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Potentiating effects of leaderless enterocin DD14 in combination with methicillin on clinical methicillin-resistant *Staphylococcus aureus* S1 strain

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26 **Abstract**

27 Biofilm formation by pathogenic bacteria as well as their resilience to antibiotic treatments
28 are a major health problem. Here, we sequenced and analyzed the genome of the clinical
29 methicillin-resistant *Staphylococcus aureus* S1 (MRSA-S1) strain, and established its
30 sensitivity to the combination of methicillin and the leaderless two peptides enterocin DD14
31 (EntDD14). Such sensitivity was assessed *in vitro* based on the MIC/FIC values as well as on
32 killing curves experiments. Moreover, combination of EntDD14 and methicillin was able to
33 reduce the biofilm formation of *Staphylococcus aureus* S1 of about ~30 %. Interestingly,
34 genes thought to be involved in the virulence of MRSA-S1, like *nuc* and *pvl* which code,
35 respectively, for nuclease and Panton-Valentine leucocidin, were shown to be downregulated
36 following treatment with EntDD14 and methicillin. Similar effects were registered for other
37 genes such as *cflA*, *cflB* and *icaB*, coding for bacterial ligands clumping factors A, B and
38 intercellular adhesion factor respectively. All these data, suggest that combinations of
39 bacteriocin and antibiotics are useful as a backup for treatment of bacterial infections.

40

41

42 **Keywords:** Methicillin resistant *Staphylococcus aureus* MRSA, methicillin, enterocin DD14,
43 synergy, anti-biofilm activity, virulence and biofilm genes expression.

44

45 1. Introduction

46 *Staphylococcus aureus* is a Gram-positive bacteria carried persistently in the anterior nares of
47 up to 30 % of human population and responsible for nosocomial and community acquired
48 infections (Sakr et al., 2018). Methicillin-resistant *S. aureus* (MRSA) is a solid variant
49 defying antibiotic-treatment. Resistance to penicillin is generally due to acquisition of genes
50 encoding penicillin binding protein (PBP2a), characterized by a low affinity to β -lactams
51 antibiotics (Stapleton and Taylor, 2002). Besides, *S. aureus* can grow in multicellular
52 communities and form biofilms on which the bacterial cells are embedded in a self-produced
53 extracellular matrix which participate to spreading and persistence of several infections,
54 including septic arthritis, endocarditis, osteomyelitis and foodborne illness (Basanisi et al.,
55 2017; Lee et al., 2018; Jayakumar et al., 2020). These biofilms can be encountered on the
56 nares, skin, mucosa, and food contact surfaces. Bacterial biofilm formation progresses
57 essentially in three steps: initial adhesion, proliferation, and detachment (Lee et al., 2018).
58 Adhesion is considered a key step in the formation of biofilm communities which is
59 facilitated by the expression of various microbial surface components which recognize
60 adhesive matrix molecules (MSCRAMMs) (Foster, 2019). These MSCRAMMs can bind to
61 one or more host extracellular matrix factors like elastin (*ebpS*), laminin (*eno*), collagen (*cna*),
62 fibronectin A and B (*fnbA* and *fnbB*), fibrinogen (*fib*) and bacterial ligands clumping factors A
63 and B (*clfA* and *clfB*) which represent the genetic backbone of biofilm formation in *S. aureus*
64 (Atshan et al., 2013). Biofilm plays a determinant role in the antimicrobial resistance as they
65 represents pharmacokinetic barrier perturbing diffusion of antibiotics and further
66 antimicrobials (Bridier et al., 2011; Craft et al., 2019). Moreover, it was demonstrated that
67 biofilm participate to the spreading of the antimicrobial resistance by the facilitating of
68 horizontal transfer between bacteria (Savage et al., 2013).

69 The increasing rate of drug resistance has become a worrisome and the number of deaths
70 associated to this plague is ~700,000 each year; a number which is foreseen to increase by
71 2050 to 10 million by year, if no action is taken now (Goel et al., 2021; O'Neill, 2016).
72 Scientists, public-health bodies and governments warn that antimicrobial resistance will be
73 next major crisis to handle. The use of bacteriocins is steadily reported as biological tools to
74 potentiate activity of deteriorating antibiotics, or replace them.

75 Bacteriocins are ribosomally synthesized antimicrobial peptides produced by Gram-negative,
76 Gram-positive and Archaea (Drider and Rebuffat, 2011; Flaherty et al., 2014; Hammami et
77 al., 2013).

78 Lactic acid bacteria (LAB) beneficial microbes can synthesize several bacteriocins able to
79 inhibit phylogenetically related bacteria (Cotter et al., 2013; Naghmouchi et al., 2007), and
80 seldom phylogenetically distant bacteria (Belguesmia et al., 2020; Madi-Moussa et al., 2021).

81 Enterocins are bacteriocins produced by LAB belonging to *Enterococcus* genus potentially
82 active against a wide range of microbes including Gram-positive and Gram-negative
83 pathogenic bacteria (Al Atya et al., 2015; Batdorj et al., 2006; Line et al., 2008). Enterocin
84 DD14 (EntDD14) is a IIB two peptides leaderless bacteriocin, produced by *E. faecalis* 14, a
85 strain that was previously isolated from meconium (Al Atya et al. 2015). EntDD14 is strongly
86 active against *Clostridia* and other Gram-positive bacteria including *Enterococcus*, and
87 weakly active against *Staphylococcus* strains (Al Atya et al., 2015; Caly et al., 2017).

88 In this work we report our observations, on the anti-*Staphylococcus aureus* activity of
89 EntDD14 and provide means to improve it, enabling therefore a potential proof-of-concept for
90 future applications. Moreover, the combination of EntDD14 + methicillin decreased growth
91 resistance of this strain to methicillin, impeded the biofilm formation and altered expression
92 of genes coding various virulence factors. Finally, the genome of methicillin-resistant

93 *Staphylococcus aureus* S1 (MRSA-S1) was sequenced and analyzed in order to locate and
94 interpret data related to virulence factors expression.

95

96 **2. Material and Methods**

97 *2.1. Bacterial strains*

98 *Enterococcus faecalis* 14, isolated from meconium (Al Atya et al., 2015), was grown in MRS
99 (de Man, Rogosa, and Sharpe) (de Man et al., 1960) or M17 medium, at 37°C for 16 to 18 h.

100 *S. aureus* ATCC 25923, MRSA USA300 and MRSA-S1, isolated from wound infection and
101 kindly provided by Dr. Gilles Prévost from Strasbourg University, France, were grown in
102 Brain Heart Infusion (BHI) broth at 37°C for appropriate experimental time, as indicated
103 below.

104 *2.2. Genome analysis of MRSA-S1 type*

105 Genome sequencing of the *S. aureus* MRSA-S1 strain was performed as previously described
106 (Vieco-Saiz et al., 2020). After total DNA extraction and purification, concentration and
107 purity were determined by fluorescence using the Qubit™ dsDNA HS Assay Kit
108 (ThermoFischer, Waltham, MA, USA), according to the manufacturer's instructions. Bacterial
109 genome was then sequenced by MicrobesNG (<http://www.microbesng.uk>) using the Illumina
110 MiSeq and HiSeq 2500 technology platforms, with matched 250 bp sequences. Sequence
111 analysis was performed using a standardized procedure involving Kraken software (Wood and
112 Salzberg, 2014). The nearest existing reference genome was determined using the Burrows-
113 Wheeler Aligner (BWA) algorithm to map sequences and assess data quality ([http://bio-](http://bio-bwa.sourceforge.net)
114 [bwa.sourceforge.net](http://bio-bwa.sourceforge.net)). Sequences obtained were assembled using the SPAdes software to get
115 the complete genome sequence (<http://cab.spbu.ru/software/spades/>).

116 Genomes alignment views were obtained using the Mauve 2.4.0 software (Darling et al.,
117 2004) and GCview (Stothard and Wishart, 2005) online tool (<http://cgview.ca/>). To complete

118 the alignment, the whole genome of the *S. aureus* ATCC 25923 (NCBI accession number
119 CP009361.1) was used as a reference. Whole genomes of the following strains were also
120 integrated in these analyses using the aforementioned softwares; *S. aureus* USA300, a
121 multiresistant pathogenic strain (NCBI accession number CP000255.1), and *S. aureus*
122 Rosencach BAA1680, resistant to methicillin and ceftiofur (NCBI accession number
123 CP010297.1). The predictive annotation of the genome was performed by the RAST server
124 (<http://rast.nmpdr.org>). RAST generated data were used to draw a Venn diagram using
125 OrthoVenn 2 software (<https://orthovenn2.bioinfotoolkits.net>) allowing visualization of
126 shared and unique protein coding genes or pseudogenes in each sequenced genome. Each
127 strain is represented by different colored oval with the number of orthologous gene clusters
128 shared by considered strains.

129 *2.3. Production and purification of enterocin DD14 (EntDD14)*

130 EntDD14 was purified using a two-step procedure described by Zgheib et al., (2021). Briefly
131 *E. faecalis* 14 was grown in 200 mL of M17 broth (Sigma Aldrich), supplemented with
132 glucose (0.5 %) and buffered with 60 mM sodium phosphate (pH 6.3), at 37°C for 24 h with
133 160 rpm continuous shaking. After harvesting by centrifugation (8,228 ×g., 4°C, 10 min), the
134 cell-free supernatant was filtrated throughout 0.2 µm filter and incubated for 24 h at room
135 temperature (20-25°C) with CM Sephadex® C-25 resin (GE Healthcare Life Sciences,
136 Chicago, IL, USA). The resin was washed with 100 mL of distilled water and 20 mL of 0.5 M
137 NaCl. The resin-bound EntDD14 was eluted with 40 mL of 1.5 M NaCl. The removal of the
138 salt from the solution containing EntDD14 was achieved by gel filtration using PD MidiTrap
139 G-10 column (GE Healthcare Life Sciences), following the manufacturer's instructions.

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141 *2.4. Checkerboard assay*

142 A pure colony of each *Staphylococcus* strain used in this study was grown overnight in BHI
143 medium at 37°C. Then, 10 µL of each overnight culture were added to the wells of 96 wells
144 cell culture plate (Cellstar) containing concentrations of EntDD14, ranging from 7.5 to 200
145 µg/mL. The minimal inhibitory concentration (MIC) was determined as defined as the lowest
146 concentration of EntDD14, which inhibit the visible growth of a microorganism after
147 overnight incubation.

148 EntDD14 interactions with methicillin were determined using checkerboard assay, as
149 previously described by (Al Atya et al., 2016). Concentrations of EntDD14 were comprised
150 between 7.5 and 200 µg/mL, while those used for methicillin ranged from 0.25 to 64 µg/mL.
151 Microplates were inoculated with *S. aureus* strains at $\sim 10^6$ CFU/mL, in a final volume of 200
152 µL per well, and incubated overnight at 37°C.

153 The fractional inhibitory concentration index (FICI) was calculated for each combination
154 using the following formula: $FICA + FICB = FICI$, where $FICA = MIC$ of drug A in
155 combination/ MIC of drug A alone, and $FICB = MIC$ of drug B in combination/ MIC of drug B
156 alone. The FICI was interpreted as follow: synergism = $FICI \leq 0.5$; indifference = $0.5 < FICI$
157 ≤ 4 ; antagonism = $FICI > 4$ (Petersen et al., 2006).

158 2.5. Killing curves

159 This experiment was performed on MRSA-S1 strain. Tubes containing BHI supplemented
160 with EntDD14, methicillin, vancomycin or combination of EntDD14 and methicillin, at
161 concentrations defined during checkboard assay, were inoculated with MRSA-S1 strain to a
162 density of $\sim 5 \times 10^6$ CFU/mL in a final volume of 5 mL and incubated at 37°C for 8 h.
163 Aliquots were removed at different periods of time (0, 2, 4, 6, and 8 h) of incubation, and then
164 serially diluted in saline solution for determination of viable cells counts. Diluted samples
165 (100 µL) were plated on BHI agar plates and colonies were counted after overnight incubation
166 at 37°C.

167 *2.6. Assessment of anti-biofilm activity by crystal violet method*

168 *S. aureus* strains were grown in a 96 wells polystyrene plate containing BHI medium for 48h
169 at 37°C, allowing formation of a mature biofilm. After which, formed biofilm, was washed to
170 remove non-adherent cells, and then treated with 200 µL of BHI containing EntDD14 at 240
171 µg/L, methicillin at 4 µg/mL, vancomycin at 2 µg/mL and combination of EntDD14 with
172 methicillin at 30 and 0.5 µg/mL respectively. The biofilm was quantified for all conditions
173 tested using the crystal violet dye as described by Xu et al. (2016).

174 *2.7. Live dead confocal microscopy*

175 MRSA-S1 strain was grown for 48h at 37°C, in a Labtek chambers (Dominique Dutscher
176 SAS, Bernolsheim, France) containing 300 µL of a sterile BHI medium. The obtained biofilm
177 was treated for 24 h as previously described with EntDD14 at 200 µg/L, methicillin 4 µg/mL,
178 vancomycin 2 µg/mL and for combination of EntDD14 with methicillin at 30 and 0.5 µg/mL
179 respectively. After gentle washing the obtained treated MRSA-S1 biofilms were stained using
180 Syto-9 and propidium iodide provided in FilmTracer™ LIVE/DEAD® kit (Invitrogen,
181 Carlsbad, CA, USA) according to the supplier recommended protocol. The obtained samples
182 were then observed under Zeiss LSM 700/780 confocal microscope associated with ZEN
183 2010 software (Oberkochen, Germany)

184 *2.8. Virulence genes expression assay*

185 A reverse transcriptase quantitative PCR (RT-qPCR) analysis was carried out to study the
186 impact of the different antibacterial agents, in a single dose or in combination.

187 MRSA-S1 strain was grown in of BHI broth (Sigma Aldrich) at 37°C and inoculated in a 24
188 wells sterile plate containing BHI sterile broth. The plate was then incubated for 48h and the
189 formed biofilm, after washing to remove non-adherent cells, was then treated with 200 µL of
190 the aforementioned formulation at sub-inhibitory concentration including EntDD14 (MIC/2 =

191 120 µg), EntDD14 + methicillin (MIC/2 = 15; 0.25 µg/mL), methicillin (MIC/2 = 2 µg/mL)
192 and PBS as a negative control. After 24h of incubation, and after washing of all wells with
193 sterile PBS, total RNAs was extracted from cells. Extraction was performed in triplicate,
194 using NucleoZol (Macherey-Nagel, Duren, Germany) and the cDNA was obtained with the
195 RevertAid RT Reverse Transcription Kit (ThermoFisher), following the instructions
196 recommended.

197 The expression of genes involved in the virulence and biofilm formation in *S. aureus* reported
198 by Galia et al. (2019) and Atshan et al., (2013) were investigated using specific primers listed
199 in [Table 1](#).

200 For each cDNA sample, the CFX Connect Real-Time PCR Detection system (Bio-Rad
201 Laboratories, Marne la Coquette, France) was used to perform amplification (using 2 µL of a
202 1:5 cDNA dilution), detection (with automatic calculation of the threshold value), and real-
203 time analysis. Then a comparative cycle time data with analysis modules from CFX
204 Manager™ Software Version 3.1, allowed to determine the relative mRNA levels for each
205 gene and in each condition used.

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Table 1

Sequences of oligonucleotide primers used for qPCR analysis for virulence and biofilm genes expression in MRSA-S1 strain.

Target	Primers Sequences (5'-3')	Amplicon size
<i>mecA</i> (methicillin resistance gene)	CAATGCCAAAATCTCAGGTAAAGTG AACCATCGTTACGGATTGCTTC	107
<i>pvl</i> (Panton-Valentine leucocidin)	AAATGCTGGACAAAACCTTCTTGG TTTGCAGCGTTTTGTTTTTCG	108
<i>nuc</i> (nuclease gene)	GGCATATGTATGGCAATTGTTTC CGTATTGCCCTTTCGAAACATT	73
<i>icaA</i> (intercellular adhesion gene)	GAGGTAAAGCCAACGCACTC CCTGTAACCGCACCAAGTTT	151
<i>icaB</i> (intercellular adhesion gene)	ATACCGGCGACTGGGTTTAT TTGCAAATCGTGGGTATGTGT	140
<i>icaC</i> (intercellular adhesion gene)	CTTGGGTATTTGCACGCATT GCAATATCATGCCGACACCT	209
<i>icaD</i> (intercellular adhesion gene)	ACCCAACGCTAAAATCATCG GCGAAAATGCCCATAGTTTC	211
<i>fnbA</i> (fibronectin binding protein A)	AAATTGGGAGCAGCATCAGT GCAGCTGAATTCCCATTTTC	121
<i>fnbB</i> (fibronectin binding protein B)	ACGCTCAAGGCGACGGCAAAG ACCTTCTGCATGACCTTCTGCACCT	197
<i>clfA</i> (clumping factor A)	ACCCAGGTTTCAGATTCTGGCAGCG TCGCTGAGTCGGAATCGCTTGCT	165
<i>clfB</i> (clumping factor A)	AACTCCAGGGCCGCCGTTG CCTGAGTCGCTGTCTGAGCCTGAG	159
<i>fib</i> (fibrinogen binding protein)	CGTCAACAGCAGATGCGAGCG TGCATCAGTTTTTCGCTGCTGGTTT	239
<i>ebps</i> (elastin binding protein)	GGTGCAGCTGGTGCAATGGGTGT GCTGCGCCTCCAGCCAAACCT	191
<i>eno</i> (laminin binding protein)	TGCCGTAGGTGACGAAGGTGGTT GCACCGTGTTTCGCCTTCGAACT	195
<i>cna</i> (collagen binding protein)	AATAGAGGCGCCACGACCGT GTGCCTTCCCAAACCTTTTGAGCA	156
16S rRNA	GGGACCCGCACAAGCGGTGG GGGTTGCGCTCGTTGCGGGA	191

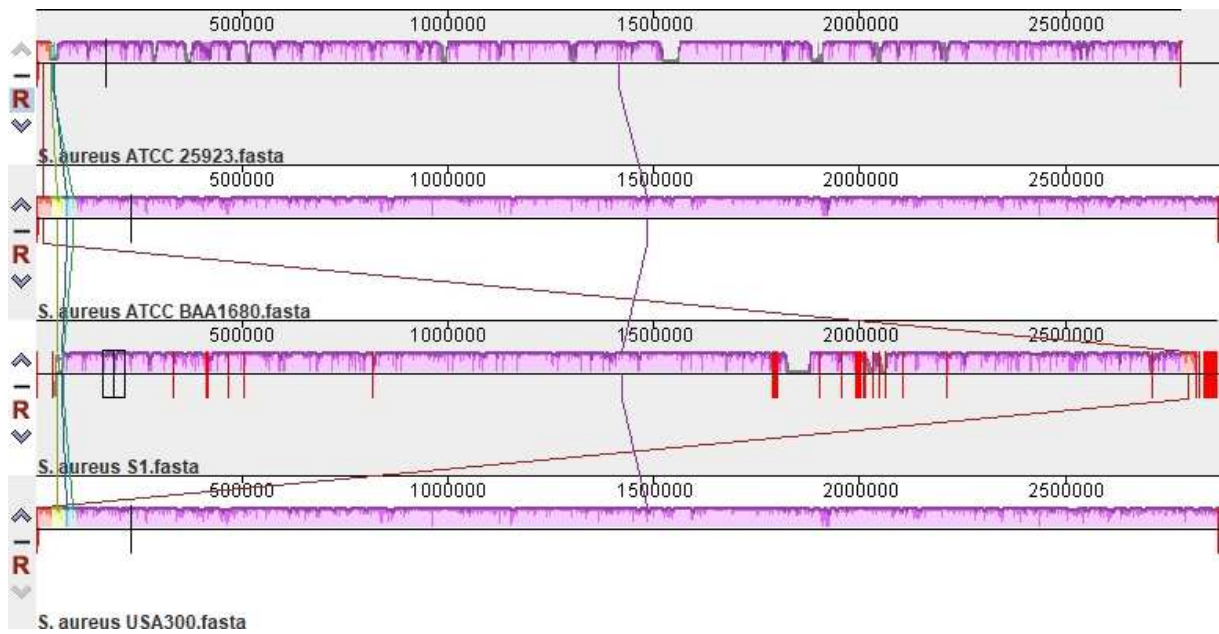
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240 3. Results

241 3.1. Snapshot on *S. aureus* MRSA-S1 genome and multiple virulence factors

242 *S. aureus* MRSA-S1 has a genome of 2,865,159 bp, with 32.7 % GC content. The N50 and
243 L50 index were 497,521 and 2 respectively. N50 index defines assembly quality in terms of
244 contiguity as it represent shortest contig length needed to cover 50 % of the genome (Castro
245 and Ng, 2017; Mäkinen et al., 2012), whereas L50 as the smallest number of contigs whose
246 length sum makes up half of genome size (Castro and Ng, 2017). The MRSA-S1 harbors
247 several genes expected to play a role in multidrug antibiotic resistance like fluoroquinolones
248 and methicillin by through efflux pumps and FmtB cell surface protein implied in oxacillin
249 and methicillin resistance (Komatsuzawa et al., 2000). Moreover, genes involved in the
250 virulence and biofilm synthesis were as well located in the genome and include fibronectin-
251 binding protein B, cell aggregation and biofilm protein SasC (Schroeder et al., 2009),
252 extracellular matrix binding protein, two component toxin Panton-Valentine leucocidin
253 (PVL), *nuc* nuclease, *cnaA* collagen binding protein, *ica* intercellular adhesion factors, *clfA*
254 and *clfB* clumping factors. Alignment of genomes from *S. aureus* MRSA-S1 and ATCC
255 25923 with Mauve 2.4.0 software enable reordering contigs of the newly sequenced MRSA-
256 S1 strain's genome. As depicted on Fig. 1, the contigs boundaries of each genome analyzed
257 are represented with red lines. The conserved distinct locally collinear blocks (LCBs) for all
258 strains are differently colored, with good positional agreement between scaffolds tiled against
259 the respective closed chromosomal sequences (Fig. 1). This genomic region encodes genes
260 which are mostly conserved in the genome sequences of other *S. aureus* strains analyzed by
261 CGview (Fig. 2), suggesting therefore that many of these genes of this variable region are
262 shared between *S. aureus* MRSA-S1, *S. aureus* USA300, *S. aureus* BAA1680 and *S. aureus*
263 ATCC 25923 strains. The Venn diagram analysis of all protein sequences annotated in the
264 whole genomes of MRSA-S1, USA300 and ATCC 25923 strains showed 2,291 common

265 putative protein coding genes representing 80 up to 90% of their predicting coding protein
266 sequences (Fig. 3).

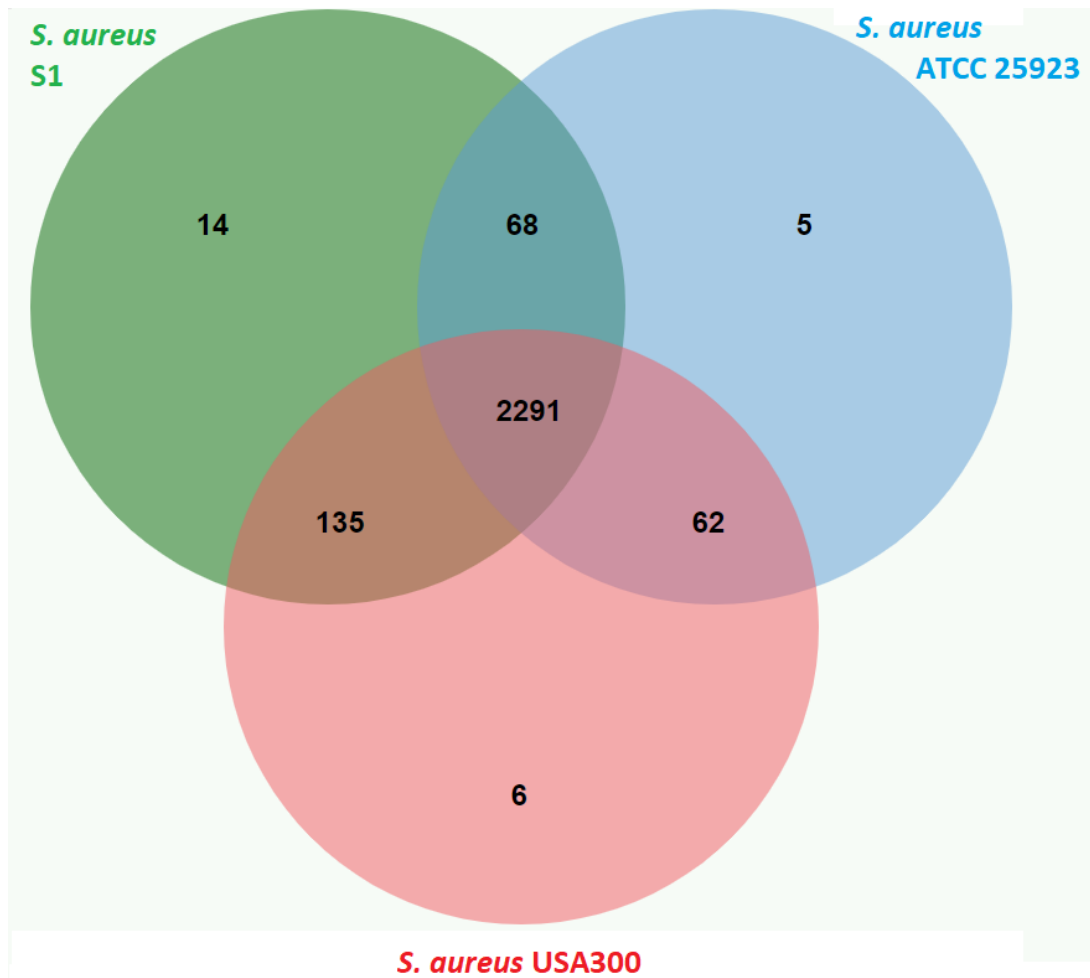


267

268 **Fig. 1.** *S. aureus* MRSA-S1 genome alignment with *S. aureus* USA300 (MRSA), *S. aureus*
269 ATCC BAA1680 (MRSA) and *S. aureus* ATCC 25923 (MSSA) genomes using Mauve
270 software

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280 **Fig. 3.** Venn diagram showing shared and unique protein coding genes (or pseudogenes) in
 281 each sequenced *S. aureus* genomes using the data generated by RAST analysis and drawn
 282 with OrthoVenn 2 online software

283

284 *3.2. EntDD14 and methicillin combination is synergistically active on MRSA strains*

285 When the methicillin antibiotic was used in combination with EntDD14, the MICs values of
 286 both molecules decreased and were below to those obtained individually. Of note, this
 287 combination enabled a synergetic effect regarding the FIC values < 0.5 registered for both
 288 EntDD14 in combination with methicillin on MRSA-S1 and USA300 strains (Table 2). In
 289 contrary, the effect of this combination was less efficient on ATCC 25923 strain with a FIC
 290 value of 0.625. Interestingly, the MIC values of methicillin for MRSA-S1 have dropped under
 291 its breakpoint point from 4 to 0.5 µg/mL (CA-SFM, 2019), when it is was associated to
 292 EntDD14, at 30 µg/mL.

293

294 **Table 2**295 Minimal and fractional inhibitory concentrations of EntDD14 and methicillin of *S. aureus*

296 strains

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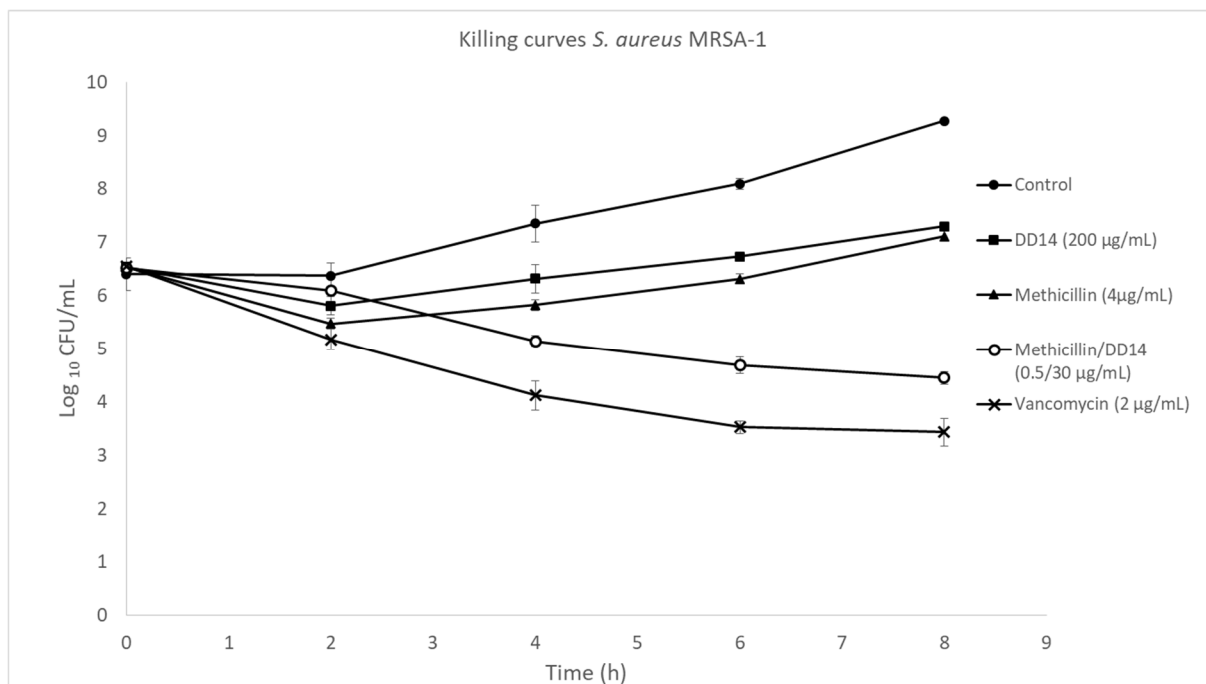
Strains	DD14 ($\mu\text{g/mL}$)	Methicillin ($\mu\text{g/mL}$)	Met/DD14 ($\mu\text{g/mL}$)	FIC
<i>S. aureus</i> MRSA-S1	>240	4	0.5/30	0.25
<i>S. aureus</i> USA300 (MRSA)	>240	16	4/30	0.375
<i>S. aureus</i> ATCC 25923 (MSSA)	>240	0.5	0.25/30	0.625

299

300 *3.3. EntDD14 associated with methicillin induces a significant loss of viable population of S.*
 301 *aureus MRSA-S1 strain*

302 The killing curves realized on MRSA-S1 strain treated with vancomycin (2 $\mu\text{g/mL}$),
 303 methicillin (4 $\mu\text{g/mL}$), EntDD14 (240 $\mu\text{g/mL}$), and the combination of EntDD14/methicillin
 304 (30/0.5 $\mu\text{g/mL}$) confirmed the synergistic effect obtained with the FIC values. As expected,
 305 methicillin, or EntDD14 alone has a weak inhibitory activity against the planktonic MRSA-S1
 306 culture compared to vancomycin, or to that of Methicillin/EnDD14 combination. In these
 307 latter cases, the number of cells expressed as Log₁₀ CFU/mL counts of MRSA-S1, diminished
 308 at least 1.5 to 3 logs after 6–8 h of incubation, leading to a killing of 95 to 99.9% of the
 309 CFU/mL count of the initial bacterial population inoculated in the medium (Fig. 4).

310

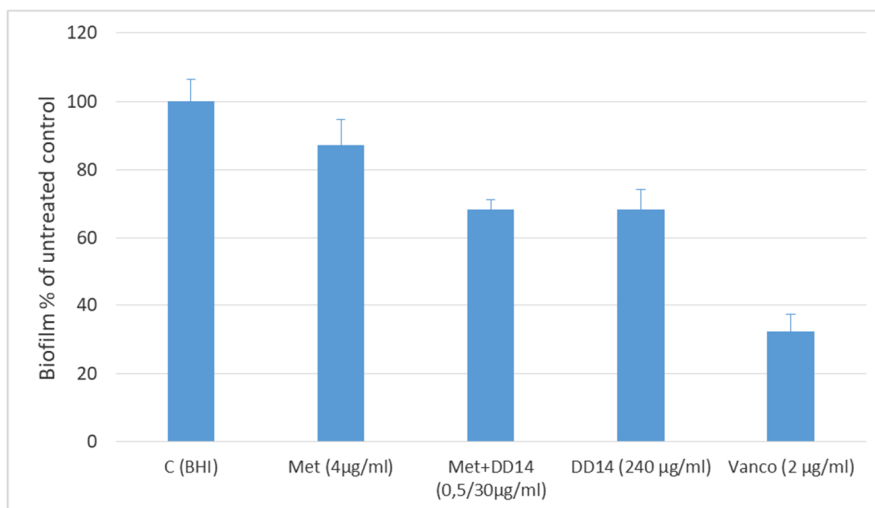


311
 312 **Fig. 4.** Time-killing kinetics on planktonic cultures of MRSA-S1 strain at 0, 2, 4, 6 and 8 h in
 313 the presence of EntDD14 (200 mg/l), vancomycin (2µg/mL) methicillin (4 µg/mL), EntDD14
 314 (240 µg/mL) and the combination with EntDD14/methicillin (30/0.5 µg/mL).

315
 316 *3.4. Anti-biofilm activity of EntDD14*

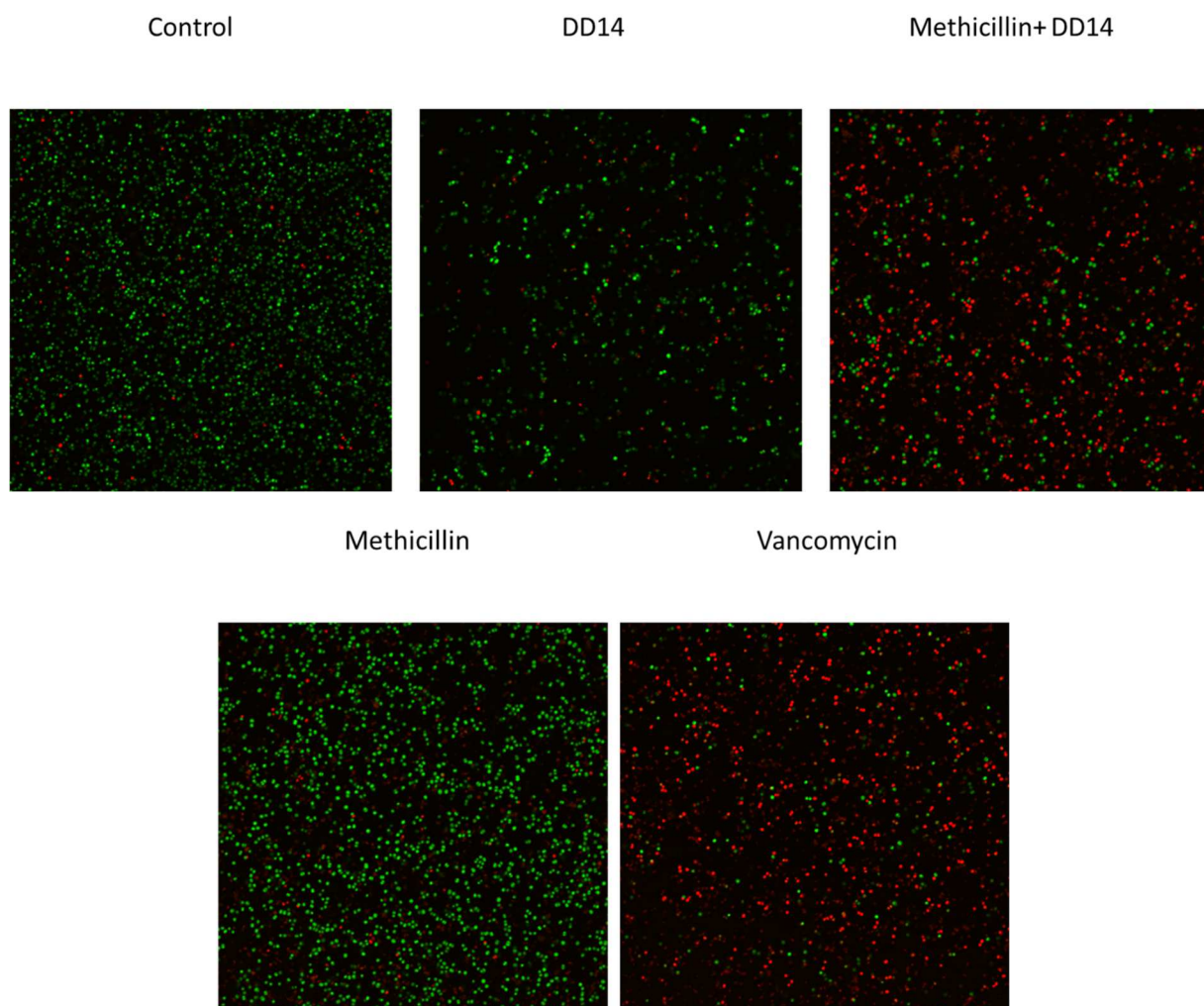
317 To determine whether EntDD14 can potentiate methicillin and therefore inhibit *S. aureus*
 318 MRSA-S1 biofilm formation, different treatments including vancomycin, methicillin,
 319 EntDD14 and combination of EntDD14 with methicillin on MRSA-S1, using previously
 320 determined MICs values were applied on 48h old MRSA-S1 biofilms.

321 As indicated on Fig. 5, methicillin and EntDD14 enabled to reduce biofilm formation of
 322 MRSA-S1 strain by ~30 %. Remarkably, EntDD14 alone showed similar results. However, an
 323 important reduction level of biofilm was observed with vancomycin, as this antibiotic was
 324 able to reduce the forming biofilm by 70 % of the initial amount. These results were
 325 confirmed by confocal microscopy analysis, which showed a higher proportion of died cells
 326 colored in red after treatment with methicillin+ EntDD14 combination, compared to
 327 methicillin alone, but less than that observed with vancomycin (Fig. 6).



328

329 **Fig. 5.** Anti-biofilm activities of EntDD14, EntDD14 in combination with methicillin and
 330 methicillin and vancomycin alone against *S. aureus* MRSA-S1 strain at MIC concentrations.



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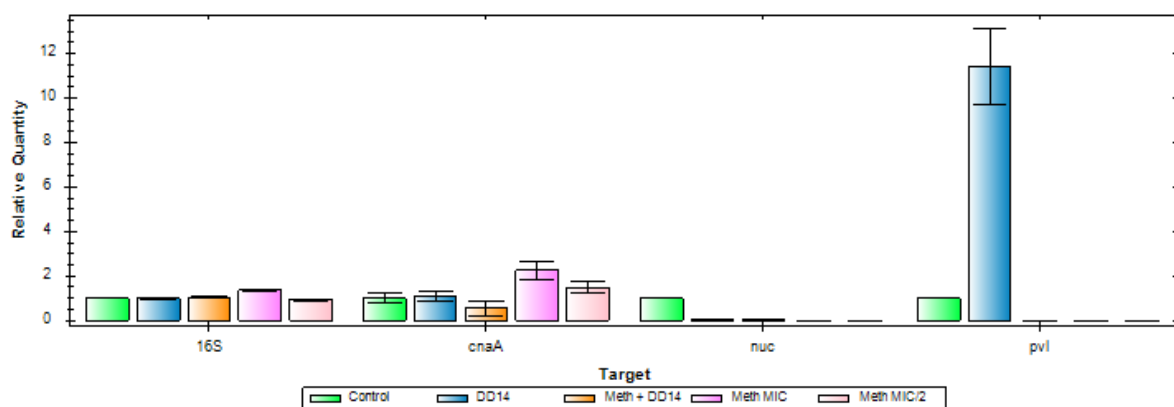
333 **Fig. 6.** Confocal microscopy views of MRSA-S1 culture untreated (control) and treated with
 334 EntDD14 (200 µg/mL) and with methicillin in combination with EntDD14 (0.5: 30 µg/mL)
 335 after Live/Dead staining using Syto-9 and propidium iodide staining.

336

337 3.5. *EntDD14* affects expression of virulence genes in *MRSA-S1* strain

338 Regarding the qPCR results, treatment of *MRSA-S1* strain with *EntDD14*, methicillin and
339 their combination induced a significant decrease of the transcripts of *nuc* and *icaB* (Fig. 7A,
340 B) almost to undetectable levels compared to the control. However, the *pvl* transcript was
341 upregulated to some point, when *MRSA-S1* was submitted to the treatments with *EntDD14*
342 alone (Fig. 7A). Similar behavior was observed with *icaA* gene, for which the transcript level
343 was higher after treatment with methicillin (Fig. 7B). Interestingly, the effect of *EntDD14* and
344 methicillin combination on *cnaA* transcript level was relatively lower, but not significantly,
345 than that of the control. In contrary, this gene was upregulated in the presence of the
346 methicillin at MIC (4 µg/mL) for which we observed 2 folds transcript level under this
347 experimental condition. Moreover, *icaC*, *epbs* and *fnbB* genes transcript levels were not
348 affected significantly by the different treatments applied during this study (Fig. 7A, B, C).
349 The transcripts of the other genes considered during this experiment were undetectable, while
350 no amplification was obtained for all conditions tested including untreated control (data not
351 shown).

352 A)



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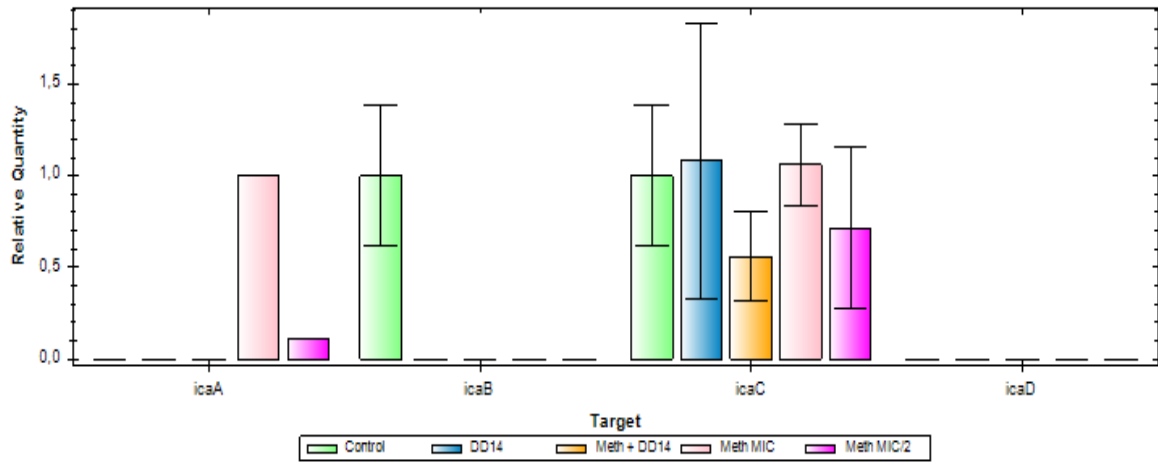
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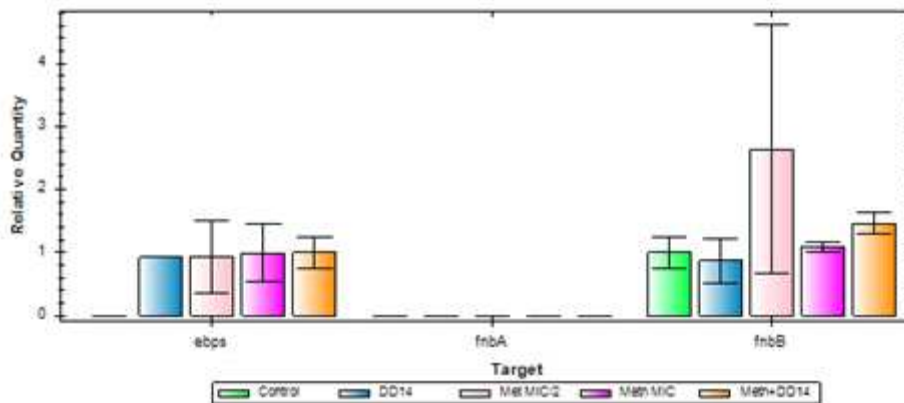
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358 **B)**



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360 **C)**



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362 **Fig. 7.** The relative of transcriptional levels of virulence and biofilm genes in the untreated
363 (control) MRSA-S1 strain and after treatment with EntDD14 at 60 $\mu\text{g}/\text{mL}$ (DD14), methicillin
364 at 4 $\mu\text{g}/\text{mL}$ (Meth MIC), methicillin at 2 $\mu\text{g}/\text{mL}$ (Meth MIC/2) and methicillin + EntDD14 at
365 0.5+30 $\mu\text{g}/\text{mL}$ (Meth+DD14).

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373 4. Discussion

374 The mechanism processes leading to biofilm formation in *S. aureus* remain to be completely
375 elucidated (Atshan et al., 2013; Foster, 2019). *Staphylococcus* pathogenic strains were often
376 reported for their strong biofilm formation abilities, which annihilate antibiotic treatments in
377 clinical contexts, compromise disinfection of contaminated medical and industrial devices
378 (Khatoon et al., 2018; Tremblay et al., 2014). *S. aureus* strains exhibiting resistance to
379 methicillin are problematic, because of cross-resistance to β -lactams and other antibiotics
380 belonging aminoglycosides, macrolides and fluoroquinolones classes (Barrett, 2004;
381 Chmielarczyk et al., 2021; Mediavilla et al., 2012).

382 Previously, we established that semi-purified enterocin DD28 (EntDD28), or enterocin DD93
383 (EntDD93) can potentiate antibiotics like kanamycin or erythromycin, by decreasing their
384 breakpoints, when tested against MRSA-S1 strain (Al Atya et al., 2016). Of note, EntDD28,
385 EntDD93 and EntDD14 are produced by different *E. faecalis* strains that were isolated from
386 meconium (Al Atya et al., 2015). EntDD28/93+erythromycin or EntDD28/93+kanamycin
387 combinations have been also reported to impede biofilm formation, by clinical MRSA-S1
388 strain, when grown on stainless steel and glass slides (Al Atya et al., 2016).

389 Here, we provide more insights and establish that purified EntDD14 can potentiate methicillin
390 and impede biofilm formation by MRSA-S1 strain. These antibacterial agents can act and
391 destabilize the cell-wall synthesis, leading to leakage of intracellular content as yet reported
392 for similar bacteriocins which form pores in the membrane of the targeted microorganisms
393 (Nissen-Meyer et al., 2010). Moreover, EntDD14 and methicillin combination decreased
394 expression of a set of virulence factors. These genes were located on the genome of MRSA-
395 S1 strain performed in the frame of this work.

396 Regarding control of MRSA strains, we established that EntDD14 and methicillin
397 combination inhibited growth of MRSA USA300 strain, but without decreasing the MIC
398 value below the breakpoint of methicillin (CA-SFM, 2019). Conversely to MRSA-S1 strain,
399 MRSA USA300 strain remained resistant to methicillin following treatment with
400 methicillin+EntDD14 combination (CA-SFM, 2019). Noteworthy, MRSA USA300 strain is
401 known as a multiresistant strain exhibiting a strong resilience and versatility for most
402 clinically used antibiotics (Higuchi et al., 2010).

403 Furthermore, combination of EntDD14 and methicillin did not lead to any synergetic effect on
404 MSSA ATCC 25923 strain. The MIC value of methicillin obtained for MSSA ATCC 25923
405 strain is 0.5 µg/mL, which already very low, this value is decreased to 0.25 µg/mL, when
406 methicillin was used in combination with EntDD14. Below 0.25 µg/mL, no activity was
407 observed as limit of the assay had been reached.

408 Moreover, EntDD14 and methicillin combination (30:0.5 µg/mL), enabled a reduction of ~30
409 % of biofilm formed by MRSA-S1 strain after 24h of incubation. Interestingly, a similar
410 reduction was observed for EntDD14 alone at 60 µg/mL, arguing a role of this bacteriocin.
411 This could be linked to “detergent activity” of bacteriocins which could disturb biofilm
412 formation but not affecting the viability and the growth of MRSA-S1 cells, based on similar
413 observations reported by Chopra et al. (2015); Mathur et al. (2018) and Shin et al. (2016).

414 Killing curves assays demonstrated that EntDD14+methicin combination, at MIC values is
415 not only inhibiting the growth of MRSA-S1 strain but also reduced the viable cell population
416 by at least 1.5 Log₁₀ CFU/mL. This result is in good agreement, with those reported by Al
417 Atya et al. (2016) when combining EntDD28 or EntDD93 with antibiotics like erythromycin
418 or kanamycin against still MRSA-S1 strain. The current concept, which consists to potentiate
419 activity of aging antibiotics with bacteriocins, mainly LAB-bacteriocins could be applied for
420 Gram positive and Gram negative bacteria associated infections (Hanchi et al., 2017;

421 [Naghmouchi et al., 2013](#)), as well as on the biofilm formation ([Zgheib et al., 2020](#)). Taken
422 together, our results suggest the ability of bacteriocin-antibiotic combinations as mean to
423 control the growth of problematic bacteria and hamper their biofilm formation. Nevertheless,
424 molecular studies showing their impact on the overall cell biology and cytology are still
425 needed. Here, we partly respond to these questions, establishing, through transcriptomics and
426 qPCR analysis, that EntDD14 and methicillin combination impacted on the level of
427 expression of several genes involved in the virulence, or the biofilm formation aptitudes of
428 MRSA-S1 strain. Among these virulence factors, we noted that expression of *icaB*
429 intercellular adhesion factor of MRSA-S1, was abolished, following all treatments applied.
430 Interestingly, an early report by Cramton et al. ([1999](#)) showed that *ica* operon is generally
431 present in *S. aureus* and is required for biofilm formation. Hou et al. ([2012](#)) found that only
432 ~11.11% of *S. aureus* strains with aptitudes to form biofilms were *icaA* gene positives.
433 Regarding genetics determinant of biofilm formation phenotypes in MRSA strains, the genes
434 encoding *icaADBC* are the most investigated biofilm related genes, beside *fnbA*, *fnbB*, and
435 *ebps* ([Atshan et al., 2013](#)).
436 However, expressions of *icaA* and *icaD* genes were not detected under the experimental
437 conditions used, while those of *icaC* gene were unaffected by the treatments applied.
438 Regulation of *icaADBC* expression is complex and varies not only between staphylococcal
439 species, but also in strain dependent-manner ([Cue et al., 2012](#)). Conditions inducing *icaADBC*
440 expression include high temperature, anaerobiosis, high NaCl concentrations, high glucose
441 levels, ethanol, and subinhibitory concentrations of some antibiotics ([Cue et al., 2012](#)). This is
442 reflected in the observation that the degree of aggregation, which can vary in strain
443 dependent-manner, and for a same strain under different conditions ([Ghasemian et al., 2015](#);
444 [Lee et al., 2018](#)). Previous studies demonstrated that *icaA* gene expression could be increased
445 by antibiotics in *S. aureus* strains due to a complex regulatory system activated in response to

446 antimicrobial substances present in the environment (Huang et al., 2014; Lázaro-Díez, et al.,
447 2016).

448 Unlike MRSA-S1 strain, most of *S. aureus* strains resistant to methicillin harbor
449 staphylococcal cassette chromosome *mec* (*SCCmec*) element which is a mobile genetic
450 element with a single determinant for methicillin resistance, namely *mecA* or *mecC* gene.
451 Both of these genes code for a modified penicillin binding enzyme (PBP2a) characterized by
452 low affinity for all β -lactams, except for cephalosporins of 5th generation, according to [Galia](#)
453 [et al. \(2019\)](#). However, these genes were not located in the genome of *S. aureus* MRSA-S1
454 strain, arguing that other mechanisms of resistance to methicillin exist in *S. aureus* MRSA-S1.
455 Indeed, cell-wall biosynthesis contributes to methicillin resistance by providing adequate
456 levels of cell-wall precursors enabling proper cell-wall synthesis in presence of β -lactams
457 ([Peacock and Paterson, 2015](#)).

458 Furthermore, the MRSA-S1 genome analysis enabled to locate fibronectin binding protein
459 (FmtB) determinant, coding for a cell wall-associated protein which confers resistance to
460 methicillin ([Kawada-Matsuo and Komatsuzawa, 2012](#); [Komatsuzawa et al., 2000](#); [Stapleton](#)
461 [and Taylor, 2002](#)).

462 Moreover, the MRSA-S1 genome analysis revealed, the *pvl* gene coding for Panton-Valentine
463 leucocidin (PVL), which is involved in the skin and soft tissue infections (SSTIs), necrotizing
464 pneumonia and osteomyelitis ([Galia et al., 2019](#)). Treatment of MRSA-S1 strain with
465 EntDD14 and methicillin decreased significantly expression level of this gene. Curiously,
466 when EntDD14 was tested alone, expression of the *pvl* gene appeared to be more important,
467 delineating a type of sensing and regulation that need to be studied in order to better
468 understand how commensals and pathogenic bacteria can interact in a same environment, like
469 the gut microbiota, when bacteriocin are produced and released. With respect to that,

470 Dumitrescu et al. (2010) reported that β -lactams increased the expression of *pvl* by activating
471 its transcription through *sarA* and *rot* regulators.

472 Interestingly, the expression of clumping factors A gene (*clfA*) was decreased, which could
473 strongly affect the proper MRSA-S1 biofilm formation. Indeed, this gene is expressed during
474 biofilm cells development and maturation, and activate bacterial adhesion and promote
475 biofilm formation (Abraham and Jefferson, 2012; Atshan et al., 2013; Herman-Bausier et al.,
476 2018). This behavior was also reported in *Clostridium perfringens*, for a formulation
477 containing EntDD14 suggesting an important the role of EntDD14 on the expression of the
478 virulence factors DNA determinants (Zgheib et al. (2021).

479 Finally, the expression of *nuc* gene, which codes for staphylococcal nuclease (Nuc), a protein
480 which facilitate escape from neutrophils of the immune system (Berends et al., 2010), was
481 also decreased following treatment of MRSA-S1 strain with EntDD14+methicillin
482 combination.

483

484 **5. Conclusion**

485 In this work we established that EntDD14 is able to potentiate activity of deteriorating
486 antibiotics, like methicillin, on *S. aureus* MRSA-S1 strain, delineating a novel approach to
487 treat health problematic bacteria. However, this proof-of-concept can be exerted in a strain-
488 dependent manner as MRSA USA300 strain remained unaffected by combination between
489 EntDD14 and methicillin. Furthermore, EntDD14 alone or in combination with methicillin
490 reduced by ~30 % the biofilm formation capability of MRSA-S1 strain, and impacted on the
491 expression of the main genes involved in biofilm formation.

492

493 **Authors contributions**

494 YB, GS and DD, Conceptualization. YB, Methodology and Investigation. YB, GS and DD,
495 Software and Validation. YB, Visualization. DD, Supervision. YB, GS and DD, Writing-
496 Original Draft, Review and Editing.

497

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500

501 **Declaration of Competing Interest**

502 The authors declare no conflict of interest.

503

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