Lactoferrin modulates the biofilm formation and *bap* gene expression of methicillin-resistant *Staphylococcus epidermidis*

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ABSTRACT

Lactoferrin is an innate glycoprotein with broad antibacterial and antibiofilm properties. Lactoferrin's autonomous antibiofilm activity against Gram-positive bacteria is postulated to involve the cell wall and biofilm components. Thus, the prevention of biomass formation and eradication of preformed biofilms by lactoferrin was investigated using a methicillin-resistant *Staphylococcus epidermidis* (MRSE) strain. Additionally, the ability of lactoferrin to modulate the expression of the biofilm-associated protein gene (*bap)* was studied. The *bap* gene regulates the production of biofilm-associated proteins responsible for bacterial adhesion and aggregation. In the *in vitro* biofilm assays, lactoferrin prevented biofilm formation and eradicated established biofilms for up to 24 and 72 hours, respectively. Extensive eradication of MRSE biofilm biomass was accompanied by the significant upregulation of *bap* gene expression. These data suggest the interaction of lactoferrin with the biofilm components and cell wall of MRSE, including the biofilm-associated protein.

Keywords: Lactoferrin, methicillin-resistant *Staphylococcus epidermidis*, biofilm prevention, biofilm eradication, biofilm-associated protein gene

Introduction

Lactoferrin is an iron-binding glycoprotein constitutively present in bodily secretions and cells, including neutrophils, macrophages, and glandular epithelial cells. Endogenous lactoferrin has immunomodulatory and antimicrobial functions both in physiological and pathological conditions (Moreno-Expósito *et al.*, 2018). *In vitro* investigation of exogenous lactoferrin had revealed antibacterial and antibiofilm activities against Gram-positive and Gram-negative pathogens. These effects were demonstrated against the planktonic (free-living) and biofilm (aggregated) forms of the bacteria.

Lactoferrin demonstrated antibacterial activity against the planktonic Gram-negative bacterium, *Salmonella typhimurium* through interactions with the lipopolysaccharide (LPS) and porin of the cell surface (Naidu *et al.*, 1993). Lactoferrin also had damaging effects on the outer membrane of *Helicobacter pylori* (Wang *et al.*, 2001).

Lactoferrin increased the twitch-dependent motility of *Pseudomonas aeruginosa* through the sequestration of iron, which subsequently led to the reduction of biofilm formation (Singh, 2004). Further antibiofilm effects were demonstrated through the degradation of a protein that mediated adherence in *Haemophilus influenzae* (Qiu *et al*., 1998), and the disconnection of *Porphyromonas gingivalis* and *Prevotella intermedia* from plastic well plates, probably through the interference of bacterial adherence (Wakabayashi *et al.*, 2009). In addition, lactoferrin reduced 24-hour-old biofilms of *Escherichia coli* through disruption of the bacterial membrane and type III secretion system (Sheffield *et al*., 2012).

The antibacterial activity of lactoferrin against Gram-positive bacteria is most often associated with the protein's iron-binding ability and cidal region. Lactoferrin was shown to inhibit the growth and killed *Staphylococcus aureus* while destructed its biofilms (Håversen *et al.*, 2010; Meyle *et al.*, 2010; Lizzi *et al.*, 2016). Lactoferrin also prevented the biofilm

formation of *Streptococcus mutans* through iron sequestration and inhibition of adhesion (Francesca *et al.*, 2004).

Leitch and Willcox (1999) established that lactoferrin improved the inhibitory and killing actions of vancomycin, against a clinical isolate of *Staphylococcus epidermidis*. The cationic lactoferrin was hypothesized to interact with the anionic components of the bacterial cell wall, which consequently assisted the killing actions of vancomycin and lysozyme (Leitch and Willcox, 1999). A decade later, Venkatesh *et al.* (2009) reported the reduction of *S. epidermidis* biofilm biomass (μm^3) and mean thickness (μm) after treatment with lactoferrin alone and in combination with vancomycin or nafcillin (Venkatesh *et al.*, 2009).

More than a decade after this 2009 report, there is still a lack of evidence explaining the antibiofilm effects of lactoferrin against Gram-positive bacteria in the absence of iron, antibiotics, antiseptics, and lysozyme. Similar data is not available for multidrug-resistant bacterial strains of clinical significance such as the methicillin-resistant *S. epidermidis* (MRSE). Hence, the abilities of lactoferrin to independently prevent MRSE biofilms and eradicate established biofilms were evaluated. In order to elucidate the potential of lactoferrin to modulate vital biofilm-related genes, expression of the biofilm-associated protein gene (*bap*) was evaluated in MRSE biofilms treated with lactoferrin.

Methods and Materials

Prevention of biofilm formation

Suspensions of methicillin-resistant *S. epidermidis* (MRSE, ATCC 49461, Microbiologics, USA) were prepared at 4×10^6 colony forming units (cfu)/mL, in tryptone soya broth. The bacteria in suspension were incubated with lactoferrin (bovine, Tatua Co-operative Dairy, New Zealand), for 2 to 24 hours at 37°C and under static conditions. The final doses of lactoferrin in the 96-multiwell plates were 512 to 4096 μg/mL. These doses of

lactoferrin corresponded to one-eighth (512 µg/mL) and up to the most effective concentration (4096 µg/mL), which eradicated preformed MRSE biofilms (Suppl., Figure S1). In this prevention assay, phosphate buffered saline (PBS) and vancomycin (1 and 2 µg/mL, Shanghai PI Chemicals, China) were the negative and positive control treatments, respectively. Doses of the vancomycin antibiotic were 1× and 2× the effective minimal inhibitory concentration determined against the MRSE.

At selected time points within the 24 hours, formed biofilms were assessed for biomass using crystal violet staining and cellular metabolic activity using resazurin staining. The crystal violet staining method was adopted from a published work (Stepanović *et al.*, 2001) and further optimised for *S. epidermidis*. In this crystal violet assay, biofilms were washed three times with PBS, fixed with methanol (99%), and stained with 0.1% aqueous crystal violet (Acros Organics, Belgium) solution for 15 minutes. Excess crystal violet was removed using PBS and dye absorbed by the biofilm biomasses was solubilised using 30% acetic acid-aqueous solution. The absorbance of the acid-dye solution was measured at 570 nm using a microplate reader (Spectramax M3, Molecular Devices, USA).

The resazurin staining method was adopted from the literature (Sandberg *et al.*, 2009; Van den Driessche *et al.*, 2014) and further optimised. In the resazurin assay, treated biofilms were washed with PBS and stained with 0.01% resazurin (Acros Organics, Belgium) aqueous solution for 3 hours at 37°C, in the dark. The fluorescence intensity of the resazurin dye solution was then measured at 560/590 nm. All experiments were performed under static conditions and the assay was repeated twice (N=9).

Prolonged 72-hour treatment of preformed biofilms

MRSE biofilms were grown to uniformity in 96-multiwell plates for 20 hours using 4×10^6 cfu/mL bacterial suspension in tryptone soya broth. Preformed biofilms were gently

washed with PBS and treated with 4096 and 8192 µg/mL lactoferrin for 24 hours. PBS and vancomycin were the control treatments. The treated biofilms were washed and re-treated for the same duration and doses twice again. At the end of 72 hours and three repeated treatments, biomasses dispersed from the treated biofilms into the broth were collected. The collected biomasses were transferred into new multiwell plates and sedimented using centrifugation at 1,500 rpm for 10 minutes at 4°C. The dispersed biomasses and remaining adherent biofilms in the wells post-treatment were stained with crystal violet and resazurin. This assay was repeated twice (N=9).

Expression of the *bap* gene

MRSE biofilms were grown to uniformity for 20 hours in 12-multiwell plates using 4×10^6 cfu/mL bacterial inocula. Biofilms were gently washed with PBS and treated with lactoferrin (4096 µg/mL). Treatment was conducted through incubation at 37°C for 20 hours, which is the earliest time point of significant biomass reduction by lactoferrin in preliminary experiments (Suppl., Figure S2). Biofilms were also treated with the combinatorial treatment of vancomycin (1 µg/mL) and lactoferrin (4096 µg/mL) and the positive control, vancomycin alone. The treated biomasses were collected using RNase-free water and scrapping. The samples were then exposed to cold acetone: ethanol solution (1:1, v/v) for 30 minutes with intermittent agitation (Beltrame *et al.*, 2015). These solvents aid in the removal of proteins and polysaccharides of the extracellular matrix present in the biofilm samples that may interfere with the cell lysis step (França *et al*., 2011). The now exposed bacterial cells were lysed using tris-ethylenediaminetetraacetic acid (EDTA) buffer and lysozyme (50 mg/mL) for 90 minutes with regular and vigorous mixing.

RNA was extracted with a column-based RNA extraction kit (Analytik Jena, Germany) using steps outlined by the manufacturer. RNA samples (300 ng) were reverse transcribed

using a complementary DNA (cDNA) synthesis kit (Bioline, UK) in thermocycling conditions recommended by the manufacturer, with optimisations. The cDNA samples were amplified and quantified in real-time using a SYBR® green-based polymerase chain reaction (PCR) kit (Bioline, UK) and subjected to a thermocycler (C1000 Touch Thermal Cycler, CFX96 Real-time system, Bio-rad, USA). 16S ribosomal RNA reference gene (*16S rRNA*) primers (5′–3′), forward: GGGCTACACACGTGCTACAA; reverse: GTACAAGACCCGGGAACGTA (França *et al*., 2011), and the biofilm-associated protein gene (*bap*) primers (5′–3′), forward: GAGCCAGATAAACAACAAGAAG; reverse: CATGCTCAGCAATAATTGGATC (Ceotto-Vigoder *et al.*, 2016) were used. The gene expression assay was repeated twice (N=9).

Statistical analysis

The two-tailed student's t-test was used to determine the statistical differences between the control and test treatment groups of all replicates (N=9). A p value of less than 0.05 ($p<0.05$) was recognised as a significant change.

Results

Lactoferrin at the doses of 512, 1024, 2048, and 4096 µg/mL, prevented the formation of MRSE biofilms for up to 24 hours, resulting in very minimal biomasses in the wells **(Figure 1)**. MRSE exposed to lactoferrin at 512 and 4096 µg/mL, the lowest and highest doses, respectively, had lesser biomass ($p<0.05$) and metabolic activity ($p<0.05$) than bacteria exposed to vancomycin.

In a preliminary study, lactoferrin eradicated pre-formed biofilm biomass most effectively at 4096 µg/mL (Suppl., Figure S1), hence this concentration was selected for subsequent studies. Preformed biofilms treated with 4096 µg/mL of lactoferrin at 24 hours

intervals for up to 72 hours had significantly lower amounts of adherent biofilms **(Figure 2A)**, while the amounts of dispersed biomasses were significantly higher compared to PBS and vancomycin. Comparing the treatment of lactoferrin at 24 and 72 hours, there was a significant increase in the amount of biomass of dispersed cells. However, these dispersed cells exhibited a significant reduction in metabolic activity **(Figure 2B)**. The reduced metabolic activities of the dispersed cells showed that lactoferrin is bacteriostatic against the reference strain of MRSE. This finding is consistent with other reported studies (González-Chávez *et al*., 2009; Moreno-Expósito *et al*., 2018). Additionally, MRSE biofilms treated with lactoferrin dislodged easily from the wells and were less dense in texture, compared to the PBS and vancomycin controls (Suppl., Figure S3).

In the evaluation of *bap* gene expressions in MRSE collected from treated biofilms, lactoferrin caused an upregulation of the gene by 22-fold **(Figure 3)**. The increased expression of *bap* was significant ($p<0.05$) compared to the biofilms treated with vancomycin alone and the combinatorial treatment of lactoferrin and vancomycin, which recorded upregulations of 1.6-fold and 1-fold, respectively.

Discussion

MRSE is a multidrug-resistant Gram-positive bacteria of significant clinical importance, causing infections through biofilm formation on invasive medical devices (Le *et al*., 2018). As the prevention of biofilm formation is established as an optimal approach to managing biofilm-related infections (Khatoon *et al.*, 2018), more studies are needed to elucidate the preventive abilities of potentially effective compounds. Although previously proven to be an antibiofilm agent, the potential of lactoferrin to prevent MRSE biofilm formation has not been reported.

In the current investigation, lactoferrin at 512 µg/mL, the lowest dose tested, prevented biofilm formation for up to 24 hours. This preventive ability might be attributed to lactoferrin's bacteriostatic activity (Suppl., Figure S4). This leads to the impediment of bacterial growth, reducing the number of bacteria that can adhere and establish biofilms. Additionally, lactoferrin was previously postulated to negatively impact the bacterial cell wall (Qiu J *et al*., 1998; Leitch and Willcox, 1999). Thus, the affected cell wall may be compromised of its components responsible for bacterial adhesion and aggregation. These two processes are vital to establishing biofilms. The reduced number of surviving MRSE due to growth inhibition and the probable impediment of adhesion regulating components may have collectively hindered the biofilm formation of MRSE by lactoferrin, under the current *in vitro* settings.

Results from the biofilm eradication assay further suggested the interaction of lactoferrin with components of the bacterial cell wall. As the biofilm forms and continue to develop and mature, alterations in nutrient availability, oxygen fluctuation, and an increase in toxic products trigger the bacterial cells trapped within the biofilm to disperse. These dispersed bacteria can survive and attach to other niches and re-form new biofilms and occupy new niches (Kaplan, 2010; Wang *et al*., 2011; McDougald *et al*., 2012). In this study, lactoferrin demonstrated the ability to eradicate established MRSE biofilms and increased the dispersal of biomasses after multiple treatments. The increased dispersal reduced the biofilm biomass and increased the susceptibility of the remaining cells in the biofilms (Wille and Coenye, 2020). As these dispersed cells are generally more susceptible to antimicrobial treatment than biofilm-residing cells (Verderosa *et al*., 2019), biofilm dispersal could be a crucial approach to managing pathogenic biofilms.

These observations also suggest the targeting of teichoic acids which are not only found on the bacterial cell wall but also in the biofilm matrix of *S. epidermidis* (Jabbouri and

Sadovskaya, 2010; Swoboda *et al.*, 2010). This interaction had been previously associated with charge, whereby the negatively charged teichoic acids are targeted by the positively charged lactoferrin (Vorland *et al.*, 1998; González-Chávez *et al*., 2009). The lactoferrin-teichoic acid interaction may have affected the teichoic acid's functions in biofilm formation, resulting in weakened biofilms.

Selected bacterial genes are also implicated in biofilm formation, thus the gene responsible for a cell wall protein, the biofilm associated protein (Bap), was further investigated. The *bap* gene was selected as Bap is present on the bacteria cell surface and may be affected by lactoferrin's interaction with the cell wall. In the *Staphylococcus* species, Bap is responsible for primary attachment to surfaces and cell-cell adhesion (Tormo *et al.*, 2005). Furthermore, disruption of the *bap* gene was previously shown to result in the loss of biofilm-forming capacity, while biofilm-negative (non-biofilm forming) *S. epidermidis* strains do not express the Bap (Tormo *et al.*, 2005; Trotonda *et al*., 2005).

Although treatment with lactoferrin resulted in minimal MRSE biomass in the wells, a significant upregulation of the *bap* gene was noted. It is thus probable that *bap* was upregulated to produce more Bap, which were affected by lactoferrin's interaction with MRSE's cell wall. To test this hypothesis, the effects of lactoferrin on the Bap structure and functions in the biofilm formation processes require further investigation. In addition, more data is needed on the specific effects of lactoferrin on teichoic acids in the biofilms of Gram-positive pathogens.

Conclusions

Lactoferrin prevented the biofilm formation of a methicillin-resistant *S. epidermidis* strain for up to 24 hours, probably through the inhibition of bacterial growth, adhesion, and aggregation. Lactoferrin also eradicated established biofilms for up to 72 hours through the

disruption and dispersion of biomass, which suggested interaction with components of the biofilm extracellular matrix responsible for structural integrity. Although a significant eradication of MRSE biofilm biomass was achieved by lactoferrin, upregulation of the *bap* gene was demonstrated. Upregulation of this gene further suggests a possible strategy of lactoferrin in targeting bacterial cell wall components that are key for biofilm formation. Additional experiments are needed to further explain the antibiofilm mechanisms of lactoferrin, specifically those targeting genes and proteins regulating biofilm formation in pathogens.

List of Abbreviations

LPS: lipopolysaccharide, MRSE: methicillin-resistant *Staphylococcus epidermidis*, *bap*: biofilm-associated protein gene, ATCC: American Type Culture Collection, cfu: colony forming units, PBS: phosphate buffered saline, EDTA: ethylenediaminetetraacetic acid, RNA: ribonucleic acid, cDNA: complementary deoxyribonucleic acid, qPCR: real-time polymerase chain reaction, Bap: biofilm-associated protein.

Declarations

Ethics approval and consent to participate/for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author, while relevant datasets are included in this published article and its supplementary files.

Competing interests

No conflict of interest is declared.

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Authors' contributions

RK conducted the experiments, analysed the data, and drafted the manuscript. CPY analysed the data and drafted the manuscript, SCC and CBY drafted and finalised the manuscript. CBY, CPY, and SCC were supervisors to RK.

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412 **List of figure legends**

413 **Figure 1.** A. Biomass and B. metabolic activity of MRSE biofilms after 24 hours of exposure
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415
416 **Figure 2.** A. Biomass and B. metabolic activity of adherent MRSE biofilms and dispersed
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419
420 **Figure 3.** *bap* gene expression in MRSE biofilm cells after 20 hours treatment with
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422 deviation bars are included.
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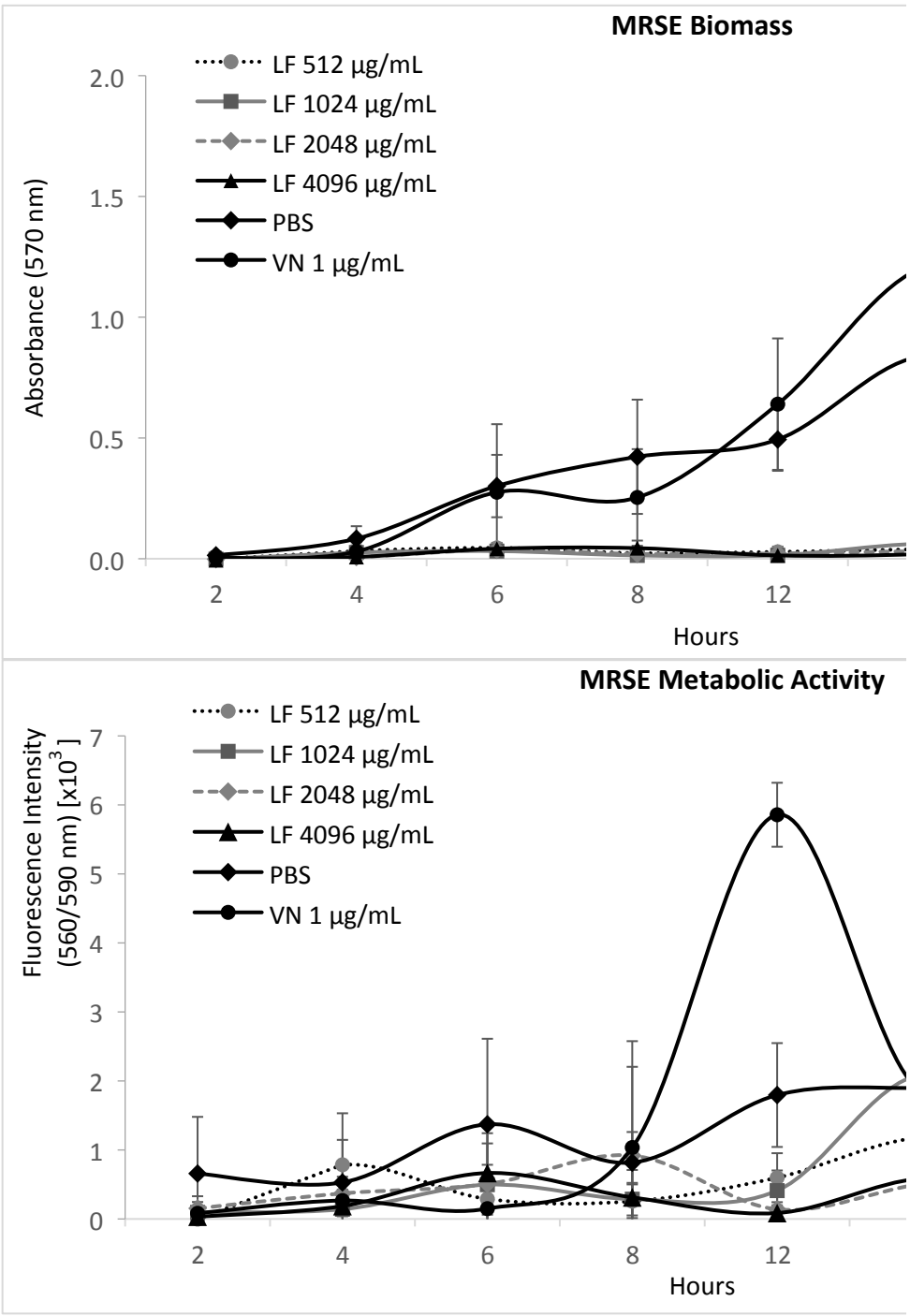
Figure 1. Biomass and metabolic activity of prevented biofilm formation



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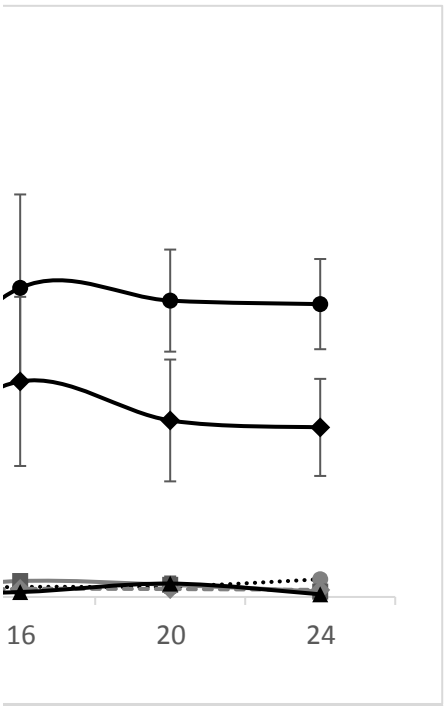

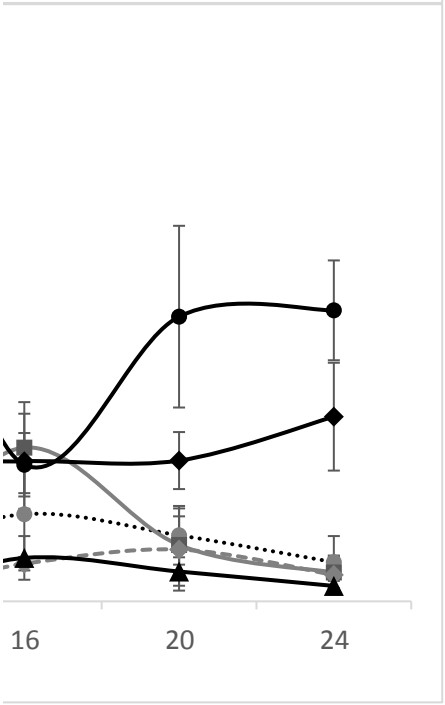

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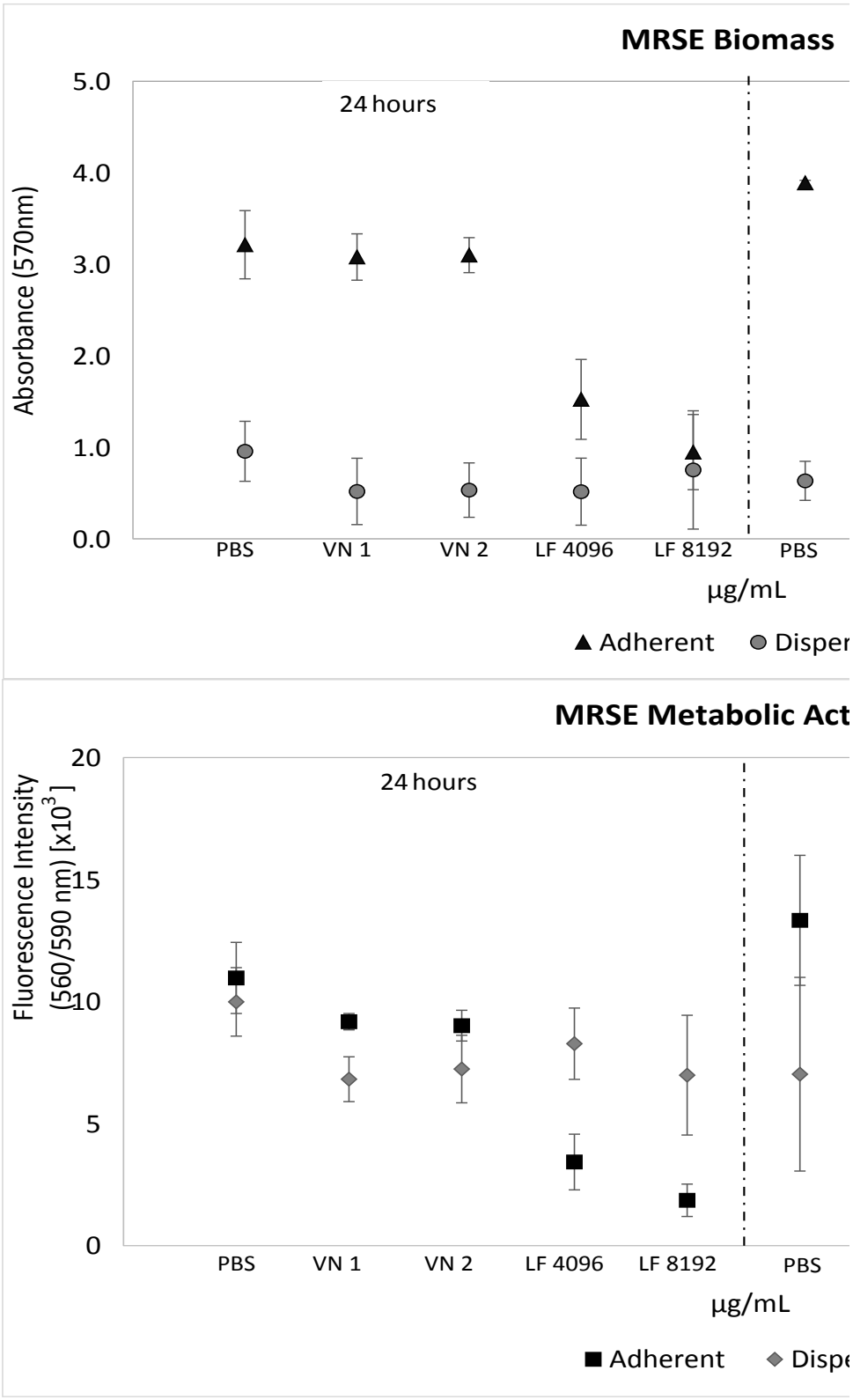
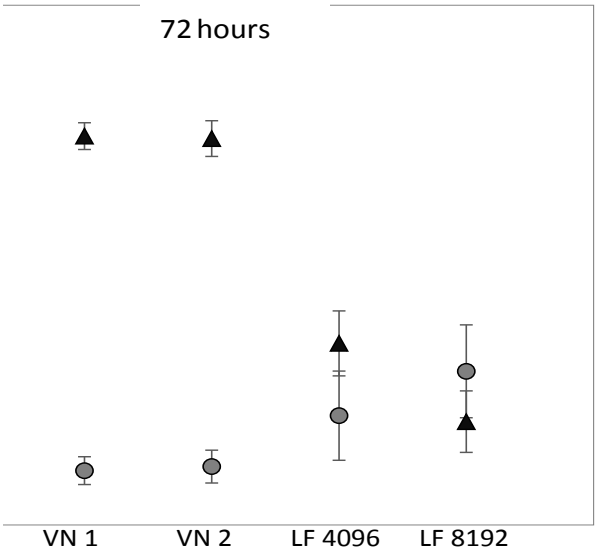
Figure 2. Biomass and metabolic activity after repeated treatment



sistant *Staphylococcus epidermidis*



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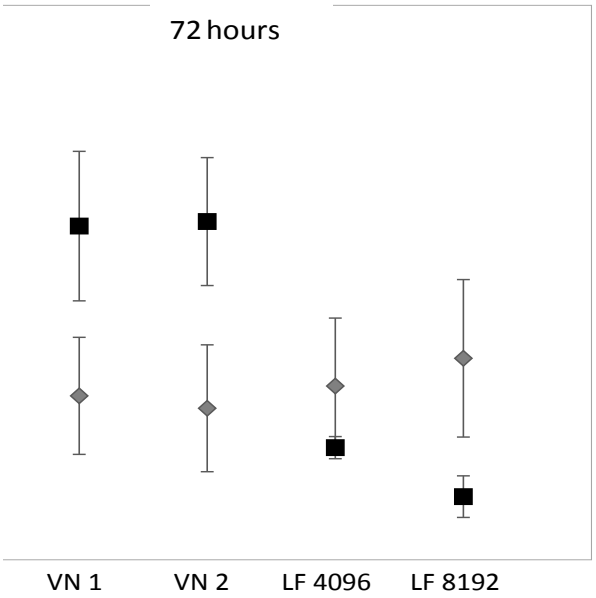

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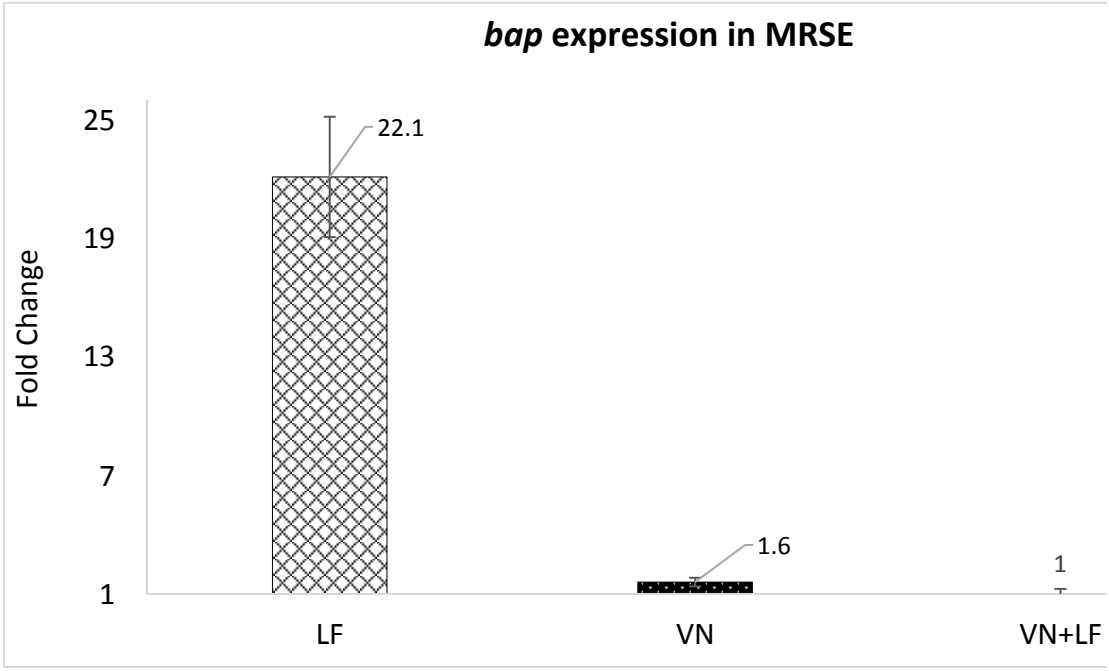
Figure 3. *bap* gene expression



sistant *Staphylococcus epidermidis*