegradable. This means it could be suitable for biotechnological, medical, and nanotechnological applications (Srikanth et al., 2015).

Despite *Bacillus* spp.'s probiotic potential, several aspects of their possible uses in the management of bovine mastitis remain unexplored. This is the first report on the antagonistic role of a commensal *Bacillus* strain colonizing the teat apex of healthy cows against mastitis pathogens. Thus, the specific goal of this work was to examine its effects on mastitis-causing *S. aureus* biofilms, the mechanisms involved in its biological activity, and its cytotoxic effects as a bioactive compound, all of which are important considerations for its prospective application in lactating cows.

2. Materials and Methods

2.1. *Staphylococcus aureus* strains from bovine intramammary infections

The assays were performed with the well-characterized *S. aureus* V329 strain(GenBank: JAGTJH000000000.1), kindly provided by Dr I. Lasa (Instituto de Agrobiotecnología, Pamplona, Spain), and with the field isolate *S. aureus* L33 (GenBank KY364357), from subclinical bovine mastitis (Isaac et al., 2017). The ability of both pathogenic *S. aureus* strains to form biofilm was previously corroborated by phenotypic and genotypic characterization (Table S1). According to the recommendations for assessment of biofilm production by staphylococci (Stepanović et al., 2007), *S. aureus* V329 was classified as strong biofilm former, while *S. aureus* L33 was considered a biofilm hyperformer strain.

2.2. Sampling and isolation of commensal bacteria from teat apex flora

Samples were collected from 168 lactating cows on a dairy farm "*Estancia Yucat*" in Villa Maria (Córdoba, Argentina). Animal sampling procedures were reviewed and approved by

the Institutional Commission for the Care and Use of Animals (CICUAL) of the National University of Villa María, and were consistent with the Guide for the Care and Use of Agricultural Animals in Research and Agricultural Teaching, 4th edition (Federation of Animal Science Societies, 2020). In addition, informed consent was obtained from a cooperation and complementarity framework agreement between the National University of Villa Maria and *Orden de la Merced "Estancia Yucat*".

The animals were previously subjected to a clinical examination and their quarters were classified as healthy or inflamed according to clinical signs and the California Mastitis Test (CMT). Quarters were defined as inflamed when CMT scores were more than or equal to 1. The side of each quarter (around 2 cm) and the orifice of each teat were swabbed with sterile cotton swabs that had been dipped into 3 mL of 0.9 % NaCl solution (Landers et al., 2010; Doyle et al., 2017). In total, 287 healthy and 385 inflamed quarters were sampled. The samples were maintained at 4°C for transportation and further processing.

For bacterial isolation, each sample was vigorously mixed and an aliquot was plated onto agar culture media. Brain heart infusion (BHI) and trypticase soy agar (TSA) were used as non-selective media. Mannitol salt agar (MSA) and McConkey agar were used to isolate staphylococci and Gram-negative bacteria, respectively. The cultures were incubated at 37°C for 48 h. Colony phenotypes were evaluated, and forty-seven morphologically diverse colonies were isolated and stored in TSB with 20 % glycerol at -80°C.

2.3. Selection and identification of commensal *Bacillus*

In 232 of the 287 healthy quarter samples (80.8%) there was an easily distinguishable colony. The colony was irregular in shape, of moderate (2–4 mm) diameter with moist and mucoid consistence. Colonies varied from membranous with an underlying mucoid matrix, through

a rough and crusty appearance as they dry. Colony characteristics were in accordance with the "licheniform" descripted in Bergey's Manual (Logan and Vos, 2015). Interesting, this colony was only found in 95 out of the 385 inflamed quarter samples (24.7%). A striking correlation was observed between the presence of *Bacillus* colony and the absence of inflammation ($p < 0.0001$ according to Fisher's exact test, Figure S1A). The inflammation exclusion effect was not complete indicating that not all animals whose teat were colonized by *Bacillus* were protected from inflammation. However, the negative association between *Bacillus* and udder inflammation was promising enough to select the strain for further studies.

Although the strain was easily recognizable as *Bacillus* genus by its morphological features, it was phylogenetically identified by 16S rRNA sequencing (Figure S1B) as previously described (Isaac et al., 2017). The nucleotide sequence obtained from *Bacillus* sp. H21 identification was deposited in the EMBL nucleotide sequence database (GenBank/EMBL/DDBJ) under the accession number OQ553822.

2.4. Growth inhibition study

The ability of *Bacillus* sp. H21 to inhibit the growth of *S. aureus* was tested following an adapted protocol of the dual culture plate technique (Bell, 1982). The *Bacillus* strain was spread on the center of a Petri dish containing solid TSA medium, in such a way that it faced the *S. aureus* strains transversely. Cross inhibition of growth was evaluated after 24 and 48 h of incubation at 37°C.

2.5. Mixed biofilms of pathogenic *S. aureus* and *Bacillus* sp. H21: interference experiments

Twenty-four-well polystyrene microtiter plates were used to assess anti-biofilm activity. For biofilm inhibition, 1 mL of a mixed *S. aureus-Bacillus* culture grown in TSB (tryptic soy broth) was added into each well, making sure that all the inocula had the same composition. The plate was incubated for 24 h at 37°C. For the eradication assay, a monospecies *S. aureus* biofilm was grown for 24 h. Planktonic bacteria were removed and the mature biofilm was washed twice with sterile phosphate-buffered solution (PBS). Finally, 1 mL of a pure culture of *Bacillus* sp. H21 was added. This culture was left to interact with the established *S. aureus* biofilm for another 24 h. In the case of the single *S. aureus* biofilm, the culture concentration was approximately 10^6 CFU mL^{-1}. The dual-species biofilm had a final concentration of 10^6 CFU mL^{-1} (5×10^5 CFU mL^{-1} for each strain). Biofilm was quantified through crystal violet staining by measuring the optical density at 570 nm (O'Toole and Kolter, 1998).

2.6. Scanning electron microscopy (SEM)

Single staphylococcal species and mixed *S. aureus-Bacillus* sp. H21 biofilms were cultured as described above, in 24-well plates with a sterile glass coverslip inside each well. After 24 h of incubation, the macroscopically visible floating biofilm and the biofilm adhered to the coverslip were collected and fixed with 2.5% glutaraldehyde for 5 h at 4°C. The samples were dehydrated through a graded series of alcohol steps (30, 50, 70 and 90 % v/v) and acetone (100%), and finally subjected to critical point drying with CO_2. After metal spraying, images were taken with a ZEISS SUPRA 55 VP scanning electron microscope (CIME, Integral Center of Electronic Microscopy, CONICET−National University of Tucumán, Argentina).

2.7. qPCR quantification of *S. aureus* in mixed *Bacillus* sp. H21-*S. aureus* cultures

Absolute quantification by qPCR was used to determine the staphylococcal content in the different phases of the mixed cultures. Standard curves were built on the basis of ten-fold serial dilutions of the *S. aureus* V329 and *S. aureus* L33 strains. These were grown in TSB, and DNA was extracted from 2 mL samples of the cultures with the DNeasy tissue kit (Qiagen). For each *S. aureus* strain, DNA concentration was plotted against the quantification cycle (Cq) on a linear standard curve.

The primer pair Sa-442 (Martineau et al., 1998) was used for PCR amplification. The reaction was performed at a final volume of 10 µL, using SsoAdvanced Universal SYBR Green Supermix (Bio- Rad) in a CFX96 Touch Time™ PCR-Real Time system (BIO-RAD, Hercules, CA, USA)**,** according to the manufacturer's instructions. The protocol consisted of initial denaturation at 98°C for 2 min, 40 cycles of denaturation for 5 s at 98°C, and combined annealing/extension for 30 s at 60°C.

For the mixed culture samples, *S. aureus* and *Bacillus* sp. H21 were co-cultured on six-well plates at a final volume of 2 mL. After 12 or 24 h of incubation, the staphylococci were counted in the three phases (biofilm adhered to the bottom of the well, planktonic cells, and floating biofilm). Cq values were compared to the standard curve to obtain of the corresponding CFU. For the eradication assay, time 0 was also included in order to quantify *S. aureus* biomass before the treatment with *Bacillus*.

2.8. Exopolysaccharide extraction and characterization

To assess EPS production by *Bacillus* sp. H21, pre-cultures of the strain at mid-exponential phase were inoculated into 500 mL flasks containing 250 mL of TSB medium. The flasks were incubated for 48 h at 37°C under static conditions, and the biofilms were manually collected afterwards. To evaluate bound and/or soluble EPSs (Sheng et al., 2010), extraction

was performed in both planktonic cells and biofilm. EPS partial purification followed instructions from Bengoa and collaborators with minor modifications (Bengoa et al., 2018). Briefly, 100 mL of 2 M NaOH were added to the cultures, which were then incubated overnight at room temperature with agitation (120-150 rpm). The cell suspensions were centrifuged for 30 min at 5000 *x*g to separate bacteria. Two volumes of cold ethanol were added to precipitate EPSs in the supernatants and in the bacterial pellets. After overnight incubation at -20°C, the samples were centrifuged for 30 min at 4°C and 5000 *x*g. Finally, the EPS pellets were dissolved in hot distilled water (90°C), autoclaved to eliminate spores, and lyophilized.

The functional groups in the final EPS samples were identified through a Fourier Transform Infrared (FTIR) analysis. For this purpose, potassium bromide tablets were used at room temperature, as well as 7 atmospheres of pressure in a Perkin Elmer 1600 FTIR device (Institute of Physical Chemistry, Faculty of Biochemistry, Chemistry and Pharmacy, National University of Tucumán, Argentina).

2.9. Determination of minimal inhibitory concentration

The minimal inhibitory concentration (MIC) of the extracted EPSs was determined through the broth microdilution method, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2018). Sterile EPSs were properly prepared and transferred into a 96-well bottom-plate with Muller-Hinton Broth (MHB), in order to obtain two-fold serial dilutions at final concentrations ranging from 0.039 to 5 mg/mL. Each well then received 5 x 10^5 CFU mL^{-1} inocula of the *S. aureus* strains. The plate was incubated at 37°C for 24 h. The MIC was defined as the lowest concentration at which no bacterial growth was macroscopically observed, considering turbidity and the formation of a pellet at the bottom

of the well. Some wells were reserved in each experiment to test EPS sterility (no *S. aureus* inoculum was added) and the viability of the inocula (no EPSs were added).

2.10. Confocal laser scanning microscopy

Biofilm architecture was studied with confocal laser scanning microscopy (CLSM) in control and EPS-treated *S. aureus* cultures. *S. aureus* biofilms were grown for 24 h at 37°C in TSB, in Nunc Lab-Tek™ chamber slides (TermoFisher Scientific). Bacterial cells within biofilms were stained with the LIVE/DEAD BacLight Bacterial Viability Kit (Life Technology) according to the manufacturer's instructions. The samples were observed under an Olympus FV1200 with a 20X upright objective (CEMINCO, Center of Micro and Nanomicroscopy, National University of Córdoba, Argentina). A sequential scan in the 488 and 546 wavelength channels was carried out for each condition, and the corresponding xy optical sections and random images were obtained. Overlapping images, orthogonal cuts and 3D biofilm structures were rendered on ImageJ software for figure composition.

2.11. *In vitro* cytotoxicity in bovine cell lines

Cytotoxicity was evaluated in bovine mammary alveolar MAC-T cells (Huynh et al., 1991) and in the BoMac cell line (Stabel and Stabel, 1995) derived from bovine macrophages as previously described Bohl and collaborators (Bohl et al., 2021). The MAC-T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 μg/mL hydrocortisone (Fada Pharma, 100 mg), 5 μg/mL bovine insulin (Betasint U-40), 100 U/mL penicillin-100 μg/mL streptomycin, and 1/100 CTS™ GlutaMAX™-I Supplement. The BoMac cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin-100 μg/mL streptomycin, and 1/100

CTS™ GlutaMAX™-I Supplement. All the cells were preserved at 37°C in a humidified atmosphere with 5% CO_2.

Then, each well in a 24-well plate was filled with 2 x 10^5 of attached cells. Active lyophilized EPSs were re-suspended in adequate medium for each cell line and different concentrations were added (from 0.15 to 10 mg/mL). The plates were incubated for 24 h. EPS-free medium was used as a control.

Cell viability was evaluated by the Thiazolyl Blue Tetrazolium Bromide method (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, MTT); Sigma-Aldrich, St. Louis, MO, USA). For this, MTT solution (0.5 mg/mL in culture medium without FBS) was added to the cells after discarding the supernatant, and the plates were incubated in the dark for 4 h at 37°C. The supernatant was discarded again and dimethyl sulfoxide (DMSO) was added to solubilize formazan crystals. Viability was determined by colorimetric quantification at 570 nm in three independent experiments.

2.12. Statistical Analysis

Statistical analysis was performed on Infostat (Universidad Nacional de Córdoba, Argentina) with one-way ANOVA, independent t-test or Fisher's exact test, as appropriate. Bonferroni's post-test was used to analyze data after ANOVA.

3. Results

3.1. *Bacillus* interference in biofilm formation by *S. aureus*

Both pathogenic *S. aureus* strains were able to form stable biofilm structures. On the other hand, *Bacillus* sp. H21 formed dense biofilm at the air-liquid interface when cultured in the conditions specified for our assay. When the *S. aureus* strains and *Bacillus* sp. H21 were combined, a heterogeneous culture was obtained, composed of three distinct phases: a

biofilm adhered to the bottom of the well, a planktonic phase, and a floating biofilm (Figure S2). In these co-cultures, *Bacillus* sp. H21 had an inhibitory effect on *S. aureus* biofilms, as detected by crystal violet staining (Figure 1A). Biofilm was reduced by approximately 52% for *S. aureus* V329 and 41% for *S. aureus* L33 with respect to the single culture controls. Despite the limitations of the technique (it enables semi-quantitative measurement of the total biofilm biomass, without differentiating staphylococcal from *Bacillus* biofilms), the results were encouraging.

To investigate bacterial behavior in the mixed cultures, the adhered and floating biofilms were studied by scanning electron microscopy (Figure 1B). As the micrographs show, the biomass of adhered biofilms produced by both *S. aureus* strains decreased notably after the addition of *Bacillus*. Interestingly, the bacterial composition of biofilm in the mixed cultures appeared to remain almost completely staphylococcal. Although extracellular material released by *Bacillus* was found, almost none of the adhered cells were identified as bacilli, which indicates no bacterial replacement.

Finally, since there was no cross inhibition in the co-cultures (data not shown), staphylococcal displacement within them was explored further through qPCR absolute quantification. *S. aureus* was quantified in the three culture phases after 12 and 24 h of co-cultivation (Figure 1C). In the single culture of *S. aureus* V329, biofilm was the predominant lifestyle (approximately 60% of the total population), but co-culturing with *Bacillus* sp. H21 brought this value down to 12.5% after the shortest treatment time. On the other hand, planktonic *S. aureus* V329 significantly increased in the mixed culture, and represented more than 85% of the total staphylococcal biomass. The antibiofilm effect of *Bacillus* sp. H21 on *S. aureus* V329 was observed even when no floating biofilm was macroscopically detected. Similar results were obtained after 24 h of co-incubation. For the same *S. aureus* strain,

adhered biofilm decreased from 64% in the single culture to 26% in the co-culture. However, S. *aureus* V329 cells were re-distributed between the planktonic phase (61%) and the floating biofilm formed by *Bacillus* sp. H21 (13%).

By contrast, hyperbiofilm producer *S. aureus* L33 turned out to be much more resistant to the antibiofilm activity of *Bacillus* sp. H21. A significant inhibition in biofilm formation (from 68% to 32%) was registered only after 24 h of treatment with *Bacillus*. Sixty-four percent of the staphylococcal cells were redistributed into the planktonic phase, and 4% into floating biofilm.

In general, *Bacillus* sp. H21 had strong non-bactericidal antibiofilm activity against both pathogenic strains, even though the effect was less significant for *S. aureus* L33. These results indicate that attacking a single virulence factor (biofilm formation) might serve as the basis for new mastitis management strategies

3.2. Disruption of pre-formed *S. aureus* biofilms by *Bacillus*

Bacillus sp. H21 was also able to significantly disrupt mature *S. aureus* biofilms (Figure 2A). After 24 h of incubation, crystal violet staining demonstrated that *Bacillus* sp. H21 eradicated approximately 60% of pre formed *S. aureus* V329 biofilms. Unexpectedly, this disruption was even more pronounced (over 90%) in biofilms formed by *S. aureus* L33.

Microscopy techniques showed that the addition of *Bacillus* sp. H21 disrupted the dense, adhesive staphylococcal biofilms without bacilli becoming incorporated into them (Figure 2B). In contrast, cells of *S. aureus* V329 and *S. aureus* L33 can clearly be seen entrapped in the floating bacilli biofilms.

The absolute quantification of *S. aureus* by qPCR confirmed these results (Figure 2C). In this case, staphylococci were counted not only after 12 and 24 h of treatment but also at time 0,

in order to quantify the initial biomass of mature biofilms. More than 60% of the initial biomass of both pathological strains was in the form of biofilm. Twelve hours of treatment with *Bacillus* were not enough to affect their density. After 24 h of treatment, the adhered biofilms of both strains were almost completely reduced; planktonic lifestyle continued largely unmodified in terms of biomass; and the now visible floating biofilm had a high percentage of the total *S. aureus* biomass entrapped in the *Bacillus* structure.

3.3. A levan-type exopolysaccharide is responsible for the anti-biofilm activity of *Bacillus* sp. H21

Considering the behavior of the *S. aureus-Bacillus* co-cultures, as well as the morphological features of the *Bacillus* sp. H21 culture, a component of the *Bacillus* biofilm matrix was inferred to be responsible for its biological activity. To corroborate this, EPSs were extracted from floating *Bacillus* biofilms and characterized by FTIR. Since levan is the main component in *Bacillus* biofilm matrices, commercial levan was used as a control (Romero et al., 2018). Approximately 140 mg of partially purified EPSs were recovered from 1 L of a 24-hour *Bacillus* sp. H21 culture. Their FTIR spectrum showed several stretching peaks corresponding to pure levan (Figure S3).

The anti-biofilm activity of this EPS was evaluated against *S. aureus* (Figure 3). A cell-free supernatant of *Bacillus* sp. H21 was included in the assay to evaluate possible active metabolites released into the culture medium, but the results were not significant (data not shown). Anti-biofilm effects by semi-purified EPS-H21 were detected only when EPS concentrations exceeded 1 mg/mL. At a concentration of 1.25 mg/mL, the EPS was able to reduce biofilm formation by almost 50% in the case of *S. aureus* V329, but this effect did not seem to be dose-dependent. A concentration of 5 mg/mL was necessary to observe a similar

inhibition for hyperproducer *S. aureus* L33. EPS-H21 performed remarkably better against mature biofilms. It disrupted mature *S. aureus* V329 biofilms almost completely, even at the lowest concentration tested. Robust *S. aureus* L33 biofilms were much more resistant, but were nevertheless reduced by between 70 and 85%.

3.4. Microbicidal and cytotoxic effects of EPS-H21

Given the high EPS concentrations needed to observe anti-biofilm effects, the MIC of EPS-H21 was determined. There were no effects on bacterial growth at concentrations ranging from 0.039 to 5 mg/mL, which indicates that the inhibitory phenotype was not associated with bacterial growth inhibition. This was confirmed through CLSM using LIVE/DEAD bacterial staining, which showed that the anti-biofilm activity of EPS-H21 was not a consequence of microbicidal effects (Figure 5).

The potential influence of EPS-H21 on the metabolic competence of bovine cells was also evaluated. As demonstrated by the MTT assays, only the highest concentration tested (10 mg/mL) affected the viability of the bovine macrophage cell line BoMac. No substantial changes were found in the viability of epithelial MAC-T cells at EPS evaluated concentrations (Figure 5).

4. Discussion

The passage of bacteria from the teat canal into the udder is the first step for the occurrence of an IMI, which may in turn lead to subclinical or clinical mastitis (Krömker and Friedrich, 2009). Although the keratin plug is the main barrier against invading pathogens, commensal bacterial species from the teat apex seem to play important roles in udder health (Braem et al., 2013). We unintentionally discovered a negative association between *Bacillus* and udder inflammation after making a microbiological evaluation of the teat apex in more than 160

lactating cows. This prompted us to explore the mediating roles that these bacteria are likely to have in the protection against IMI-associated pathogens. *S. aureus* was selected as the target, since it is a zoonotic pathogen that causes the most prevalent forms of bovine mastitis, which do not respond to antibiotic therapy and therefore lead to chronic infections (Brouillette et al., 2004; Monistero et al., 2018; Attia Algharib et al., 2020).

S. aureus usually requires multi-targeted therapies because of its many virulence factors, among which biofilm formation is key (Moormeier and Bayles, 2017). *Bacillus* species, on the other hand, are often robust biofilm producers. They reside mainly in the soil and have different functions in natural and industrial environments (Randrianjatovo-Gbalou et al., 2017). When *Bacillus* sp. H21 was tested against *S. aureus*, the ability of both bacteria to form biofilms created a culture made up of three phases: a biofilm adhered to the bottom and composed mainly of *S. aureus* cells, a mixed planktonic phase, and a floating *Bacillus* biofilm. The *in vitro* interactions between *Bacillus* and *S. aureus* depended on the inoculum conditions. Our results indicate that *Bacillus* sp. H21 is able to inhibit *S. aureus* biofilm formation by changing its biofilm lifestyle preference into a planktonic one. When antagonizing mature biofilms, *Bacillus* sp. H21 seemed to capture pathogen cells in its own biofilm matrix. The colonization ability of *S. aureus* could be reduced as a result, which means that novel mastitis treatment and prevention strategies could be based on this inhibitory effect.

Piewngam and collaborators (Piewngam et al., 2018) also described *S. aureus* exclusion mediated by *Bacillus*. According to our results, this phenomenon occurs through direct interference. Similarly, Min et al. (Min et al., 2022) found that *Bacillus* reduced colonization by *S. aureus* and alleviated mastitis caused by this pathogen in an *in vivo* mouse model. They also observed that it was able to antagonize *S. aureus* biofilms *in vitro* in a non-bactericidal

manner. The value of this non-lethal activity resides in the fact that it may potentially be harnessed to enhance antibiotic efficiency without eliciting resistant phenotypes (Musk Jr. and Hergenrother, 2006; Stowe et al., 2011). Moreover, since the *Bacillus* strain used in our study was isolated from the teat apex of healthy animals, it is naturally adapted to this niche and thus a promising candidate for teat disinfection. Pre- and post-milking teat disinfection based on chemical compounds is the most frequently used prevention practice on dairy farms. It is an excellent tool for mastitis control and reduces bacterial contamination in milk (Barkema et al., 1998; Fox et al., 2006; Suriyasathaporn and Chupia, 2011; Zucali et al., 2011). Previous research has shown the potential benefits of external probiotic applications, as an alternative to commercially available chemical disinfectants which may leave residues in milk (Dahl et al., 2003; Borucki Castro et al., 2012).

Despite the growing interest in the use of *Bacillus* species as probiotics, little is known about the mechanisms involved in their activity against pathogens. *Bacillus* spp. are known to antagonize Gram positive and Gram negative pathogens by producing diverse antimicrobial compounds. Several of them also engage in competition by exclusion, a phenomenon in which beneficial bacteria prevent the adhesion and colonization of potentially harmful microorganisms (Mingmongkolchai and Panbangred, 2018). The morphology of some *Bacillus* spp. biofilms suggests that their extracellular matrix components fulfill biological functions, but these have not been extensively studied either (Marvasi et al., 2010).

Since EPSs are important components in the architecture of *Bacillus* biofilms (Romero et al., 2018), we evaluated whether the EPSs produced by *Bacillus* sp. H21 were at least partly responsible for the strain's anti-biofilm activity against *S. aureus*. The results confirmed that a firmly-adhered EPS produced by *Bacillus* sp. H21 was able not only to inhibit biofilm formation by both *S. aureus* strains isolated from bovine mastitis, but also to disrupt the

structure of their mature biofilms. Likewise, other authors found that inhibition of biofilm formation was not related to bactericidal activity by EPSs (Abid et al., 2019). Instead, the initial attachment and auto-aggregation of bacterial cells may be inhibited by partially affecting their surface properties (Kim et al., 2009). On the other hand, the potential of *Bacillus* EPSs to disrupt mature biofilms and the mechanisms involved have rarely been assessed (Spanò et al., 2016). Further studies are needed to better understand this ability, and to determine whether EPS combinations with commercial antibiotics could optimize therapies against resistant biofilm-associated infections.

Given the nature of most *Bacillus* EPSs, we suspected that the purified EPS from *Bacillus* H21 was a levan-type polysaccharide. FTIR characterization confirmed that it was made up mostly of levan; the rest of its content might correspond to associated proteins (Romero et al., 2018). Levan, a fructose homopolymer, is the best studied EPS produced by *Bacillus subtilis* (Srikanth et al., 2015). Its biocompatible and biodegradable properties could position it as a versatile alternative to commercial synthetic polymers. It has also proven to have prebiotic effects that modulate microbiota, although its pharmaceutical and medical applications require further research (Srikanth et al., 2015). In some assays, the anti-biofilm activity of levan was dose-independent. This has important implications for bioprospection, since biological effects were possible at minimal effective concentrations. Our study showed that the polymer has no cytotoxicity in bovine mammary epithelial cells and macrophages, so it may be apt to be administered in animals. Further *in vivo* experiments are nevertheless needed to confirm the preventive/therapeutic activity of EPS-H21.

5. Conclusion

This research has probed *in vitro* the potential of commensal *Bacillus* that colonize the teat apex of healthy cows to be used in anti-mastitis strategies. The isolated strain, *Bacillus* sp.

H21, was able to disrupt biofilm formation, one of the most important virulence factors of pathogen *S. aureus*, without affecting bacterial viability or having cytotoxic effects in bovine cell lines. Preventing pathogens that are entering the udder from forming biofilm could reduce the likelihood of new IMI, while the elimination of mature pathogenic biofilms could work as a complement of classic antimicrobial therapeutic treatment in animals that have already been diagnosed. These findings highlight the bioprospective potential of *Bacillus* sp. H21, either as a probiotic in its own right or through the purification of its bioactive compounds, and they contribute to the ongoing search for new strategies to mitigate increasing resistance in biofilm-forming pathogens that affect food-producing animals.

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Declarations of interest:

None

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Figure Captions

Figure 1. Inhibition of pathogenic *Staphylococcus aureus* biofilm formation by commensal *Bacillus* sp. H21. **(A)** Biofilm formation quantified by crystal violet staining. Control without treatment represents 100 %. Values show means from triplicate wells ± SEM. Asterisks denote values that are significantly different from untreated controls ($p < 0.05$). (**B**) Scanning electronic microscopy micrographs of biofilms from the *S. aureus*-*Bacillus* sp. H21 co-cultures after 24 hours. The first row shows micrographs from control monocultures, while the second and the third rows show micrographs of floating biofilm and biofilm adhered to the bottom, respectively, after co-cultivation with *Bacillus* sp. H21. Scale bar: 2 µm (**C**) *S. aureus* quantification by qPCR in the three phases of the mixed culture after 12 and 24 h of co-incubation with *Bacillus* sp. H21: biofilm adhered to the bottom, planktonic phase, and floating biofilm. Floating biofilm was detected only after 24 h. (*) denotes significant differences between the biomass of the different *S. aureus* phases; (**) denotes significant differences between total co-cultured *S. aureus* biomass and untreated single *S. aureus* controls ($p < 0.05$). *p* values were obtained using one-way ANOVA followed by Bonferroni post-hoc analysis.

Figure 2. Eradication of mature pathogenic *Staphylococcus aureus* biofilm by commensal *Bacillus* sp. H21. **(A)** Biofilm formation quantified by crystal violet staining. Control without treatment represents 100 %.Values show means from triplicate wells ± SEM. Asterisks denote values that are significantly different from untreated control ($p < 0.05$). (**B**) Scanning electronic microscopy micrographs of biofilms from the *S. aureus*-*Bacillus* sp. H21 co-cultures after 24 hours. The first row shows micrographs of the control monocultures, while the second and the third rows show micrographs of floating biofilm and biofilm adhered to the bottom, respectively, after co-cultivation with *Bacillus* sp. H21. Scale bar: 2 µm (**C**) *S. aureus* quantification qPCR in the three phases of the

mixed culture after 12 and 24 h of co-incubation with *Bacillus* sp. H21xz: biofilm adhered to the bottom, planktonic phase and floating biofilm. Quantification of initial biofilm (0 h) was also graphed to show initial *S. aureus* biofilm before treatment with *Bacillus* sp. H21. Floating biofilm was detected only after 24 h of co-cultivation. (*) denotes significant differences between biomass in the different *S. aureus* phases; (**) denotes significant differences between total co-cultured *S. aureus* biomass and untreated single *S. aureus* controls ($p < 0.05$). *p* values were obtained using one-way ANOVA followed by Bonferroni post-hoc analysis.

Figure 3. Anti-biofilm activity of the purified levan-type EPS from *Bacillus* sp. H21 **(A)** Inhibition of biofilm formation by *S. aureus* V329 and L33, in the presence of different concentrations of semi purified EPS. **(B)** Eradication of *S. aureus* V329 and L33 biofilms after treatment with different concentrations of the EPS. Data is shown as residual biofilm after treatment. In both panels, **A** and **B**, 100% represents untreated (0 mg/mL EPS) *S. aureus* cultures. Biofilm was quantified through crystal violet staining. Statystical analysis was carried out using two-way ANOVA followed by Bonferroni post-hoc. Treatments sharing letters indicate no significant differences ($p > 0.05$).

Figure 4. Bacterial viability and 3D structure of biofilms formed by *S. aureus* V329 and *S. aureus* L33 in the presence of semi-purified EPS-H21 (1 mg/mL). Untreated controls correspond to PBS treatment. Co-culture of *S. aureus* strains with *Bacillus* sp. H21 was also tested. Only adhered biofilms composed mainly of staphylococcal cells were studied. The LIVE/DEAD BacLight Bacterial Viability Kit was used, in which red staining indicates cells with damaged membranes that are considered to be dead, and green staining cells with intact membranes. Scale bar is represented for the z-axis of each image (20 µm). In all cases, the edges of the x- and y-axes have a total length of 212 µm. Microphotographs (20X) of one of three independent experiments are shown.

Figure 5. Effects of EPS-H21 on the viability of bovine cells. Different EPS-H21 concentrations were tested for 24 h to evaluate their cytotoxic effect on MAC-T (mammary alveolar) and BoMac (macrophages) bovine cell lines by the Thiazolyl Blue Tetrazolium Bromide method. Values are shown as mean ± SEM of OD570nm. * denotes significant differences with respect to the untreated control ($p < 0.05$) according to two-way ANOVA followed by Bonferroni post-hoc analysis.

'**Declarations of interest:**

none'

Highlights

Teat-apex colonizing *Bacillus* was negatively associated to udder inflammation in cows

Bacillus sp. H21 produces an exopolysaccharide that antagonizes *S. aureus* biofilms

H21 strain has bioprospective potential as a probiotic and for its bioactive molecules

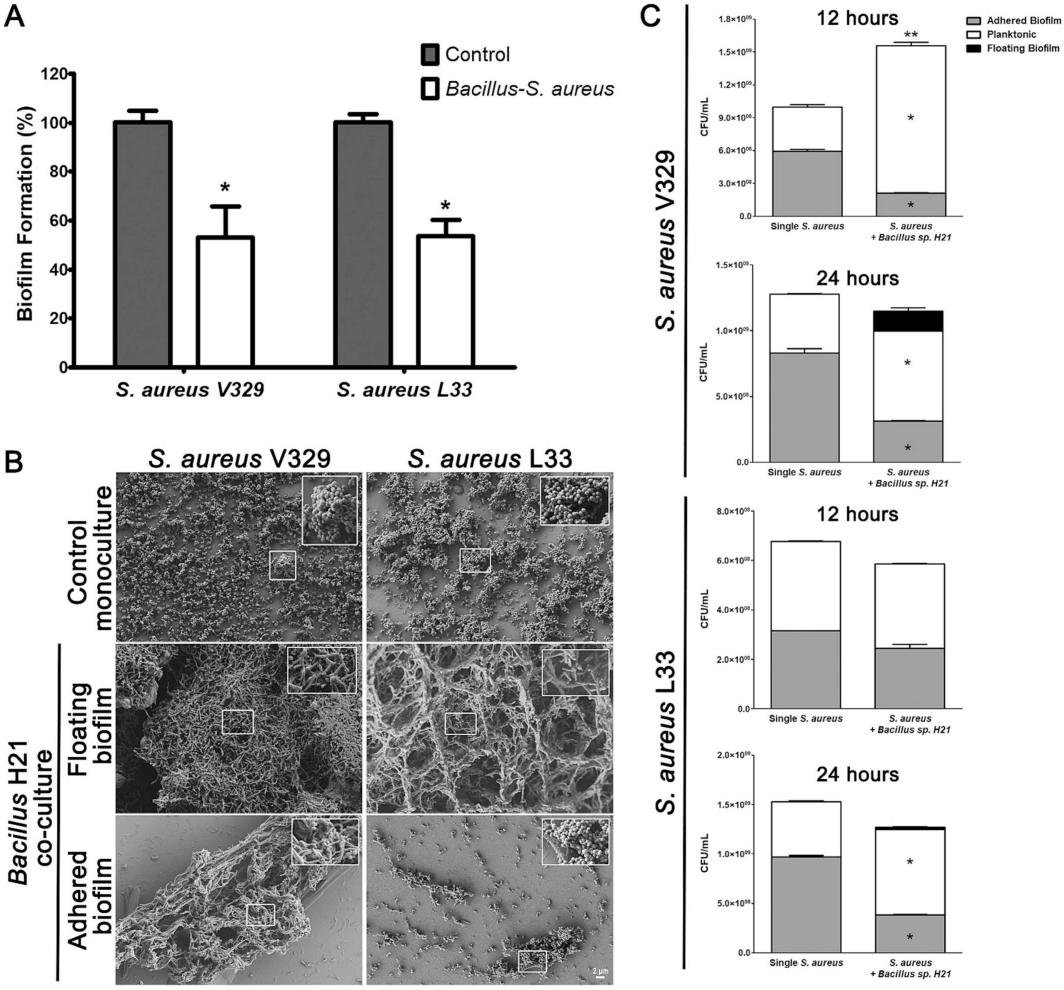
A
Control
Bacillus-S. aureus
Biofilm Formation (%)
120
100
80
60
40
20
0
*
*
S. aureus V329
S. aureus L33
B
S. aureus V329
S. aureus L33
Control monoculture
Bacillus H21 co-culture
Floating biofilm
Adhered biofilm
2 µm
C
S. aureus V329
12 hours
Adhered Biofilm
Planktonic
Floating Biofilm
CFU/mL
**
*
*
Single S. aureus
S. aureus + Bacillus sp. H21
24 hours
CFU/mL
*
*
Single S. aureus
S. aureus + Bacillus sp. H21
S. aureus L33
12 hours
CFU/mL
Single S. aureus
S. aureus + Bacillus sp. H21
24 hours
CFU/mL
*
*
Single S. aureus
S. aureus + Bacillus sp. H21

Figure 1

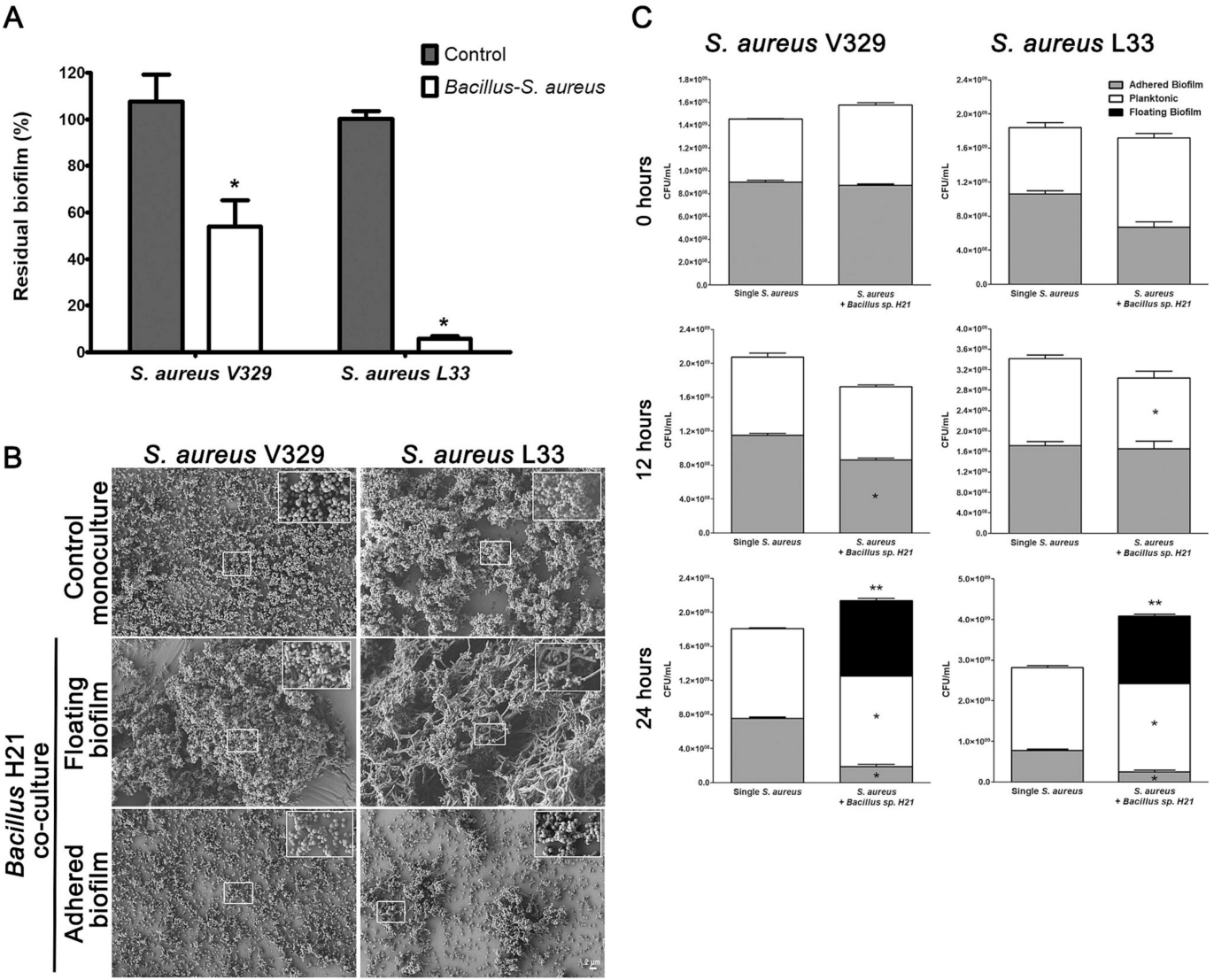
A
Control
Bacillus-S. aureus
Residual biofilm (%)
120
100
80
60
40
20
0
*
*
S. aureus V329
S. aureus L33
B
S. aureus V329
S. aureus L33
Control monoculture
Bacillus H21 co-culture
Floating biofilm
Adhered biofilm
2 µm
C
S. aureus V329
S. aureus L33
Adhered Biofilm
Planktonic
Floating Biofilm
0 hours
12 hours
24 hours
CFU/mL
Single S. aureus
S. aureus + Bacillus sp. H21
*
**

Figure 2

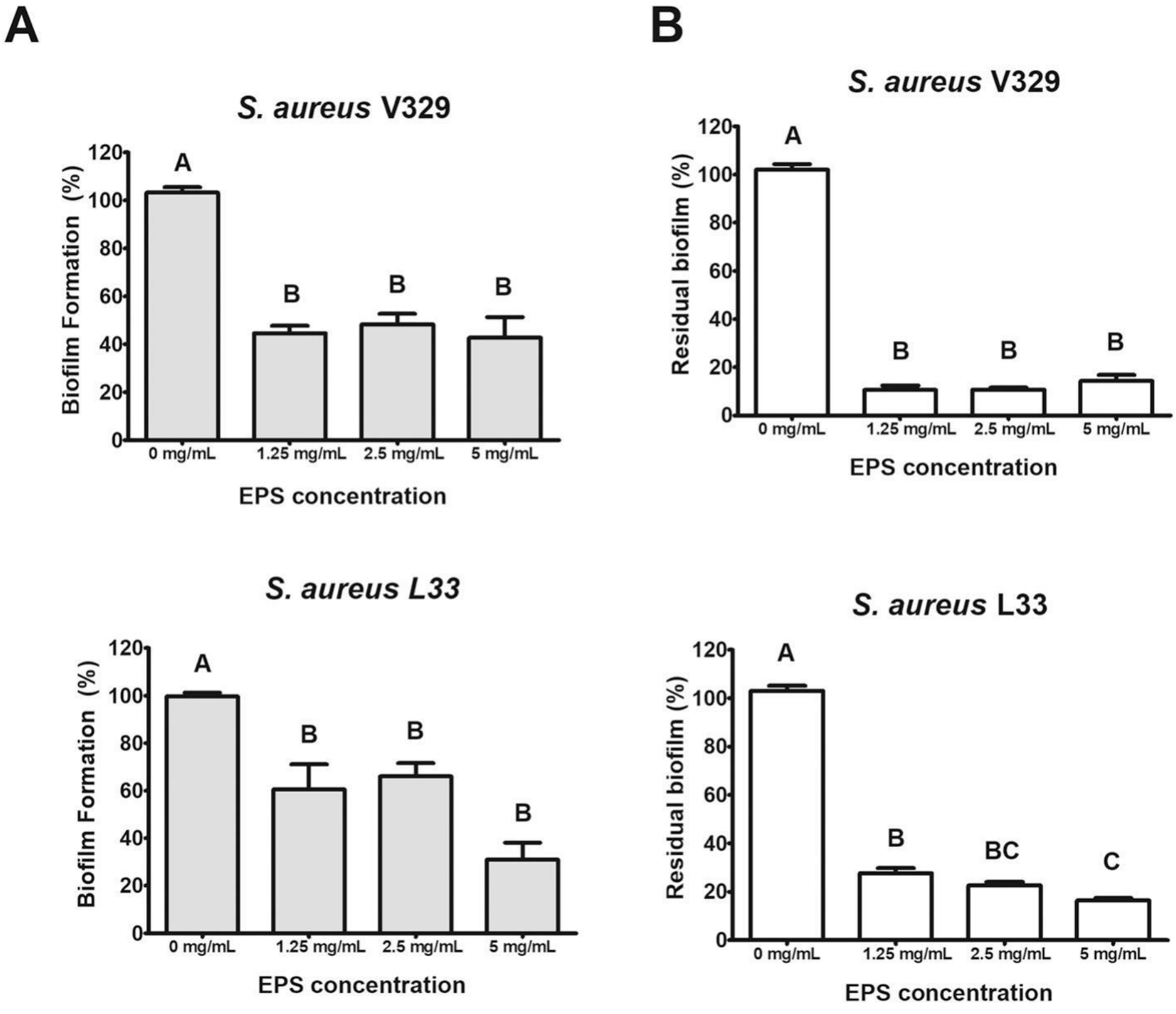
A
S. aureus V329
Biofilm Formation (%)
120
100
80
60
40
20
0
A
B
B
B
0 mg/mL
1.25 mg/mL
2.5 mg/mL
5 mg/mL
EPS concentration
B
S. aureus V329
Residual biofilm (%)
120
100
80
60
40
20
0
A
B
B
B
0 mg/mL
1.25 mg/mL
2.5 mg/mL
5 mg/mL
EPS concentration
S. aureus L33
Biofilm Formation (%)
120
100
80
60
40
20
0
A
B
B
B
0 mg/mL
1.25 mg/mL
2.5 mg/mL
5 mg/mL
EPS concentration
S. aureus L33
Residual biofilm (%)
120
100
80
60
40
20
0
A
B
BC
C
0 mg/mL
1.25 mg/mL
2.5 mg/mL
5 mg/mL
EPS concentration

Figure 3

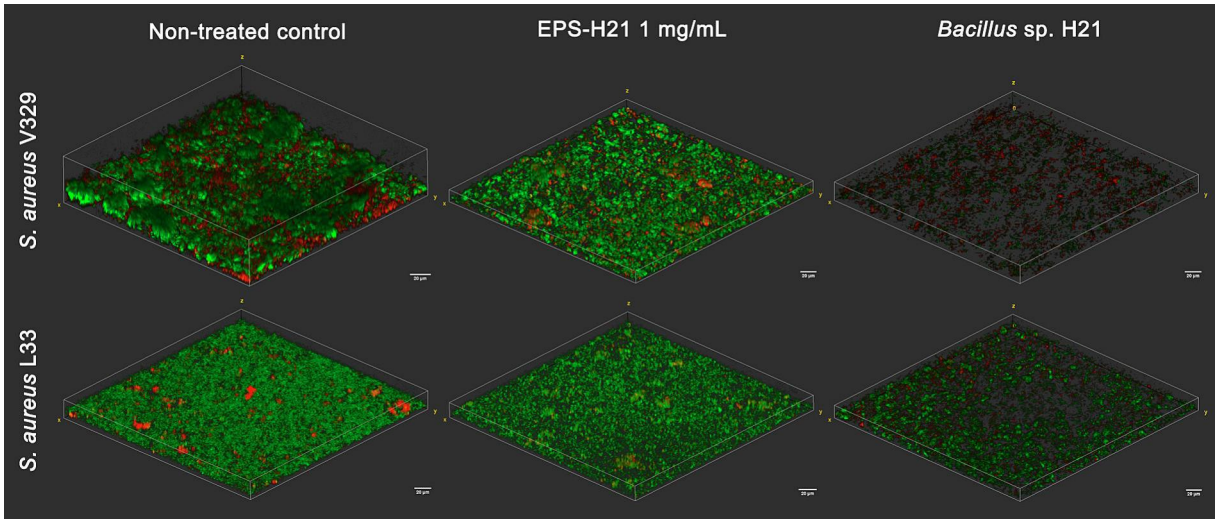
Non-treated control
EPS-H21 1 mg/mL
Bacillus sp. H21
S. aureus V329
S. aureus L33

Figure 4

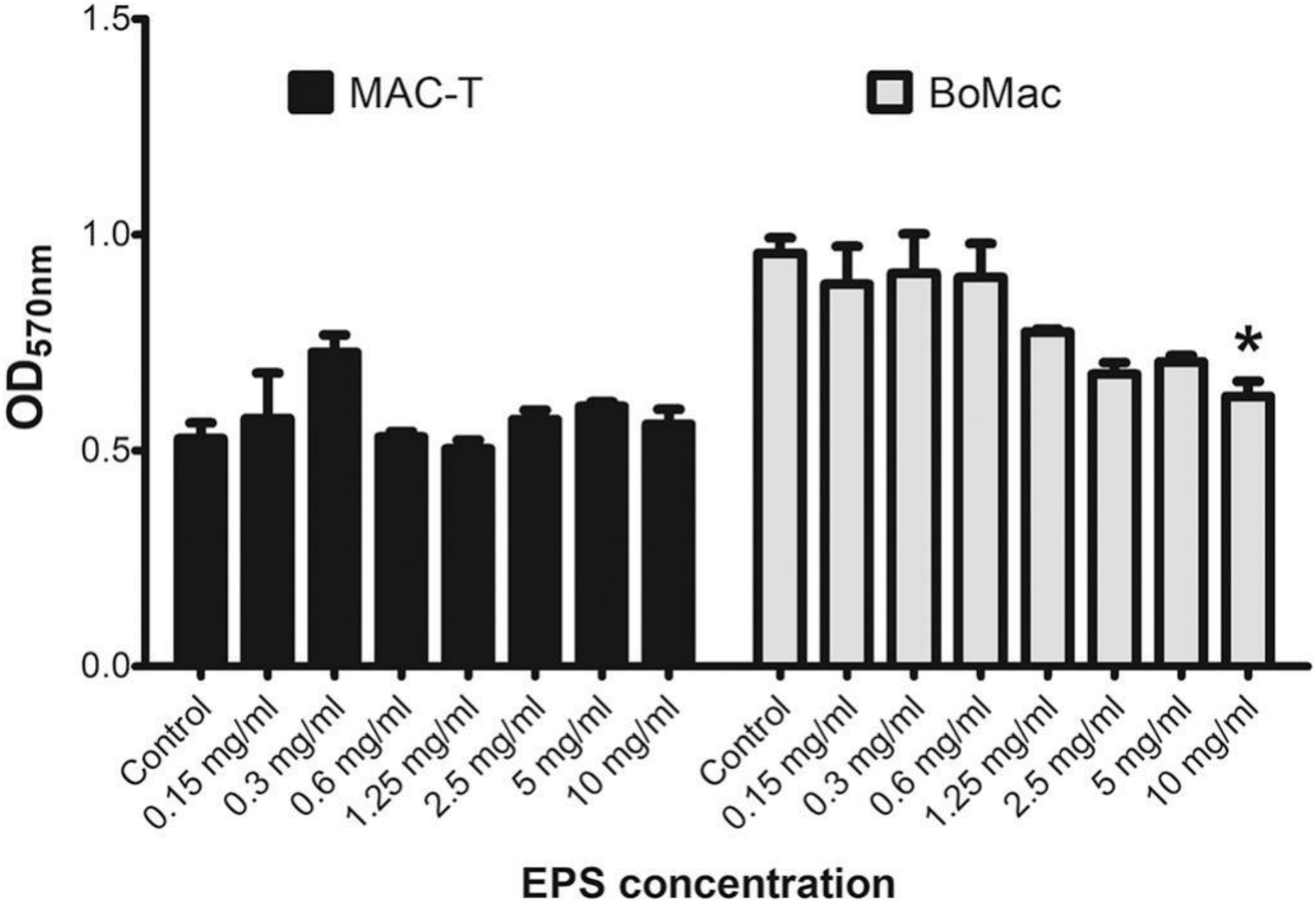
1.5
1.0
0.5
0.0
OD570nm
MAC-T
BoMac
*
Control
0.15 mg/ml
0.3 mg/ml
0.6 mg/ml
1.25 mg/ml
2.5 mg/ml
5 mg/ml
10 mg/ml
Control
0.15 mg/ml
0.3 mg/ml
0.6 mg/ml
1.25 mg/ml
2.5 mg/ml
5 mg/ml
10 mg/ml
EPS concentration

Figure 5