Lugdulysin of *Staphylococcus lugdunensis*, a metalloprotease that inhibits and disrupts protein biofilm of *Staphylococcus aureus*

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Abstract

Background

Staphylococcus lugdunensis is a commensal skin microorganism that, unlike other coagulase-negative staphylococci, presents increasing clinical importance. This species yields a metalloprotease called lugdulysin that may contribute to its higher degree of virulence. This study aimed to determine the biochemical characterization of the lugdulysin produced by *S. lugdunensis* clinical isolates and investigate its effect on the formation and disruption of biofilm of *Staphylococcus aureus* isolates. The protease was isolated and characterized for its optimal pH and temperature, activity in the presence of inhibitors and enzymatic kinetics. The influence of metal cofactor supplementation on proteolysis was also evaluated, with and without inhibitors. Finally, the protease capacity to inhibit and disrupt biofilms of different *S. aureus* lineages and biofilm matrix was analyzed.

Results

The protease optimal pH and temperature were 7.0 and 37° C, respectively. EDTA inhibited the protease, and the activity was not recovered by divalent ion supplementation. In addition, divalent ions did not change enzymatic activity without inhibitors, which was stable for up to 3 hours. Its structure was determined via homology modelling. The protease significantly inhibited the formation and disrupted established biofilms of *S. aureus* isolates with protein biofilm.

Conclusions

This study confirmed features of the lugdulysin metalloprotease and showed that this *S. lugdunensis* virulence factor may be a new competition mechanism and/or modulation of the staphylococcal biofilm.

Background

Coagulase-negative staphylococci (CoNS) are usually found in the microbiota of the skin and mucosa and has become an opportunistic pathogen due to their ability to colonize invasive medical devices, causing bloodstream infection [1]. Although most CoNS tend to have more latent pathogenicity, *Staphylococcus lugdunensis* is an exception with a higher virulence degree [2, 3]. This pathogen is the second leading cause of CoNS endocarditis [4, 5], and it is well-known for its pathogenicity in bone and joint infections [2, 6, 7]. *Staphylococcus lugdunensis* can adhere to cell-surface proteins such as fibronectin and fibrinogen and produce toxins, likewise *S. aureus* [8–10]. Moreover, this species also carries *agr* [11], *fbl* [9, 12], *atlL* [13], *vwbl* [14] and *slush* [9, 15] genes that encode factors capable of contributing to its virulence.

In vitro studies have identified proteolytic factors that may explain clinical manifestations of staphylococcal infections [7, 16]. Lately, Argemi et al. [7] described a metalloprotease produced by *S. lugdunensis* clinical isolates named lugdulysin. Lugdulysin, is a hyicolysin-like protease, a metalloprotease found in *Staphylococcus hyicus* associated with osteoarticular infections [7, 17]. The involvement of metalloproteases in human diseases occurs due to their ability to remodel the extracellular matrix, as observed in osteomyelitis, tumor invasion, metastasis, and vascular inflammatory diseases [18–20]. Many studies support the hypothesis that proteases could play a

role in the formation and stability of microbial biofilms from many species [21, 22]. In *S. aureus* isolates, these enzymes can limit the growth and detachment of biofilm by *sarA*- and *agr*-mediated mechanism, respectively [22, 23]. Proteases are also necessary to induce *Staphylococcus epidermidis* biofilm formation by processing the Aap protein [24]. Additionally, Connely et al. [25] have demonstrated that *Bacillus subtilis* lacking extracellular proteases could not produce biofilms [25]. In all cases, the biofilm matrixes were mainly composed of proteins like occurs in the biofilm of *S. lugdunensis* isolates [9, 26, 27].

Despite the initial characterization of lugdulysin carried out by Argemi et al. [7], its chemical and structural characteristics remain unknown. Hence, this study aimed to elucidate biochemical aspects of lugdulysin and investigate its influence on the formation and disruption of biofilm from *S. aureus* isolates belonged to different lineages and presenting different biofilm matrices.

Results

Protease purification and expression identification

Bands of 50kDa obtained after electrophoresis were excised from PAGE and MALDI-TOF MS and the search for homologous sequence indicated that it corresponded to putative neutral metalloprotease produced by *S. lugdunensis* (Table 1). The subcellular localization prediction by Gpos-mPLoc and PSortB describes the sequence as extracellular (data not shown). The protein has a pre-protein region with yet unknown function [7]. The secondary structure, according to I-TASSER, shows that the protein is composed of 64.73% in loop, 13.27% in sheet and 21.97% in helix, with no transmembrane domains (Fig. 1). The protease sequence was also analyzed for its genetic ontology (Fig. 2). Due to the presence of the *HEXXH* domain, the enzyme requires a zinc molecule in its catalytic site to be active. The isoelectric point of the enzyme is 4.99. Therefore, the enzyme is possibly active whilst negatively charged.

Table 1

Identification of the metalloprotease using purified secreted proteins from *Staphylococcus lugdunensis* supernatant by searching for homologous sequences with the Mascot software online database

Score	Mass (kDa)	Matches	Description
385	23972	13	Chain A, use of the neutron diffraction HD exchange technique to determine the conformational dynamics of trypsin
192	26093	5	Chain A, bovine trypsin complexed with Rpr131247
212	66198	16	Keratin 1 [<i>Homo sapiens</i>]
109	66230	6	Keratin, type II cytoskeletal 2 epidermal [<i>Pan troglodydes</i>]
173	62320	8	Cytokeratin 9 [<i>Homo sapiens</i>]
162	62932	7	Predicted: keratin, type I cytoskeletal 10 isoform X 1 [<i>Pan troglodydes</i>]
53	48667	2	Predicted: low quality protein: polymerase-2-like [<i>Thamnophis sirtalis</i>]
52	50020	1	Neutral metalloprotease [<i>Staphylococcus lugdunensis</i>]
46	38050	1	Hypothetical protein [uncultured <i>Microgenomates</i> bacterium Rifle_16ft_4_minimus_37633]
42	32424	2	Predicted: trypsin alpha-like [<i>Stomoxys calcitrans</i>]
37	31095	1	Hypothetical protein [<i>Paenibacillus</i> sp. Root52]
35	24467	2	Chain E, Crystal structures of rat anionic trypsin complexed with the protein inhibitors Appi and Bpti
28	31710	1	Predicted: low quality protein: trypsin II-P29-like [<i>Haliaeetus albicilla</i>]
22	53182	1	Predicted: uncharacterized protein LOC105797379 [<i>Gossypium raimondii</i>]
21	28005	2	CG8299 [<i>Drosophila melanogaster</i>]
17	206260	3	Zinc finger Isd1 subclass family protein [<i>Tetrahymena thermophila</i> SB210]

Staphylococcus lugdunensis metalloprotease is highlighted in bold with the respective match, score, and its predicted mass (kDa). Other proteins with more matches are common contaminants inherent to the methodology.

Protease biochemical characterization

The proteolytic enzyme activity from 546s *S. lugdunensis* isolate identified by mass spectrometry was measured by a colorimetric biochemical assay using azocasein as substrate. The EDTA caused a significant reduction in proteolytic activity, with a statistically significant decrease ($p < 0.01$) compared to the activity without inhibition (Fig. 3A). There was no recovery in activity with any metals supplemented after EDTA inhibition (Fig. 3B). The figure shows activity with 5 mM EDTA and no metals added as a control. Then, 1 mM calcium, magnesium, zinc, and manganese were added in the other bars showing no significant change between themselves and control. Metal saturation in the medium was not able to recover the enzymatic activity of lugdulysin. Not only was there no recovery of activity after EDTA inhibition by metal ion supplementation, as there was no significant change in proteolytic activity with different metal supplementation without inhibition. In Fig. 3C, the first bar shows unsupplemented protease activity, while the other bars show the same protease supplemented with 1 mM

calcium, magnesium, zinc, and manganese. There is no significant difference between unsupplemented and supplemented protease activity. A negligible activity level was observed from pH 2.0 to 4.0, with a slight increase in activity from pH 6.0 and reaching peak activity at pH 7.0, reducing at pH 8.0 (Fig. 3D).

- Regarding the influence of temperature (Fig. 3E), it is notable that the highest proteolytic activity are shown between 37°C and 40°C, with temperatures above or below these values exhibiting considerable decrease. The proteolytic activity begins immediately, with absorbance above control in 10 minutes of reaction (Fig. 3F). The activity reached a plateau around one and a half hour, with no significant increase up to 3 hours of activity. The determination of K_m and V_{max} was obtained with the substrate saturation in the enzymatic activity. The values obtained from a non-linear regression, are shown in Figs. 3G and 3H.

Disruption and inhibition of biofilm production by proteases

Trypsin can determine the biofilm composition between protein and non-protein biofilm, as it degrades specifically the proteins present in it. Figure 4A shows the effect of trypsin or lugdulysin on biofilm formation in six staphylococcal isolates. Trypsin inhibited the formation of more than 70% of the *S. lugdunensis* protein biofilm and between 60% and 70% of the *S. aureus* protein biofilm. Lugdulysin metalloprotease showed a reduction above 50% for *S. aureus* protein biofilm and between 20% and 40% for *S. lugdunensis* biofilm. Less than 10% inhibition in *S. aureus* polysaccharide biofilm was observed.

Finally, the protease disruptive potential in pre-formed biofilm was evaluated. Trypsin disrupted between 40% and 50% of *S. aureus* protein biofilm and more than 70% of *S. lugdunensis* biofilm. Lugdulysin promoted a reduction from 40–50% in *S. aureus* protein biofilm, similar to trypsin, but no biofilm reduction was observed for *S. lugdunensis*. Contrastingly, the impact of lugdulysin in *S. lugdunensis* biofilm was shallow compared to trypsin ($p < 0.0001$). Non-protein matrix biofilm isolates did not shown any reduction with the addition of trypsin or lugdulysin (Fig. 4B).

Discussion

Lugdulysin could be involved in *S. lugdunensis* pathogenicity according to previous findings. However, its fundamental biochemical properties had not yet been determined. Based on the genomic annotation of *S. lugdunensis*, only one metalloprotease presents a molecular weight of 37 kDa produced by this species, as briefly proposed by Argemi et al. [7]. The present study supports this finding, where the extracellular proteins were concentrated, and the proteolytic activity was confirmed by degradation of azocasein. This assay was also used to investigate the influence of EDTA, a metalloprotease inhibitor that extinguished the enzyme's proteolytic activity.

Furthermore, the similarity of the protease to the previously characterized enzyme (MEROPS Accession number MER0001182) was confirmed by mass-spectrometry peptide sequencing. As there was no previous characterization of this enzyme, various functional properties, such as its activity lifespan, optimal pH and temperature were investigated, the influence of inhibitors and supplementation with metallic ions. We further analyzed different functional properties and through *in silico* analysis, providing information regarding cell location, isoelectric point, putative cleavage preference and the 3D structure of lugdulysin. Zinc-ion binding sites were identified, which was already expected by the presence of the *HEXXH* domain. Predict Protein server identified putative metalloendopeptidase activity, cleaving L-amino acid-rich peptides at inner parts of peptides

instead of amino or carboxy-terminal [28]. Given the presence of a signal peptide, the protease is likely secreted, as previously suggested by Argemi [7].

In the present study, EDTA significantly reduced the proteolytic activity, confirming the lugdulysin as a metalloprotease [29, 30]. In our attempt to recover its activity after inhibition, there was no observable recovery after adding Ca^{2+} , Mg^{2+} , Zn^{2+} or Mn^{2+} . It is possible that its structure is affected by the metal removal, which could prevent the recovery of the proteolytic activity [31]. It is known that metalloproteases respond diversely to different metallic ions. The response of the lugdulysin to ions is similar to aureolysin, another staphylococcal metalloprotease produced by the *S. aureus* [32]. For both metalloproteases, after inactivation with EDTA, ions were also unable to restore the protease activity. On the other hand, hyicolysin, a metalloprotease of *S. hyicus*, has its activity entirely recovered by adding zinc and cobalt, and partially restored with Mg^{2+} , Ca^{2+} and Cu^{2+} [17]. These data indicate a close relationship with *S. aureus*, as already shown among other features presented by both species, such as adhesins and hemolysins [9].

The biochemical characterization indicated that, despite its structural similarity with hyicolysin [7], lugdulysin shares more biochemical similarities with aureolysin. For instance, the optimum pH and temperature of hyicolysin are 7.4 to 7.9 and 55 °C, respectively [17], while for lugdulysin and aureolysin is 7.0 and 37 °C. Indeed, whilst the clinical relevance of hyicolysin remains unclear, it is known that aureolysin play an essential role in the pathogenesis of *S. aureus* infections. This metalloprotease can modulate the pathogenesis of osteomyelitis by triggering alterations in bone turnover [16], promotes the escape of immune response, inhibiting the complement system by degradation of C3 component [33] and cleaves staphylococcal surface-associated proteins allowing the transition from an adherent to an invasive phenotype [34]. Thus, the biochemical properties of lugdulysin, like aureolysin, could be related to the emergence of *S. lugdunensis* as pathogenic bacteria implicated in severe infections, especially endocarditis and osteoarticular infections [18].

The effect of proteases against biofilms of *S. aureus* is already well established in the literature [35, 36]. Here, lugdulysin significantly reduced the *S. aureus* pre-formed protein biofilm, similar to trypsin, inhibiting the production of protein biofilm by *S. aureus*. Diversely, the same outcome did not occur in *S. lugdunensis* isolates. To this species, lugdulysin did not affect the pre-formed biofilm, causing only a slight inhibition in biofilm production. These results are surprising because not even the biofilm of *S. aureus* is so resistant to own metalloprotease as the biofilm of *S. lugdunensis* appears to be resistant to lugdulysin. *In vitro* studies demonstrated that aureolysin significantly disrupt the *S. aureus* pre-formed protein biofilm [22, 36]. Even more, Abraham & Jefferson [36] found that the inactivation of the aureolysin gene augments *S. aureus* biofilm production. Nonetheless, some species produce proteases that can protect and modulate the biofilm formation whereas attack biofilms of bacteria from other species [37, 38]. However, to our knowledge, there are no reports to date of any staphylococcal protease that simultaneously acts to protect the biofilm of the species that produces it and causes damage to the biofilm of another species. The resistance of the *S. lugdunensis* protein biofilm to its protease could demonstrate a possible new mechanism that may generate a competitive advantage among staphylococci.

Our study presents some limitations. First, biochemical studies are needed for a better comprehension of lugdulysin characteristics, such as substrate specificity. Second, we have not established the mechanism by which lugdulysin affects *S. aureus* biofilm. It remains unclear whether it degrades the protein matrix or acts on

proteins involved in biofilm gene expression. Also, the effect of lugdulisin on *S. aureus* was only evaluated *in vitro*, which limits the understanding of the role of this metalloprotease as a virulence factor.

Conclusions

This report identified optimal conditions of function and stability of the lugdulysin produced by *S. lugdunensis* isolates from human clinical specimens. Moreover, we showed the striking effect of this protease on the formation and dispersion of biofilm from different *S. aureus* isolates, a pathogen of significant clinical relevance which biofilm has been associated with antimicrobial resistance and invasive medical devices related infections. These preliminary findings show that lugdulysin may be a new mechanism of competition and/or modulation of the staphylococcal biofilm.

Methods

Bacterial isolates

Previous published bacterial isolates of *S. lugdunensis* (541s and 546s) and *S. aureus* (63a, 1636a, 1176a and 1348a) [9, 39, 40] were selected for the study. These isolates belonged to the Laboratory of Hospital Infection collection at the Federal University of Rio de Janeiro. They presented distinct genotypic profiles determined by the PFGE technique. In addition, the biofilm characteristics and biochemical composition were also determined previously, according to Ferreira et al. [41] (Table 2).

Table 2
Characteristics of the staphylococci isolates used in the present study

Species	Isolate number	Clinical specimen	Methicillin susceptibility	Clonality/ ST	Biofilm matrix composition	Biofilm production	Reference
<i>S. aureus</i>	63a	nares	resistant	BEC/239	PRT + PSC	Strong	[40]
	1636a	blood	resistant	USA100/5	PRT + eDNA	Strong	[39]
	1176a	urine	sensitive	USA800/5	PSC	Strong	[40]
	1348a	blood	sensitive	USA400/1	PSC	Strong	[40]
<i>S. lugdunensis</i>	541s	blood	sensitive	A	PRT	Strong	[9]
	546s	blood	sensitive	A	PRT	Strong	[9]
BEC – Brazilian endemic clone; ST – sequence type; PRT – proteic; PSC – polysaccharide.							

Protease isolation

Colonies of *S. lugdunensis* 546s isolate grown on Triplic Soy Agar (TSA) (Becton Dickinson, USA) were transferred to 1L Tryptic Soy Broth (TSB) (Becton Dickinson) and subjected to overnight incubation at 37°C under 200rpm. After growth, isolates were centrifuged for 25 minutes at 7000xg (4°C). The supernatant with the protease was subjected to vacuum pump filtration (GAST - IDEX Health & Science, LLC, USA) with a 0.22µm membrane (Sartorius Stedim Biotech SA Frankfurt., Germany). The recovered supernatant was then subjected to a tangential

flow filtration procedure (Millipore Lab Scale TFF System, Millipore, Mass., USA) using an initial volume of 1L and reducing to approximately 30 mL for approximately 4 hours. The supernatant was concentrated using a 50 kDa ultrafiltration membrane (Pellicon XL 50 - Millipore), and lyophilized. The dry weight was determined and the material was resolubilized in Tris-HCl 20 mM pH 7.0.

Protein profile analysis

The concentrated supernatant was analyzed using SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) with 15% acrylamide according to Laemli [42] and stained according to Neuhoff [43]. The coomassie-stained gel was then destained and submitted to in-gel digestion using trypsin 20 µg / mL (Sigma-Aldrich), adapted from Shevchenko [44]. The trypsinized peptides were analyzed in a MALDI-TOF/TOF type mass spectrometer (Microflex LT, Bruker, USA) [44]. Mascot Distiller software was used to analyze the processed bands for MALDI-TOF/TOF analysis and raw data (Matrix Science, version 2.2.1.0). Protein identification was performed by searching for homologous sequences in online Mascot software (Table 2). *In silico* analysis of the metalloprotease was performed with the sequence deposited by previous characterization [7], available at UniProt under the accession number A0A133QCC8. Proteases domain and other biochemical characteristics were proposed using the automated Predict Protein (<https://predictprotein.org/>), using a database of up-to-date public sequences, performing alignments, and predicting protein function and structure [45]. Prediction of cell location was performed using automated domain service, while analysis of other biochemical characteristics was performed using the automated service to predict subcellular localization of Cell-PLoc proteins, Gpos-mPLoc, for prediction specifically in Gram-Positive bacteria [46]. Those data correlated to data obtained in PSortB, which performed the same analysis [47]. The putative structure was obtained via homology modeling using the protein prediction webserver I-TASSER [48].

Protease biochemical characterization

To determine proteolytic activity, a colorimetric method determined by Lei and coworkers [29] was carried out, with adaptations. As a positive control, trypsin (Sigma-Aldrich) (initial concentration 20 µg/mL) was used, and, for the negative control, the protease received immediate addition of 10% trichloroacetic acid (TCA). The activity units (U) that were determined as the variation of 440 nm absorbance per mg of protein per hour. This protocol was used to evaluate the effect of inhibitors on protease, with the addition of 5mM EDTA in the reaction [47]. Various divalent metals were supplemented in the reaction medium to evaluate the metal influence on proteolytic activity, following proteolytic activity method [29] and adding 1 µL of 1M metallic salt. In this study, Calcium Chloride (VETEC, Brazil), Zinc Chloride (Isofar, Brazil), Manganese Chloride (Carlo Erba, Italy) and Magnesium Chloride (Sigma-Aldrich, USA) were used. To assess the recovery of proteolytic activity, the azocasein method [29] was carried out with EDTA-inhibited enzyme. Various metallic salts were supplemented in the reaction medium to overcome the EDTA chelating potential following the abovementioned method. The optimum pH was determined with various buffers with different pH were used. The selected buffers were pH 2.0 KCl-HCl buffer, pH 4.0 acetic acid/sodium acetate buffer, pH 6.0 and pH 8.0 phosphate buffer, 20 mM. The method was conducted as per the previously described method [29] with 37°C incubation. To evaluate optimum enzyme operating temperature, the azocasein method [29] was performed at pH 7.0 in different temperatures: 10°C, 20°C, 30°C, 40°C, 50°C, 60°C, 90°C. The rate of substrate cleavage was determined with a substrate serial dilution curve following, for 15 minutes. The concentrations of azocasein used were: 0.04, 0.08, 0.16, 0.32, 0.640 and 1.28 mg/mL. Michaelis-Menten and Lineweaver-Burk curves were plotted based on these data to determine K_m and V_{max} for azocasein.

Protease influence on biofilm formation

Metalloprotease influence on biofilm formation was analyzed by the quantitative micromethod to evaluate biofilm production described by Stepanovic [49] with modifications. Twenty microliters of a bacterial suspension in sterile distilled water corresponding to the 0.5 McFarland standard were added in triplicate to the wells containing 180 µl of tryptic soy broth, TSB (Becton, Dickinson and Company; Sparks, MD, USA), supplemented with 1% glucose (Isofar; Duque de Caxias, RJ, Brazil). Finally, 15 µL of freeze-dried protease diluted in PBS (20 µg/mL) were added in triplicate for each. The plates were incubated at 37°C for 24h. Then, wells were washed twice with sterile PBS buffer (pH 7.2), dried for 1 h at 60°C, and stained with 200 µL of a 0.1% safranin solution (w/v, in water) for 15 min. After, wells were washed twice again, and 200 µL of a 95% ethanol solution was added. Absorbance (OD492nm) was read after 30 min of incubation at room temperature. The culture medium without bacterial inoculum was used as a negative control and trypsin (20 µg/mL) was used as the positive control. All experiments were performed in triplicate at 3 independent times.

Protease influence on pre-formed biofilm

To observe the protease influence in pre-formed biofilm, the biofilm formation was initially performed on 96-well plate as previously described [49] with modifications. After the bacterial suspension is added to the wells and the microplate were incubated at 37°C for 24h. The medium was discarded, and three washing steps with 200 µL sterile PBS buffer, pH 7.2 (Laborclin, Brazil) were performed to remove unbound cells. Then, 135 µL of PBS 100 mM pH 7.5 and 15 µL of freeze-dried protease (20 µg/mL) were added in triplicate for each isolate, and the 96-well plate was submitted to a second incubation at 37°C for 1 = h. After, the medium was discarded, another wash was performed with 200µL PBS to remove unbound cells, followed by incubation at 60°C for 1 hour, and wells were stained with 200 µL of a 0.1% safranin solution (w/v, in water) for 15 min. After, wells were washed twice again, and 200 µL of a 95% ethanol solution was added. Absorbance (OD492nm) was read after 30 min of incubation at room temperature. The culture medium without bacterial inoculum was used as a negative control, and trypsin (20 µg/mL) was used as the positive control. All experiments were also performed in triplicate at three independent times.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Figures

Figure 1

Predicted 3D structure of the *Staphylococcus lugdunensis* neutral metalloprotease by I-TASSER homology modelling. TM-score = 0.44 ± 0.14 ; RMSD = 12.5 ± 4.3 Å. HEXXH (HEYQH) domain highlighted in red (1A). Residues HIS241, HIS245 (highlighted in yellow) and GLU242 (highlighted in beige) are the catalytic triad, zoomed in Figure 1B. GLU268 was also highlighted as a yellow stick as it is a known catalytic residue conserved among M30 proteases. The catalytic zinc ion is represented by a grey sphere pointed by an arrow. The structure is C- α trace coloured according to sequence from dark blue (N terminus) to red (C terminus). Distances measured from the catalytic zinc to the catalytic amino acids were 2.8, 3.1 and 4.0 Å for the residues HIS241, HIS245 and GLU268, respectively. Figures were drawn using PyMOL (www.pymol.org).

Figure 2

Molecular functions of the lugdulysin metalloprotease determined by gene ontology via the Predict Protein database. The protease has two main domains, the HEXXH responsible for zinc (and other divalent ions) binding and the catalytic triad responsible for the peptidase activity. As per the highlighted boxes, the peptidase domain has aminopeptidase and endopeptidase activities, which are metal dependent. Moreover, the protease has also peptide binding capacity, which may be correlated with its activity.

Figure 3

Aspects associated with the lugdulysin activity purified from *Staphylococcus lugdunensis* supernatant using azocasein as substrate. A) Inhibition of metalloprotease activity with EDTA 5 mM (control = without EDTA) (p = 0.0045). B) Proteolytic activity recovery test with metallic ions supplementationCa²⁺, Mg²⁺, Zn²⁺or Mn²⁺. No

significant difference was found in relation to EDTA inhibition ($p = 0.9803, 0.9530, 0.9763$ and 0.9772 respectively). C) Effect in the enzymatic reaction by the addition of Ca^{2+} , Mg^{2+} , Zn^{2+} or Mn^{2+} . Supplementation with metallic ions did not present significant difference when compared to no supplementation as control ($p = 0.9930, 0.8772, 0.9950$ and 0.1715 respectively). D) Optimal pH determination. The X-axis represents the pH values tested, while y-axis shows the enzymatic activity in U. E) Determination of the optimal temperature conditions. X-axis represents temperature range used; y-axis shows the enzymatic activity in U. F) Enzymatic activity vs time. X-axis represents the timespan used; y-axis shows enzymatic activity in units of absorbance. G) Lugdulysin Michaelis-Menten constant determination. H) Double reciprocal Lineweaver-Burk graph.

Figure 4

Influence of the lugdulysin or trypsin on *S. aureus* and *S. lugdunensis* biofilms. A) Effect of trypsin or lugdulysin non biofilm formation. Trypsin inhibited more than 70% of the *S. lugdunensis* protein biofilm (isolates 541s and

546s) and between 60 and 70% of the *S. aureus* protein biofilm (isolates 1636a and 63a). There was less than 10% inhibition in polysaccharide biofilm (*S. aureus* isolates 1348a and 1176a). Lugdulysin metalloprotease showed reduction >50% of *S. aureus* protein biofilm and between 20% and 40% of *S. lugdunensis* biofilm. B) Effect of trypsin or lugdulysin metalloprotease on disruption of pre-formed biofilm. Lugdulysin and trypsin showed similar effects on *S. aureus* biofilm (1636a and 63a isolates), with reduction from 40% to 50%, and no biofilm reduction was found for *S. lugdunensis* biofilm when the lugdulysin was used. Trypsin inhibited more than 70% of *S. lugdunensis* protein biofilm (isolates 541s and 546s). Asterisks refer to comparison of treatment (lugdulysin or trypsin), with ** referring to the value of $p < 0.001$ and *** as $p < 0.0001$.