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1 **Control of *Staphylococcus epidermidis* biofilm by surfactins**
2 **of an endophytic bacterium *Bacillus* sp. 15F**

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24 ABSTRACT

25 The present paper deals with the preparation and annotation of a surfactin(s) derived from a
26 culture of the endophytic bacterium *Bacillus* 15F. The LC-MS analysis of the acetonitrile
27 fraction confirmed the presence of surfactins Leu/Ile7 C15, Leu/Ile7 C14 and Leu/Ile7 C13
28 with $[M+H]^+$ at m/z 1036.6895, 1022.6741 and 1008.6581, respectively. Various
29 concentrations of the surfactin(s) (hereafter referred to as surfactin-15F) were used to reduce
30 the adhesion of *Staphylococcus epidermidis* S61, which served as a model for studying
31 antibiofilm activity on polystyrene surfaces. Incubation of *Staphylococcus epidermidis* S61
32 with 62.5 $\mu\text{g/ml}$ of surfactin-15F resulted in almost complete inhibition of biofilm formation
33 ($90.3 \pm 3.33\%$), and a significant reduction of cell viability (resazurin-based fluorescence was
34 more than 200 times lower). The antiadhesive effect of surfactin-15F was confirmed by
35 scanning electron microscopy. Surfactin-15F demonstrated an eradication effect against
36 preformed biofilm, causing severe disruption of *Staphylococcus epidermidis* S61 biofilm
37 structure and reducing viability. The results suggest that surfactins produced by endophytic
38 bacteria could be an alternative to synthetic products. Surfactin-15F, used in wound dressings,
39 demonstrated an efficient treatment of the preformed *Staphylococcus epidermidis* S61
40 biofilm, and thus having a great potential in medical applications.

41 **Keywords:** surfactin, *Staphylococcus epidermidis*, biofilm, antiadhesive, eradication,
42 dressings.

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48 **1. Introduction**

49 Endophytes are microorganisms that colonize and survive in the internal tissues of healthy
50 plants without causing any negative effects on the host [1,2]. One of the key benefits they
51 provide to the plant is the production of bioactive metabolites [3]. Endophytic bacteria are
52 considered as potential reservoirs of myriad bioactive antimicrobial compounds that can be
53 used in medical and industrial and agricultural applications [4–6].

54 Many studies report *Bacillus* species as endophytic bacteria in higher plants with a diversity
55 of secondary metabolites production [7–9]. *Bacillus* species are known to produce a variety of
56 bioactive lipopeptides [10,11], among which surfactins are among the most potent
57 biosurfactants [12]. Surfactins are cyclodepsipeptides incorporating a β -hydroxy-fatty acid
58 unit in the macrocycle. The β -hydroxy-fatty acid units may differ in length (from C13 to C16)
59 and may be grafted in positions 2, 4 or 7 on Ala, Val or Leu amino acids in the peptide ring
60 [13]. Surfactin could be applied in various biopharmaceutical and medical applications [14]
61 due to its anti-inflammatory [15], antibacterial [16], antifungal [17], antiviral [18], anticancer
62 [19] and antibiofilm activities [20], as well as its ability to heal wounds demonstrated by more
63 recent research [21–23].

64 Bacterial biofilms continue to be a major health problem, playing a role in more than 80 % of
65 bacterial infections [24]. Biofilms are a community of microorganisms adhered to a surface or
66 each other and encased in a matrix of extracellular polymeric substances that is self-produced
67 [25]. This matrix acts as a support of the three-dimensional structure of the biofilms and
68 protects them from extreme conditions (temperature, radiation, acidic or alkaline pH, high
69 salinity, high pressure and desiccation) [26–28]. Bacteria in biofilm behave differently from
70 the same planktonic microorganisms and exhibit a high antibiotic resistance [25,29,30]. This
71 resistance is intimately related to the unique physiology and structure of biofilms [31].

72 Antibiotic penetration is physically limited by the chemical composition of the biofilm's EPS
73 matrix, which acts as a physical barrier. It is composed of several anionic and cationic
74 molecules, including uronic acids, proteins, glycoproteins, glycolipids, eDNA, etc [32,33].
75 Furthermore, hydrolases generated by biofilms, including β -lactamase, can degrade antibiotics
76 [34]. The high cell density and spatial proximity of bacteria within the biofilm promote a
77 favorable environment for horizontal gene transfer and subsequently an increase in antibiotic
78 resistance [35]. Genes are transferred horizontally through three different mechanisms: direct
79 contact between donor and recipient cells (conjugation), absorption of extracellular nucleic
80 acid by competent cells (transformation), or transfer of mediated DNA by a bacteriophage
81 (transduction) [36]. Moreover, oxygen limitation in the biofilm deeper layers, generating a
82 metabolically less active or inactive state, also plays an important role in biofilm resistance to
83 antibiotics that only target metabolically active bacteria [37]. Therefore, it is necessary to find
84 alternative compounds to antibiotics in order to eradicate or reduce biofilms.

85 *Staphylococcus epidermidis* , a bacterium commonly found on human skin, can become
86 pathogenic when it adheres to to indwelling medical devices such as catheters, prosthetic
87 joints, and pacemakers. This bacterium is primarily responsible for device-associated
88 Infections, including central line-associated bloodstream infections (CLABSIs), catheter-
89 associated urinary tract infections (CAUTIs), and prosthetic joint infections. These infections
90 can lead to serious complications, including sepsis and device failure [38]. *S. epidermidis* has
91 a unique ability to adhere to surfaces and form biofilms which provides protection against the
92 host's immune response and antibiotics, thereby making these infections difficult to treat.
93 Biofilms formed by *S. epidermidis* often exhibit resistant to antibiotics due to factors such as
94 reduced antibiotic penetration, altered gene expression, and the presence of persister cells.
95 Strains of Methicillin-resistant *S. epidermidis* (MRSE) pose a particularly significant
96 challenge [39].

97 Different treatment strategies have been proposed to control *S. epidermidis* biofilm-associated
98 infections, including the use of combination antibiotic therapy. In fact, antibiotics are often
99 used to combat antibiotic resistance. Options may include vancomycin, daptomycin, linezolid,
100 and rifampin. However, antibiotic susceptibility testing is crucial to guide treatment. In some
101 instances, antibiotic lock therapy, which involves instilling antibiotics into catheter lumens, is
102 used to treat or prevent biofilm-related infections [40]. Furthermore, researchers are exploring
103 novel approaches to disrupt biofilms, including the use of enzymes (e.g., dispersin B) and
104 antimicrobial peptides [41]. Additionally, the development and use of medical devices with
105 antimicrobial coatings can help reduce biofilm formation [42]. Current research is focused on
106 understanding the molecular mechanisms of *S. epidermidis* biofilm formation and developing
107 new strategies to prevent and treat biofilm-related infections. This includes studying the role
108 of quorum sensing, gene regulation, and biofilm-specific factors. Moreover, engineers and
109 researchers are working on developing implantable devices that are less prone to biofilm
110 formation. This includes materials that resist bacterial adhesion and novel coatings with
111 antimicrobial properties [43,44].

112 In this regard, the objectives of the current study were to isolate endophytic bacteria from
113 various plant leaves not overly exploited, produce and characterize the secondary metabolites,
114 evaluate their antibacterial activity as well as their ability to inhibit and eradicate the
115 development of *S. epidermidis* biofilms and finally the development of antibiofilm dressing
116 model.

117 **2. Material and methods**

118 *2.1. Plant collection and isolation of endophytes*

119 To isolate endophytic bacteria, healthy fresh leaves were collected from several locations in
120 Tunisia. Leaves of *Cupressus sempervirens* and *Juniperus oxycedrus* were gathered from Sidi

121 Bouzid, located in central Tunisia (35°02' N, 9°30' E). Leaves of *Nerium oleander* and
122 *Arthrocnemum glaucum* leaves were collected from Sidi Mansour, Sfax, located in the
123 central-eastern Tunisia (34°46'25.4"N 10°48'38.3"E). Additionally, leaves of *Abies alba* L.
124 and *Nerium oleander* were collected from Mount Bou Kornine, Hammam-Lif in northern
125 Tunisia (36° 42' 18"N, 10° 20' 00"E) (Fig. S1 in supplementary material).

126 The approach of Araújo et al. was adopted (with some modifications) to disinfect leaf
127 surfaces and eliminate any form of contamination [45]. Briefly, healthy leaves were washed
128 with sterile water to remove surface debris. The leaves were then immersed in 70% ethanol
129 for three minutes, followed by five-minute immersion in 5% aqueous sodium hypochlorite
130 (NaOCl), and a final one-minute re-immersion in 70% ethanol. This process surface-sterilized
131 the samples. They were then rinsed with sterile distilled water. To verify the effectiveness of
132 the disinfection process, a sample of the final rinse was plated onto the surface of Potato
133 Dextrose Agar (PDA) medium, which served as a control. Thereafter, the leaves were
134 aseptically cut into small segments using a sterile scalpel blade. These fragments were then
135 placed in a ratio of 4 to 5 fragments per sterile 2 ml Eppendorf tubes, each containing 700 µl
136 PDB (Potato Dextrose Broth) medium. The tubes were incubated at 30 °C for 7 days until
137 growth forms appeared. After incubation, a volume of the Eppendorf tubes was plated onto
138 Petri dishes containing PDA culture medium and incubated at 30 °C. Subsequently, distinct
139 bacterial colonies were individually selected, and each bacterial strain was separately
140 transferred to PDA plates and stored at 4 °C for further investigations.

141 2.2. Identification of endophytic microorganisms

142 Molecular identification of the isolated endophytic bacteria was performed using the 16S
143 rDNA sequencing method. The commercially available WiZard Genomic DNA purification
144 Kit (Promega) was used to extract the genomic DNA from the purified endophytic bacteria.
145 Molecular identification was assessed by amplifying and sequencing the 16S rDNA,

146 according to the method described in our previous report [46]. Briefly, PCR was performed
147 with universal primers 16S-27F and 16S-1492R and PCR products were sequenced using
148 primer 907r (5'-CCGTCAATTCCTTTRAGTTT3'). The 16S rDNA sequences data were
149 analyzed and aligned with BioEdit software and compared to the NCBI GenBank database by
150 Blast analysis to find identities between sequences. The phylogenetic tree was constructed
151 using MEGA 4.0 software.

152 2.3. *Extraction of secondary metabolites*

153 The production of secondary metabolites by the isolated endophytic bacteria *Bacillus* 15F was
154 carried out by culturing on PDB for 48 h at 30 °C in a rotary shaker with constant shaking at
155 160 rpm. After incubation, the culture was centrifuged, and the cell-free supernatant was used
156 for the extraction of extracellular metabolites with an equal volume of ethyl acetate by cold
157 percolation [47]. The supernatant-solvent mixture was mixed and allowed to rest until two
158 immiscible phases were formed. The upper phase (ethyl acetate) was collected, and the
159 solvent was then removed under vacuum at 40 °C using a rotary evaporator. The crude extract
160 obtained was stored at 4 °C until further use.

161 2.4. *Annotation of Bioactive Compounds by LC–MS Analysis*

162 The solid–phase extraction (SPE) method was used to fractionate the ethyl acetate extract
163 using the C18 Phenomenex strata-X column. The column was first conditioned by eluting
164 three volumes of acetonitrile. The crude extract was then deposited on the column surface.
165 The column was subsequently eluted with different portions of mobile phase, first with 100%
166 acetonitrile (yielding the surfactin-15F fraction) and then with a binary mixture of
167 dichloromethane/methanol (50/50 – 3 column volumes). The acetonitrile (hereinafter named
168 surfactin-15F) and dichloromethane/methanol fractions were collected and dried under
169 vacuum. All solvents used were of HPLC grade. The surfactin-15F fraction was diluted at 5

170 mg/mL, in methanol, and a volume of 2 μ L was injected. The UHPLC-MS/CAD analyses of
171 the extracts were performed using a Dionex Ultimate 3000 UHPLC system in which the
172 effluents were split (1:1) towards a HESI HRMS Q-Exactive focus mass spectrometer and a
173 Corona Veo charged aerosol detector (Thermo Scientific) under the control of Xcalibur
174 software. A Hypersil GOLD C18 column (150 mm \times 2.1 mm) with 1.9 μ m particle size
175 (Thermo Scientific) was used for chromatographic separation. The flow rate was set at 0.5
176 ml/min. Extracts were eluted using a two-component solvent system, with eluent A being
177 water and eluent B acetonitrile, both solvents being modified with 0.1% formic acid. A
178 gradient profile was used, starting at 5 % of B, remaining constant for 1 min, and then
179 increasing linearly to 100 % B over 15 min. The percentage of B eluent was held constant at
180 100 % for 9 min and then returned to initial conditions over 1 min followed by 4 min of
181 equilibration. The electrospray interface (ESI) polarity was continuously switched between
182 positive and negative polarity with a detected mass range between 100 and 1500 Da.

183 2.5. *Antibacterial activity*

184 2.5.1. *Bacterial strains*

185 Antibacterial activities were tested against a variety of bacteria strains, including *Bacillus*
186 *subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 9144, *Staphylococcus epidermidis* S61,
187 *Pseudomonas aeruginosa* M1, *Salmonella enterica* CIP 80.39, *Agrobacterium tumefaciens*,
188 *Micrococcus luteus* and *Listeria monocytogenes*. These bacterial strains were cultured
189 overnight in LB medium at 30 $^{\circ}$ C and 160 rpm. Following the incubation period, bacterial
190 suspensions with a concentration of 10^6 CFU/ml were prepared by diluting the culture with
191 LB medium.

192 2.5.2. *Determination of MIC and MBC*

193 The antibacterial activity of surfactin-15F was assessed using the microdilution method in 96-
194 well plates, following the guidelines of the National Committee for Clinical Laboratory
195 Standards [48] with some modifications. Initially, surfactin-15F was dissolved in LB with 10
196 % DMSO, and a series of twofold dilutions were prepared in a 96-well microplate. Each well
197 was filled with 100 μ l of diluted surfactin-15F, 80 μ l of the LB medium and 20 μ l of the
198 inoculum. The final DMSO concentration was maintained 2.5% across all wells, including the
199 control well (which did not contain surfactin). The microplates were then incubated at 30 °C
200 for 24 h. Post-incubation, 20 μ l of MTT (0.5 mg/ml) dissolved in water was added to the well.
201 The Minimum Inhibitory Concentration (MIC), which is the lowest concentration that inhibits
202 bacterial growth, was determined by the change in MTT color from yellow to purple. The
203 Minimum Bactericidal Concentration (MBC) values were ascertained by streaking 10 μ l of
204 each bacterial inoculum on LB plates. The (MBC) values were defined as the lowest
205 concentration that could reduce and kill more than 99.9% of the original inoculum.

206 2.6. *Effect of surfactin-15F on S. epidermidis S61 biofilm*

207 2.6.1. *Antiadherence activity*

208 As for antibacterial activity, surfactin-15F was dissolved in 10% DMSO to evaluate its
209 antiadherence capacity. A stock solution of Surfactin-15F was prepared at a concentration of
210 2 mg/ml as in TSB containing 10% DMSO. Sterile two-fold dilution series were then
211 performed to obtain a concentration range from 0.062 mg/ml to 0.007 mg/ml. The
212 antiadherence activity was performed in 96 flat-bottom plates against *S. epidermidis* S61, a
213 known biofilm-forming strain [46]. Each well was filled with 100 μ l of the extract at different
214 concentrations, 50 μ l of the S61 bacterial suspension (resulting in a final optical density of 0.1
215 at 600 nm in each well) and 50 μ l of glucose (yielding a final concentration of 2.25 % in each
216 well). Inoculum-free wells served as blanks, while wells fed with TSB containing 10%
217 DMSO and inoculum but devoid of surfactin served as controls. The biofilm biomass was

218 quantified using the crystal violet assay, as previously described [42], following a 24-hour
219 incubation period at 30°C. The percentage of biofilm inhibition was represented using the
220 following formula:

221

222 **% Biofilm inhibition= [(OD (growth control) – OD (sample))/OD (growth control)] x100**

223 *2.6.2. Eradication activity*

224 *2.6.2.1. Crystal Violet Method*

225 Biofilm eradication on polystyrene plates was assessed following the method proposed by
226 Kavanaugh and Ribbeck [49] with some modifications: 200 µl of *S. epidermidis* S61 culture,
227 adjusted to an OD of 0.1 at 600 nm with TSB medium (supplemented with 2.25 % glucose),
228 was pipetted into each well of the plate. The plates were incubated at 30 °C for 24 h under
229 static conditions. Following incubation, the TSB medium and planktonic cells were then
230 discarded by aspiration, and each well was washed twice with sterile sterile Phosphate-
231 Buffered Saline (PBS). Subsequently, 200 µl of surfactin-15F (dissolved in TSB containing
232 10% DMSO and ranging in concentration from 15.6 to 250 µg/ml) was added to each well. As
233 negative controls, wells devoid of surfactin (supplemented with TSB containing 10% DMSO)
234 were used. The plates were reincubated for 24 hours at 30 °C. Post-incubation, the biofilm
235 biomass was quantified using the crystal violet assay, as previously described [42]. The
236 activity was expressed as the percentage of biofilm eradication, calculated using the following
237 formula:

238

239 **Biofilm eradication= [(OD (growth control) – OD (sample))/OD (growth control)] x100**

240

241 *2.6.2.2. Resazurin assay*

242 The resazurin assay was employed to measure the viability within the *S. epidermidis* S61
243 biofilm. Resazurin, a blue dye, undergoes a transformation to fluorescent pink resorufin in the
244 presence of living bacteria [50]. The biofilm, which had been treated with surfactin-15F in 10
245 % DMSO, along with the control (biofilm treated with TSB medium supplemented with the
246 same solvent), were rinsed with PBS and then filled with 100 µl of resazurin (7-hydroxy-3H-
247 phenoxazin-3-one 10-oxide) (AppliChem GmbH, Germany) at a concentration of 10 µg/ml.
248 Following a 30 min-incubation in the dark, the fluorescence (excitation at 550 nm and
249 emission at 590 nm) was measured using a Varioscan Flash, Thermo scientific [51].

250 2.6.2.3. Determination of CFU reduction

251 *S. epidermidis* S61 biofilm formation was performed in round glass coverslips immersed in 6
252 flat-bottom plates for 24h at 30°C. After incubation, coverslips were washed with
253 physiological water to eliminate planktonic cells and treated with surfactin-15F at
254 concentrations of 62.5 and 250 µg/ml (dissolved in TSB containing 10% DMSO). As negative
255 controls, wells devoid of surfactin (supplemented with TSB containing 10% DMSO) were
256 used. After 24 h of treatment at 30°C, coverslips were removed and washed with
257 physiological water. Following the detachment of *S. epidermidis* biofilm detachment by both
258 sonication and vortex, a series of 1/10 dilutions were performed with physiological water and
259 100 µl of each dilution were plated in TSA medium. After 24 h at 30°C, the number of viable
260 cells was determined and the results are shown as a log₁₀ reduction in CFU following
261 surfactin treatment relative to the CFU of the untreated control [52].

262 2.6.3. Biofilm imaging

263 2.6.3.1. Fluorescence microscopy

264 The eradication effect of surfactin-15F against *S. epidermidis* S61 biofilm was validated using
265 Fluorescence microscopy. Biofilms were grown overnight on circular glass coverslips,
266 subsequently washed with PBS and treated with surfactin-15F (62.5 µg/ml dissolved in TSB
267 containing 10 % DMSO). Coverslips treated solely with TSB containing 10 % DMSO served
268 as controls. After an overnight treatment, the planktonic cells were removed from the biofilms

269 by washing them with PBS. A Live/Dead Light bacterial viability kit (Molecular Probes,
270 Invitrogen) was then used for staining. The kit included two dyes: Propidium iodide (PI),
271 which stains dead bacteria red, and SYTO 9, which stains all living bacteria in green [46].
272 Following staining, the coverslips were washed with distilled water to remove excess
273 fluorochromes . The biofilms were visualized under a Zeiss Axio Imager Fluorescence
274 Microscope equipped with an ApoTome.2 and a 63× oil immersion objective. Z-stack pictures
275 at 1 μm intervals were captured to reconstruct three-dimensional representations of the
276 biofilms.

277 2.6.3.2. Scanning electron microscopy (SEM)

278 Scanning electron microscopy (SEM) was used to visualize the biofilm of *S. epidermidis* S61
279 and to confirm the antiadherence effect of surfactin-15F, as determined by the crystal violet
280 assay. Adapting the method of Kerekes et al. [53] with some modifications, samples for SEM
281 were prepared in stainless-steel coupons placed in 24-well flat bottom plates. Following an
282 overnight co-incubation of *S. epidermidis* S61 with surfactin-15F at 62.5 μg/ml in 10 %
283 DMSO at 30 °C, the planktonic cells were removed, and the coupons were then washed with
284 PBS. Coupons treated with TSB supplemented with 10 % DMSO, but without surfactin
285 served as controls. The biofilms were fixed over a period of four hours using 2.5 % (v/v)
286 glutaraldehyde in PBS. Subsequently, the fixed biofilms were washed with PBS and dried
287 using ethanol concentrations ranging from 30% to 100%. After a 24-hour drying period, the
288 coupons were gold-coated using a fine coat JFC-1100 E Ion Sputtering Device from JEOL,
289 Japan. The biofilm was then imaged at 15 kV at magnifications of ×2,000 and ×5,000 using
290 the JSM-5400 scanning electron microscope (JEOL, Japan).

291 2.7. Hemolytic activity

292 The capacity of surfactin-15F to trigger the release of hemoglobin from human erythrocytes
293 was assessed using a method developed by Luo et al. [54] with slight modifications. Briefly, 1
294 ml of the freshly collected blood was centrifuged at 4000 rpm for 5 min. After removing the
295 supernatant, the pellet was gently washed twice with PBS buffer (10 mM, pH 7.4). The
296 erythrocytes were then resuspended in approximately 4 % PBS. To evaluate the hemolytic
297 activity, 100 µl of the erythrocyte solution was added to the same volume of the surfactin-15F
298 at different concentrations (ranging from 2 to 0.062 mg/ml). The mixture was incubated at 37
299 °C for 1 hour. After centrifugation at 4000 rpm for 10 min, the optical density (OD) of the
300 supernatant was measured at a wavelength of 450 nm [54]. Two controls were used: a
301 negative control consisting of PBS, and a positive control comprising Triton X-100 (0.1%).
302 The percentage of hemolysis was calculated using the following equation:

303

$$304 \quad \text{Hemolytic activity (\%)} = [(OD_{\text{surfactin}} - OD_{\text{PBS}}) / (OD_{\text{Triton X-100}} - OD_{\text{PBS}})] \times 100$$

305

306 2.8. *Membrane biofilm model*

307 *S. epidermidis* S61 biofilm was grown on three distinct types of sterile membranes: cellulose
308 filter paper with a porosity of 10 µm, cellulose nitrate membrane filter with a pore size of
309 0.45 µm and a nylon membrane also with a pore size of 0.45 µm [55]. Briefly, biofilm
310 formation was carried out on these membranes using TSB culture medium supplemented with
311 2.25 % (v/v) glucose and an S61 bacterial inoculum at an OD_{600 nm} of 0.1. Biofilm
312 formation was assessed after 24, 48 and 72 h. Post-incubation, the TSB medium was
313 discarded, and the membranes were carefully washed with PBS to eliminate planktonic
314 bacteria. The filters were then air-dried at room temperature and stained with 0.2 % crystal

315 violet for 10 min. Any excess of crystal violet was rinsed off with water. The intensity of the
316 violet staining is directly proportional to the biofilm formation on these membranes.

317 2.9. *Antibiofilm dressing model*

318 Sterile dressings were utilized as biomaterials to create an antibiofilm coating for treating *S.*
319 *epidermidis* S61 biofilm, which was established on the nylon membrane. This experiment
320 aimed to assess the efficacy of a surfactin-based dressing in reducing or eliminating bacterial
321 biofilms. In particular, 2x2 cm squares of hydrophilic bleached cotton gauze (Bandlux) were
322 cut and sterilized via autoclaving. Subsequently, these squares were immersed in 250 μl of
323 surfactin at concentrations of 62.5 and 125 $\mu\text{g/ml}$, resulting in dressings impregnated with
324 surfactin-15F at densities of 3.9 $\mu\text{g/cm}^2$ and 7.8 $\mu\text{g/cm}^2$, respectively.

325 Following the formation of the S61 biofilm on the nylon membrane surface over a period of
326 24 h, the membranes were placed in TSA dishes and covered with surfactin-15F dressings
327 [55]. The biofilms coated with the dressings were then incubated at 30 °C for 24 h. This
328 treatment was replicated four times for each examined concentration. A control dressing,
329 impregnated solely with water without the addition of surfactin-15F, was used in a similar
330 manner to cover the *S. epidermidis* S61 biofilm. After the exposure period, the dressings (both
331 treatment and control) were carefully removed and placed on TSA medium at 30 °C for 24 h.
332 Another dressing of the same concentration was then suspended in 10 ml of sterile
333 physiological saline for 1 h with agitation. This shaking step is necessary to detach the biofilm
334 from the dressings. Following the detachment of the biofilm, a series of 1/10 dilutions were
335 performed with physiological water. Finally, 100 μl of each dilution was plated on TSA
336 medium and incubated at 30 °C for 24 h to determine the number of viable cells. The results
337 were expressed as Colony Forming Unit (CFU) [55].

338 2.10. *Statistical analysis*

339 All experiments were carried out at least 3 times. The results were expressed as mean values
340 with the standard error. Statistical analyses were performed using Student's t-test to compare
341 the control and treated samples at a significance level of 5%.

342 **3. Results and discussion**

343 *3.1. Isolation and identification of endophytic bacteria*

344 Healthy leaves were randomly collected from various plants across different regions of
345 Tunisia, resulting in the isolation of seventeen bacterial strains. The absence of any microbial
346 growth in the control plates confirmed the elimination of epiphytic microorganisms upon
347 surface sterilization of leaves. The isolates obtained from *Cupressus sempervirens*, *Juniperus*
348 *oxycedrus*, *Arthrocnemum glaucum*, *Nerium oleander* (from Sidi mansour, Sfax), *Nerium*
349 *Oleander* (from Mount Bou Kornine, Hammam-Lif) and *Albies Alba* plants were designated
350 as letter AR, RR, PR, F, N and Sp, respectively. BLAST analysis of the sequenced 16S rDNA
351 bacterial genes showed similarities among various bacteria to the genus *Bacillus*. However,
352 three bacteria 20Sp, 6Sp and 8N were found to belong to the genera *Stenotrophomonas*,
353 *Enterobacter* and *Pseudomonas*, respectively (Fig. 1). All sequences have been provided as
354 supplementary material.

355 *3.2. Annotation of compounds*

356 The ethyl acetate extract of strain 15F was subjected to SPE, and the surfactin-15F fraction
357 was characterized by LC-MS analysis. The chromatographic profile of Surfactin-15F,
358 depicted in Fig. 2, was compared with that of the acetonitrile fractions of the PDB extract to
359 point out the molecules produced exclusively by the strain *Bacillus* sp. 15F. The charged
360 aerosol detector-based profile showed a small peak at 15.46 min (compound **1**), a larger peak
361 at 15.88 min corresponding to the most abundant molecules in the sample (compound **2**), and
362 another important peak at 16.25 min (compound **3**) (Fig. 2). Detailed MS data for compounds

363 **1-3** are summarized in Table 1. Molecules, that dominated the sample and eluted between
 364 15.46-16.25 min with molecular mass ions from m/z 1007.6 to 1035.6 were annotated as
 365 homologous surfactins when their molecular formulas were compared to the list of known
 366 surfactins [13,56]. The surfactins were eluted with approximately 100 % acetonitrile. These
 367 results align with [57], which showed that elution of surfactins can be performed with 85 to
 368 100 % acetonitrile in reversed phase HPLC [57]. The presence of 3 homologous surfactins
 369 with different fatty acid

370 **Table 1. Analytical data for the 3 major surfactins in Surfactin-15F.**

Cmpd. #	t_R (CAD, min)	m/z		Molecular formula	Annotation
		ESI ⁻	ESI ⁺		
1	15.46	1006.6442 ([M-H] ⁻)	1008.6581 ([M+H] ⁺)	C ₅₁ H ₈₉ N ₇ O ₁₃	Leu/Ile7 C13 surfactin
			1030.6411 ([M+Na] ⁺)		
2	15.88	1020.6597 ([M-H] ⁻)	1022.6741 ([M+H] ⁺)	C ₅₂ H ₉₁ N ₇ O ₁₃	Leu/Ile7 C14 surfactin
			1044.6552 ([M+Na] ⁺)		
3	16.25	1034.6751 ([M-H] ⁻)	1036.6895 ([M+H] ⁺)	C ₅₃ H ₉₃ N ₇ O ₁₃	Leu/Ile7 C15 surfactin
			1058.6708 ([M+Na] ⁺)		

371
 372 combinations was confirmed by both Negative (ESI⁻) and Positive (ESI⁺) ionization modes
 373 (Table 1, Fig. S2, S3 and S4 in supplementary material). The structure of surfactin C13 (R¹ =
 374 C4H9), C14 (R¹ = C5H11), C15 (R¹ = C6H13) was presented in Fig. 3. According to the
 375 literature, the detection of surfactins with m/z ranging from 1000 to 2000 can be performed
 376 using ESI-MS [57]. Surfactins are more easily detected in ESI⁻, and the use of formic acid
 377 facilitates their protonation in ESI⁺ [58]. The analysis of the sample also revealed the presence
 378 of the corresponding C13 surfactin (compound **1**), with the presence in negative ionization
 379 mode of the [M-H]⁻ ion at m/z 1006.6442. In the ESI⁺ mode, its [M+H]⁺ ion was at m/z
 380 1008.6581 and its [M+Na]⁺ ion at m/z 1030.6411 (Table 1). Regarding compound **2** in
 381 particular, the presence of a protonated molecular ion ([M+H]⁺) at m/z 1022.6741, its sodium

382 adducts $[M+Na]^+$ at m/z 1044.6552 and $[M-H]^-$ at m/z 1020.6597 suggested that **2** should be
 383 annotated as Leu/Ile7 C14 surfactin. Compound **3** was found to be a homolog of **2**, possessing
 384 an additional CH_2 group. Hence, **3** was annotated as Leu/Ile7 C15, based on the protonated
 385 molecular ions $[M+H]^+$ and its sodium adduct $[M+Na]^+$ at m/z 1036.6895 and 1058.6708,
 386 respectively, and its $[M-H]^-$ ion at m/z 1034.6751 (Table 1). According to the literature, a
 387 surfactin is usually produced as a mixture of three, four or five isoforms [59–62]. The
 388 obtained results are consistent with those of [56], which demonstrated the production of
 389 surfactin derivatives including Leu/Ile7 C13 and Leu/Ile7 C14 by *Bacillus subtilis* isolate
 390 LSFM-05. Other studies also reported the production of C13, C14 and C15 surfactin
 391 homologs by the genus *Bacillus* [10,63].

392

393 3.3. Antibacterial activity

394 The antibacterial activity of surfactin-15F was measured against several actively growing
 395 bacterial strains and the MICs and MBCs values are summarized in Table 2. All the bacterial
 396 strains tested exhibited good growth in the control experiment (2.5 % DMSO). A
 397 concentration of 2 mg/ml of surfactin-15F did not effectively inhibit the growth of
 398 *Agrobacterium tumefaciens*, *Staphylococcus aureus* ATCC 9144 and *Pseudomonas*
 399 *aeruginosa* M1. The most potent antibacterial activity was observed against *S. epidermidis*
 400 S61 with the lowest MIC and MBC values of [0.0625-0.125] and 0.5 mg/ml, respectively.

401

402 **Table 2. Antibacterial activities of surfactin-15F dissolved in 2.5% DMSO against**
 403 **several bacterial strains.**

Surfactin-15F		
Bacteria	MIC*	MBC*

<i>Bacillus subtilis</i> ATCC 6633	[0.25-0.5]	2	404
<i>Staphylococcus aureus</i> ATCC 9144	>2	>2	405
<i>Staphylococcus epidermidis</i> S61	[0.062-0.125]	0.5	406
<i>Pseudomonas aeruginosa</i> M1	>2	>2	407
<i>Salmonella enterica</i> CIP 80.39	[1-2]	>2	408
<i>Agrobacterium tumefaciens</i>	>2	>2	409
<i>Listeria monocytogenes</i>	[0.5-1]	>2	409

410

411

412

413 * MIC; MBC (mg/ml).

414 The antibacterial propriety of surfactin is attributed to its ability to penetrate the membranes
415 of various types of bacteria. This leads to surfactin exhibiting detergent-like activity and
416 creating a permeable environment for the lipid bilayer, which subsequently results in the
417 dissolution of the membrane cells [16,64].

418 3.4. Effect of surfactin-15F on *S. epidermidis* biofilm

419 3.4.1. Antiadherence activity

420 In this work, *S. epidermidis* S61, a biofilm-forming bacterium, was used as a model isolate to
421 investigate the antiadherence activity of surfactin-15F. Surfactin-15F dissolved at
422 concentrations ranging from 7.8 to 62.5 µg/ml in TSB containing 10 % DMSO, inhibited
423 biofilm formation with a reduction in OD from 1.85 (control) to 0.65 and 0.16, respectively
424 (Fig. 4). The highest antiadherence effect (90 %) was observed at a concentration 62.5 µg/ml,
425 but surfactin-15F exerted a strong (> 50 %) antiadherence effect even at the lowest tested
426 concentration (Fig. 4.a). As shown in Fig. 4.b, the resazurin test demonstrated that surfactin-
427 15F at 62.5 µg/ml induced almost total reduction of cell viability (only $(0.003 \pm 0.0002) \times 10^3$
428 compared to the untreated control $(0.7 \pm 0.051) \times 10^3$).

429 Previous studies have highlighted the potential of surfactins as inhibitors of microbial
430 adhesion and biofilm formation. For instance, Liu et al. [65] reported the ability of surfactins
431 produced by *Bacillus subtilis* to inhibit 40 % of *S. aureus* ATCC65389 biofilm formation at a
432 concentration of 8 µg/ml. At a concentration of 32 µg/ml, the antiadhesive effect exceeded 80
433 % [65]. Similarly, Zeraik and Nitschke [66] reported that 0.1 % (w/v) of surfactins produced
434 by *Bacillus subtilis* LB5a reduced the biofilm formation of *Listeria monocytogenes* ATCC
435 19112, *S. aureus* ATCC 25923 and *Micrococcus luteus* ATCC 4698 with inhibition rates in
436 the range of 63–66% , 47-56%, 34-42% at 4, 25 and 35 °C, respectively [66].

437 Another study, conducted by Meena and coworkers showed that 100 µg/ml of surfactins from
438 *Bacillus subtilis* KLP2015 reduced *S. aureus* ATCC 6538 biofilm by 58 % [67].

439 Numerous studies have investigated the reduction of bacterial adherence following the
440 treatment of surfaces with biosurfactants [62, 63]. According to the literature, the antiadhesive
441 effect of a biosurfactant is attributed to its ability to adsorb to a solid surface by changing the
442 hydrophobicity of the surface. The nonpolar part interacts with the hydrophobic surface, while
443 the polar part is exposed to the aqueous environment, resulting in a decrease in the
444 hydrophobicity of the surface. This change impairs microbial adhesion to this surface and thus
445 reduces biofilm development [68]. According to Zeraik and Nitschke [66], the antiadherence
446 effect favored by surfactin treatment, an anionic surfactant, could be due to electrostatic
447 repulsion between bacteria and surfactin molecules adsorbed on the polystyrene surface and a
448 reduction in hydrophobic interactions. These authors showed that the surface hydrophilicity
449 increased after conditioning with a surfactant, and therefore, the adhesion of microorganisms
450 decreased.

451 3.4.2. Antiadherence confirmation by scanning electron microscopy

452 The effect of surfactin-15F on *S. epidermidis* S61 biofilm was confirmed by scanning electron
453 microscopy. As shown in Fig. 5, a notable difference was observed between the control and

454 the treated biofilm. Representative images of the untreated biofilm (Fig. 5.a) showed a thick
455 biofilm composed of many layers of adherent cells, while the surface treated with 62.5 µg/ml
456 (Fig. 5.b) and 15.6 µg/ml of surfactin-15F (Fig. 5.c) showed few adherent cells and a decrease
457 in biofilm formation. On the other hand, we noticed a slight swelling of bacterial cells after
458 the treatment with surfactin-15F compared to the cells within the untreated biofilm (control
459 with 10 % DMSO). Surfactin-15F thus resulted in almost complete destruction of the matrix
460 with the appearance of signs of cell stress and cell debris after bacterial lysis. These results
461 reconfirm the antiadhesive potential of surfactin-15F against *S. epidermidis* S61 biofilm.

462 3.4.3. Biofilm eradication

463 The ability of surfactin-15F to eradicate preformed biofilms of *S. epidermidis* S61 was also
464 investigated. A mature biofilm was treated with surfactin-15F at concentrations ranging from
465 15.6 to 250 µg/ml. The efficacy of biofilm eradication was assessed using both crystal violet
466 and resazurin tests. Compared to the untreated biofilm (control), surfactin-15F at a
467 concentration of 15.6 µg/ml destroyed approximately 70 ± 4.8 % of the *S. epidermidis* S61
468 biofilm (data not shown).

469 Furthermore, evaluation of cell viability in treated and untreated biofilms showed that
470 surfactin-15F significantly reduced the viability of the *S. epidermidis* S61 biofilm.
471 Specifically, at a concentration of 62.5 µg/ml, surfactin-15F exhibited a fluorescence
472 intensity of $(0.07 \pm 0.01) \times 10^3$, whereas the control (treated with 10 % DMSO) showed a
473 fluorescence intensity of $(0.7 \pm 0.06) \times 10^3$ (Fig. 6).

474 The effectiveness of surfactin in reducing the viability within *S. epidermidis* S61 biofilm was
475 also confirmed by the determination of Log reduction after surfactin treatment. Surfactin-15F
476 at concentrations of 250 and 62.5 µg/ml was effective in reducing more than 99,9% of biofilm
477 with Log reduction of 5.69 and 4.6, respectively (Fig. 6. b). According to Kundu et al., a
478 decrease of $\geq 3 \log_{10}$ ($\geq 99.9\%$), was deemed indicative of effective disinfection [69].

479 The antibiofilm activity of surfactin against *S. epidermidis* is attributed to several
480 mechanisms. According to the literature, surfactins insert into lipid bilayers, dissolve the
481 phospholipid phase, chelate monovalent and divalent cations, and alter the membrane
482 permeability through channel formation or membrane dissolution, similar to detergents [70].
483 Some studies have shown that surfactins can form channels within biofilms that disrupt
484 membrane integrity and permeability to ion (including Ca^{2+} and K^{+} ions), ultimately leading
485 to membrane damage and cell death [71,72]. Biosurfactants (BS) hinder biofilm formation by
486 altering cell-to-cell adhesion capacity, disrupting membranes, inhibiting the electron transport
487 chain and limiting the amount of energy required by cells [73].

488 3.4.4. Eradication activity confirmation by fluorescence microscopy

489 Fig. 7 presents three-dimensional fluorescence images of *S. epidermidis* S61 biofilm. Notably,
490 a stark contrast exists between the untreated biofilm control and the biofilm treated with
491 surfactin-15F. In the control (Fig. 7.a), an abundant and dense biofilm (stacked layers of
492 cells), with the coexistence of a large number of viable bacteria (green) and a less quantity of
493 dead bacteria (red), was observed. However, after treatment with surfactin-15F, the majority
494 of the biofilm biomass is removed, leaving only a few bacterial cells, mostly dead, attached to
495 the cover slip surface (Fig. 7.b). These results confirm the efficacy of surfactin-15F in
496 disrupting the structure of *S. epidermidis* S61 biofilm and reducing cell viability.

497 3.5. Hemolytic activity

498 Given the sensitivity of human cells to hemolysis induced by certain compounds, it is crucial
499 to investigate the hemolytic properties of surfactin-15F before considering its potential use in
500 future medical applications. Hemolytic activity of surfactin-15F on human red blood cells, a
501 widely used model to assess the hemolytic properties, was evaluated [74]. The results of
502 hemolysis are shown in Fig. 8. This assay showed that hemolytic activity is dose dependent.

503 At concentrations of 0.5 to 2 mg/ml, surfactin-15F exerted a strong hemolytic effect with the
504 percentage of hemolysis exceeding 80 %. The IC₅₀, defined as the concentration capable of
505 destroying 50 % of red blood cells, was of the order of 0.26 mg/ml. However, even at low
506 concentrations, surfactin-15F caused mild hemolysis. No detectable hemolysis was observed
507 at concentrations less than or equal to 62.5 µg/ml. Yan et al. showed that the hemolytic
508 activity of surfactin produced by *Bacillus subtilis* OKB105 was barely noticeable at the
509 concentration of 20 µg/ml [75]. Other studies revealed that surfactin had a significant
510 hemolytic impact, which might limit its application as a medicinal drug. However, given the
511 significance of this molecule and its remarkable bioactive qualities, some research was
512 concentrated on the reduction of the surfactine hemolysis either by chemical modification of
513 the molecule skeleton and then the generation of surfactin linear analogs and derivatives [76–
514 78] or by genetic engineering [79,80].

515 3.6. *Membrane biofilm model*

516 The biofilms formed on the membranes for 24 h, 48 h and 72 h of incubation, and stained
517 with crystal violet are shown in Fig. 9. Across different incubation times, all surfaces of the
518 filter papers exhibited a violet coloration without any visible bacteria attached to them (Fig.
519 9.a,b,c). This suggest that the filter paper does not provide sufficient support for *S.*
520 *epidermidis* S61 biofilm formation, and the observed purple color on the filter papers may
521 result from the absorption and adsorption of crystal violet by the entire surface of the filter
522 rather than biomass of the biofilm.

523 When evaluating *S. epidermidis* S61 biofilm formation on the cellulose nitrate membrane
524 stained with crystal violet, the 24h membrane showed more intense violet staining compared
525 to the membranes incubated for 48 h and 72 h, with no distinct appearance of bacterial cells
526 on the membrane surface (Fig. 9.d,e,f). The differences in color intensity of the membranes
527 over the incubation period indicate a priori biofilm formation by the S61 strain.

528 However, the nylon membranes showed purple coloration at different incubation times, with
529 visible bacteria adhering to the surface (Fig. 9.g,h,i). Fig. 9.g illustrates a uniform layer of
530 adherent bacteria with an intense purple color covering the entire surface of the membrane,
531 demonstrating biofilm formation by *S. epidermidis* S61. Thus, the bacterium is able to form a
532 thick biofilm on the nylon membrane, particularly the first 24 h of incubation.

533 The ability of the bacterium *S. epidermidis* S61 to form a biofilm on various types of
534 membranes can be attributed to multiple factors. The adhesion of bacteria to a surface is
535 influenced by the hydrophobic / hydrophilic nature, or the presence of charges on the bacterial
536 walls or the surface of the substrate. However, other parameters, particularly the
537 physicochemical properties of the bacterial cells and the quality of the surfaces, can also
538 impact bacterial adhesion [81]. As per the literature, the hydrophobicity of the bacterial
539 surface is a crucial property that contributes to the adhesion of bacteria to both living and non-
540 living surfaces [82,83]. The higher the hydrophobicity of the bacteria, the greater their ability
541 to aggregate on a surface [84]. *S. epidermidis* S61, which exhibited strong hydrophobicity as
542 determined by solvent adhesion methods (MATS), has demonstrated the capability to form
543 biofilms not only on hydrophobic substrates such as polystyrene plate [46] but also on
544 hydrophilic substrates like cellulose and nylon nitrate membrane.

545 It has been reported that bacteria with hydrophobic proprieties have a higher affinity to adhere
546 to hydrophobic materials. However, some bacterial strains can form biofilms on both
547 hydrophilic and hydrophobic substrates. Cerca et al. [85] demonstrated that there is no
548 correlation between the hydrophobic character of the bacterial surface and its initial ability to
549 bind to hydrophilic or hydrophobic substrates [85]. *S. epidermidis* can adhere to hydrophilic
550 membranes such as cellulose nitrite and nylon, with a greater affinity for nylon. This confirm
551 that several factors are involved in adhesion and biofilm formation that are not related to
552 hydrophobic/hydrophilic character. The nylon membrane used had a cationic character with a

553 positive charge. Considering that negative charges dominate the bacterial cell surface and that
554 S61 has a basic and electron donor character [46], the adhesiveness of S61 and its ability to
555 form a biofilm on the nylon membrane could be explained by physicochemical and
556 electrostatic interactions between cell membrane and nylon membrane. Van Loosdrecht et al.
557 [86] demonstrated that the adhesion of bacteria to the surface depends not only on the
558 hydrophobicity of the cells, but also on Brownian motion, Van der Waals attractions,
559 gravitational forces and electrostatic surface charges [86].

560 3.7. *Surfactin-15F dressings and their in vitro efficacy for treatment of pre-formed S61* 561 *biofilm on a nylon membrane*

562 Based on the previous results, the nylon membrane emerged as the optimal support for the
563 formation of *S. epidermidis* S61 biofilm. The effectiveness of surfactin dressings on this
564 biofilm was quantitatively and qualitatively assessed when the biofilm, formed on the nylon
565 filter, was 24 hours old. As shown in Fig. 10.a, the application of dressings impregnated
566 surfactin-15F at concentrations of $7.8 \mu\text{g}/\text{cm}^2$ (dressing 1) and $3.9 \mu\text{g}/\text{cm}^2$ (dressing 2)
567 resulted in the removal of the majority of the biofilms. Following the formation of biofilm
568 and its treatment with the surfactin-15F-impregnated dressings, only a few colonies were
569 observed to grow on the dressings that were in contact with the surface of the TSA medium.
570 In contrast, the control group, which did not receive any surfactin-15F treatment, exhibited
571 significant growth of *S. epidermidis* after the dressing made contact with the culture medium
572 (Fig. 10.a).

573 This effect was also qualitatively confirmed by visualizing the residual biofilms on the nylon
574 membrane stained with crystal violet. Indeed, the membranes treated with the surfactin
575 dressings showed a reduction in bacterial cell condensation compared to the control,
576 particularly in the middle and at the end of the membrane. This was characterized by a
577 decrease in violet staining (Fig. 11). This effect was more pronounced in the case of treatment

578 with dressing 1 (Fig. 11.b), confirming that the effect of surfactin-15F is dose-dependent. The
579 decrease in the biomass of the biofilm adhering to the nylon membrane thus corroborates the
580 previous results. These results demonstrate the *in vitro* efficacy of surfactin-15F dressings
581 against *S. epidermidis* S61 biofilm.

582 To quantify these results, the number of colony forming units (CFU) in the dressings was
583 measured. The results expressed as $\text{Log}_{10}(\text{CFU})$ as a function of treatment concentration are
584 reported in Fig. 10.b. Treatment of the biofilm with dressings containing 7.8 and 3.9 $\mu\text{g}/\text{cm}^2$
585 of surfactin-15F resulted in a significant decrease in the number of colonies compared to the
586 untreated biofilm and subsequently a decrease in biofilm growth after treatment. This
587 decrease is more pronounced in the treatment with dressing 1 (7.8 $\mu\text{g}/\text{cm}^2$) confirming that the
588 effect of surfactin-15F is dose-dependent. Since these concentrations have no hemolytic
589 effect, their applications in dressings for the treatment of infections associated with *S.*
590 *epidermidis* biofilms should be experimented *in vivo*, and represents a safe and promising
591 therapeutic strategy.

592 **4. Conclusion**

593 Biofilm resistance and antibiotic resistance are some of the most significant problems in
594 medicine. This study showcased the potential of endophytes, represented by the strain
595 *Bacillus* sp. 15F, to produce antiadhesive molecules, surfactin(s), that counter the formation
596 of *S. epidermidis* S61 biofilms formation. The surfactin(s) exhibited significant antibacterial
597 and antibiofilm activity without causing a hemolysis effect. This activity was further validated
598 by the development of surfactin-containing dressings, which demonstrated notable efficacy
599 against the mature biofilm of *S. epidermidis* S61. The model of surfactin-based dressing gave
600 promising results that encourage further research and evaluation of their effectiveness, *in vitro*
601 and *in vivo*, in wound repair and healing, while reducing the negative effects of biofilm
602 infections. Moreover, given their antibacterial and antibiofilm performances, the surfactins
603 can be used in futur studies as antiadhesive coating for medical implants such as catheter and
604 prostheses and as a cleaning agent to suppress and eradicate bacterial biofilm from medical

605 instruments. The combination of surfactin and nanoparticles may also offer an interesting
606 strategy for combating biofilms and bacterial resistance.

607 Overall, the surfactin(s) produced by the endophyte *Bacillus* sp. 15F hold promising potential
608 for use in medical, hygienic, and therapeutic fields.

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Figures

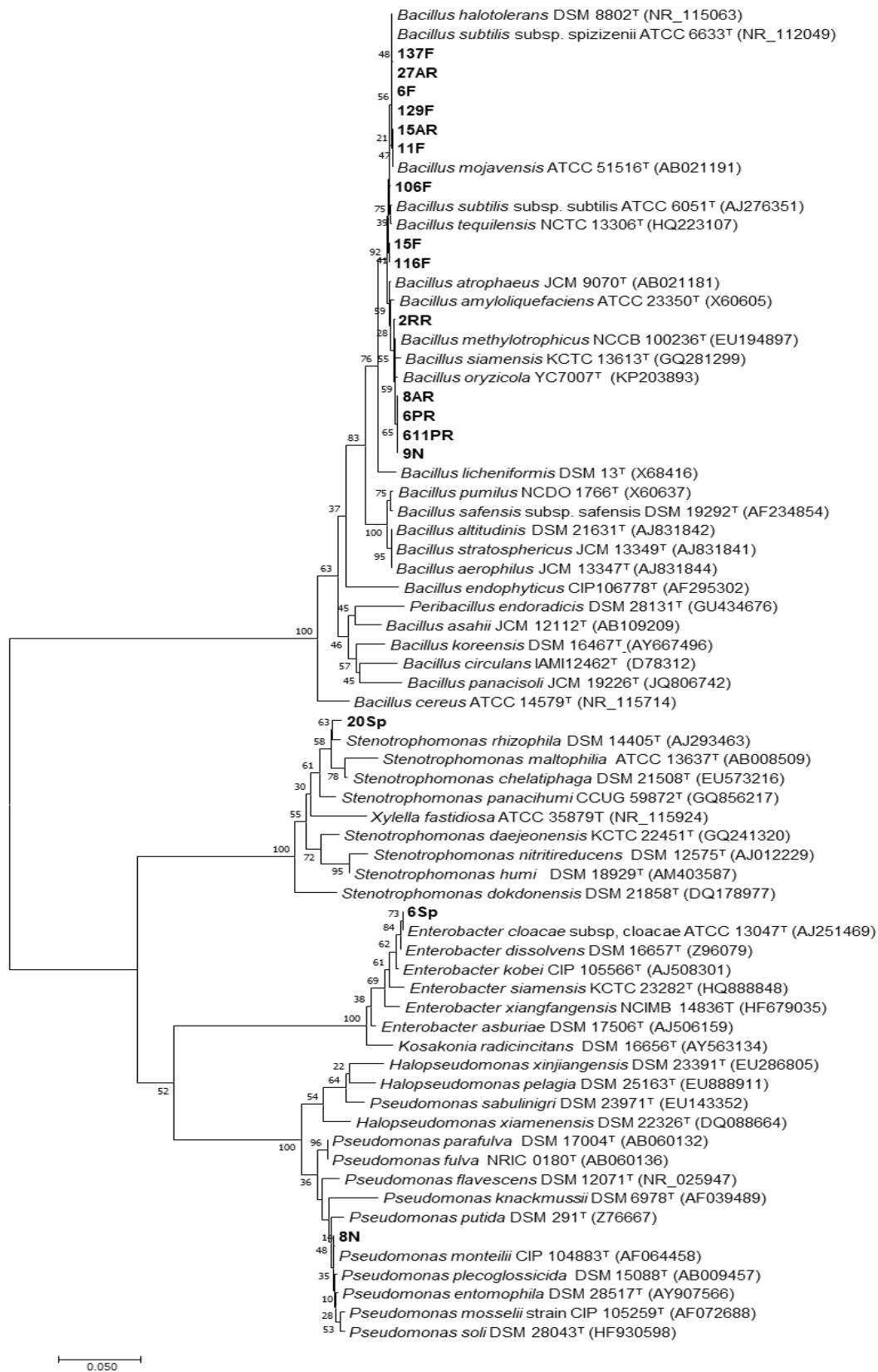


Fig. 1. Evolutionary relationships of taxa.

The evolutionary history was inferred using the Neighbor-Joining method [87]. The optimal tree with the sum of branch length = 1.27497657 is shown. The percentage of replicate trees

in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches [88]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [89] and are in the units of the number of base substitutions per site. The analysis involved 70 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 721 positions in the final dataset. Evolutionary analyses were conducted in MEGA4 [90].

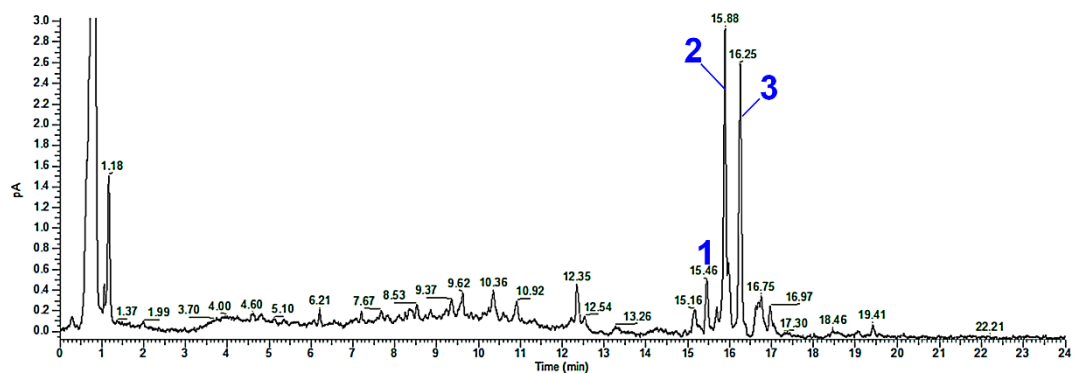


Fig. 2. UHPLC-CAD profile of surfactin-15F.

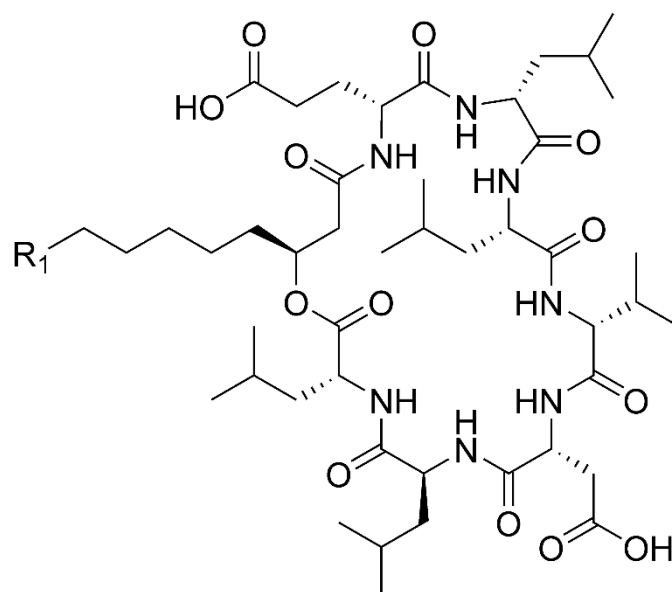


Fig. 3. The structure of surfactin C13 (R₁ = C₄H₉), C14 (R₁ = C₅H₁₁), C15 (R₁ = C₆H₁₃)

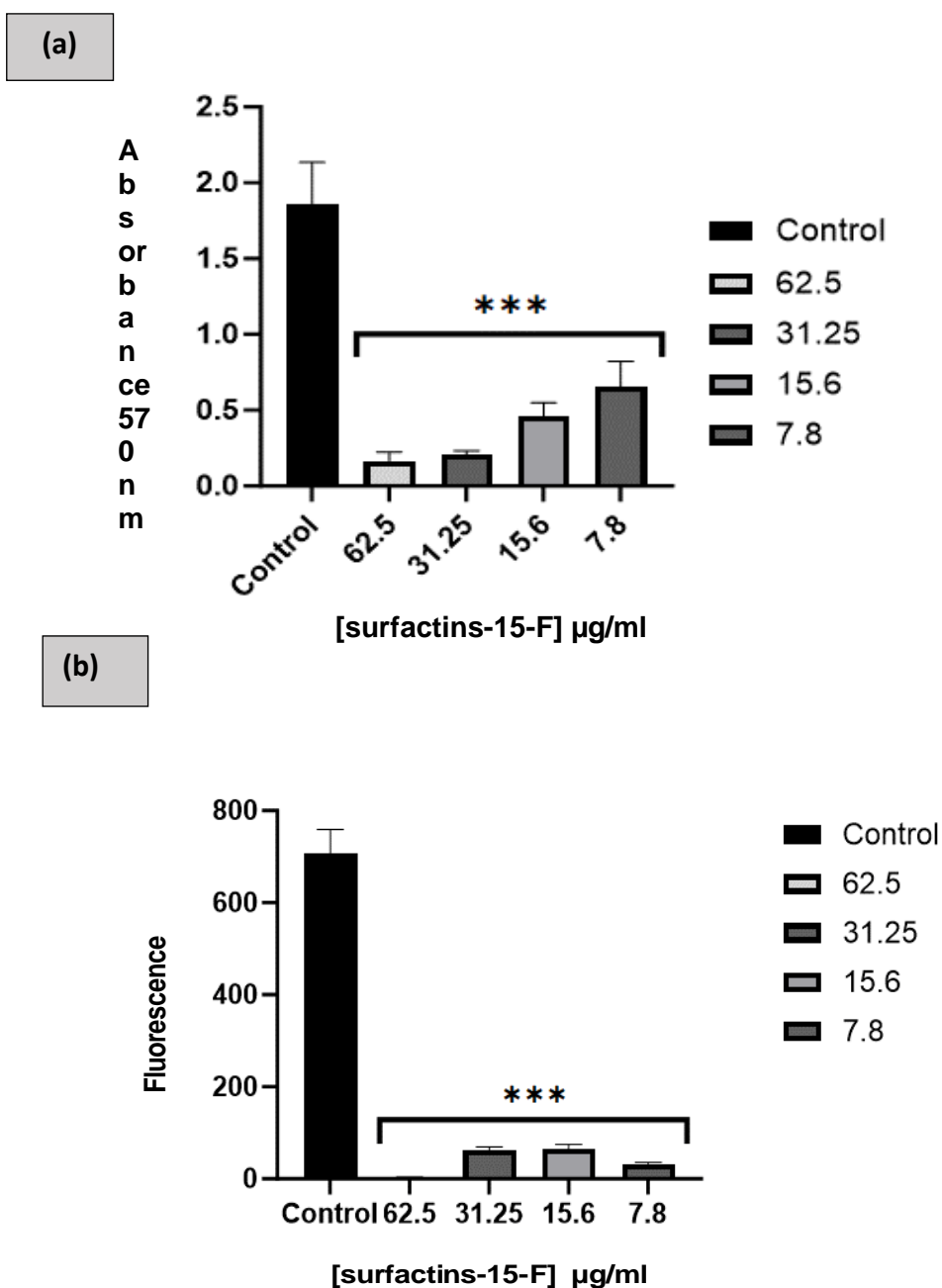


Fig. 4. The ability of surfactin-15F produced by *Bacillus* 15F in inhibiting biofilm formation of *Staphylococcus epidermidis* S61. (a) showed the results obtained from the crystal violet test for the antiadhesive effect of surfactin-15F dissolved in 10% DMSO. (b) showed the viability obtained, using resazurin method, of the control and the treated biofilm with surfactin-15F dissolved in 10% DMSO. The data show the average of five independent replicates \pm standard error. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ by student's paired test.

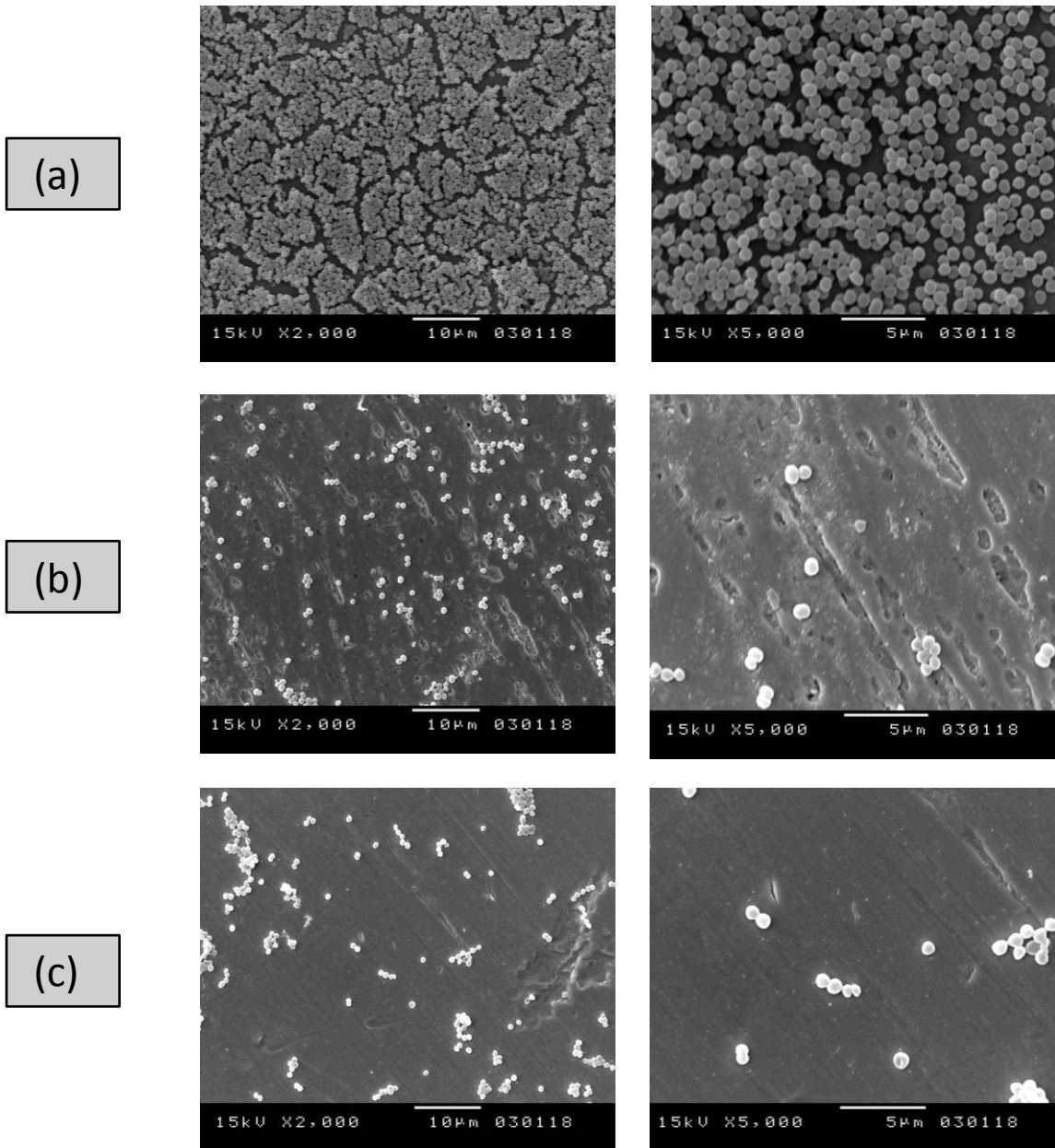
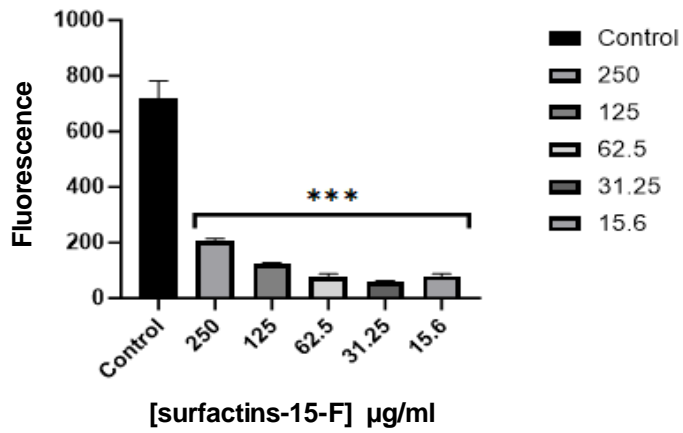


Fig. 5. Scanning electron microscopy images of 24 h biofilms of *Staphylococcus epidermidis* S61 formed on stainless steel surface (a) untreated biofilm (control), (b) biofilm after 24 h of contact with 62.5 µg/ml of surfactin-15F (treated) and (c) biofilm after 24 h of contact with 15.6 µg/ml of surfactin-15F (treated)

(a)



(b)

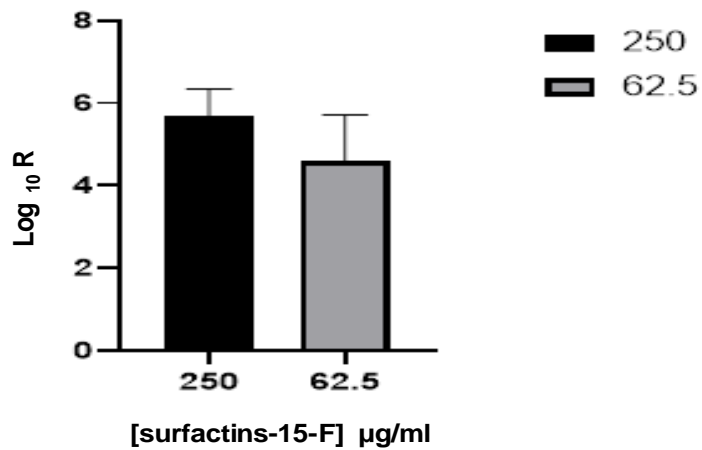


Fig. 6. (a) Evaluation of cell viability within *Staphylococcus epidermidis* S61 biofilm by resazurin test. Results are presented as the mean \pm standard deviation of three. * $p < 0.001$ from control values. (b) Log reduction of CFU counting after the treatment of *S. epidermidis* S61 biofilm with surfactin.**

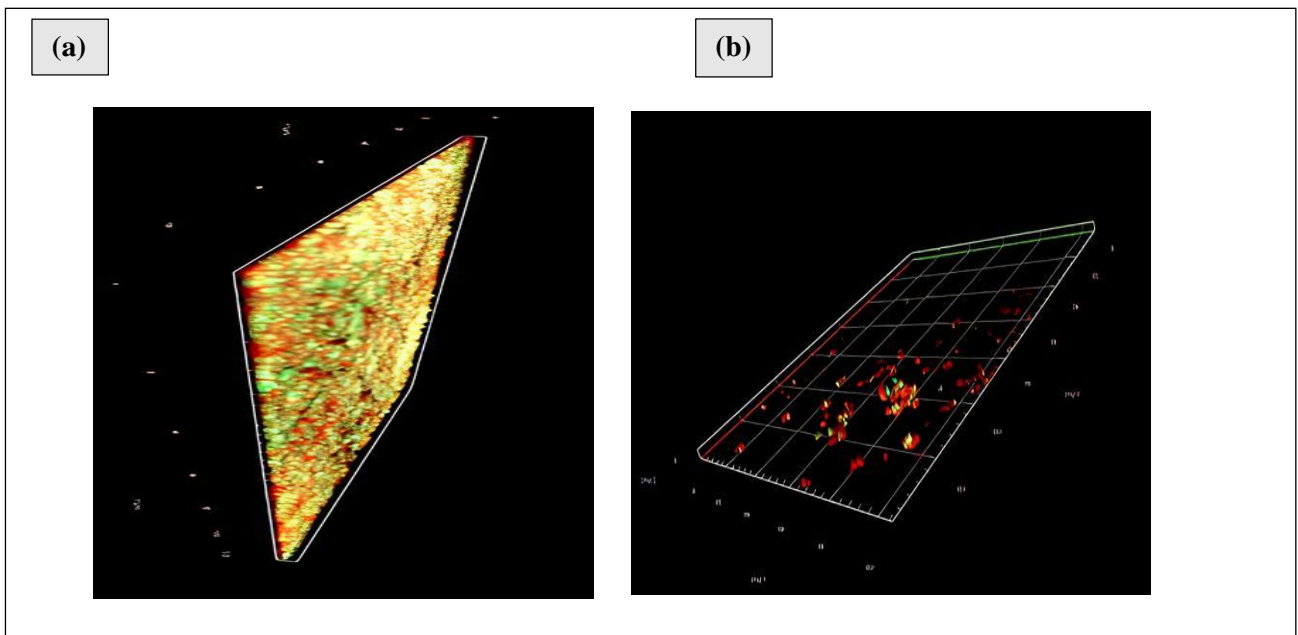


Fig. 7. Fluorescence Apotome imaging, *Staphylococcus epidermidis* S61. a) Biofilm control (with 10% DMSO. b) Biofilm treated with 62.5 µg/ml of surfactin-15F. Live bacteria appears in green and dead bacteria in red.

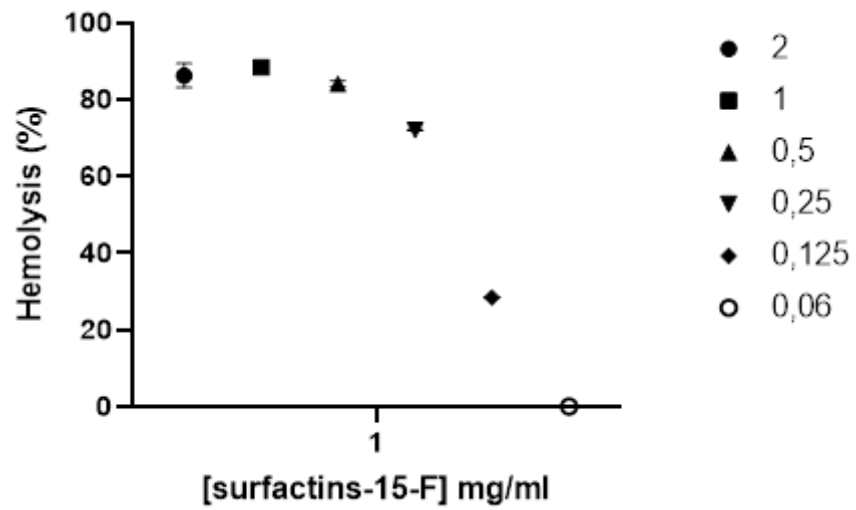


Fig. 8. Hemolytic activity of surfactin-15F as a function of its concentration measured at 450 nm.

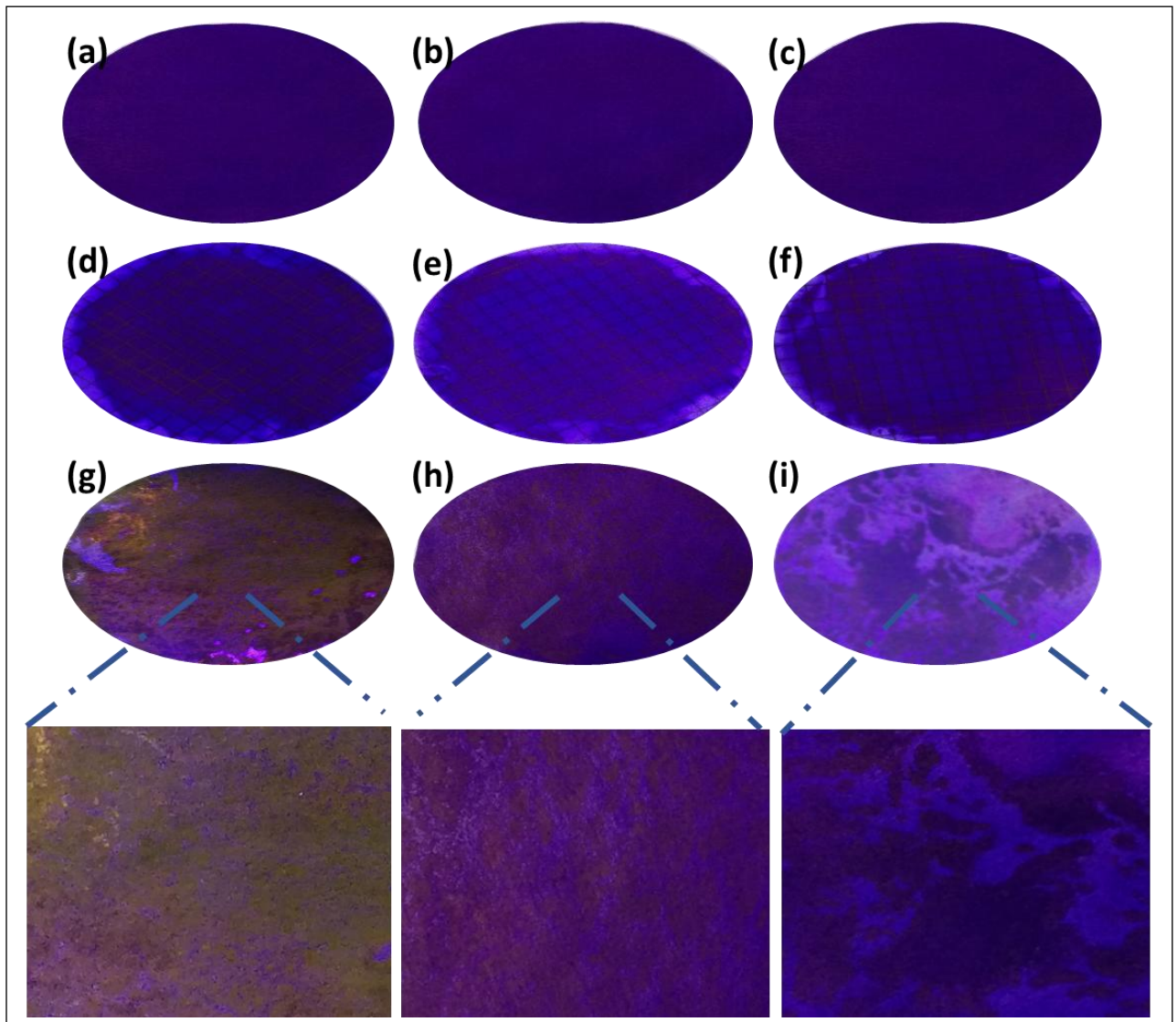
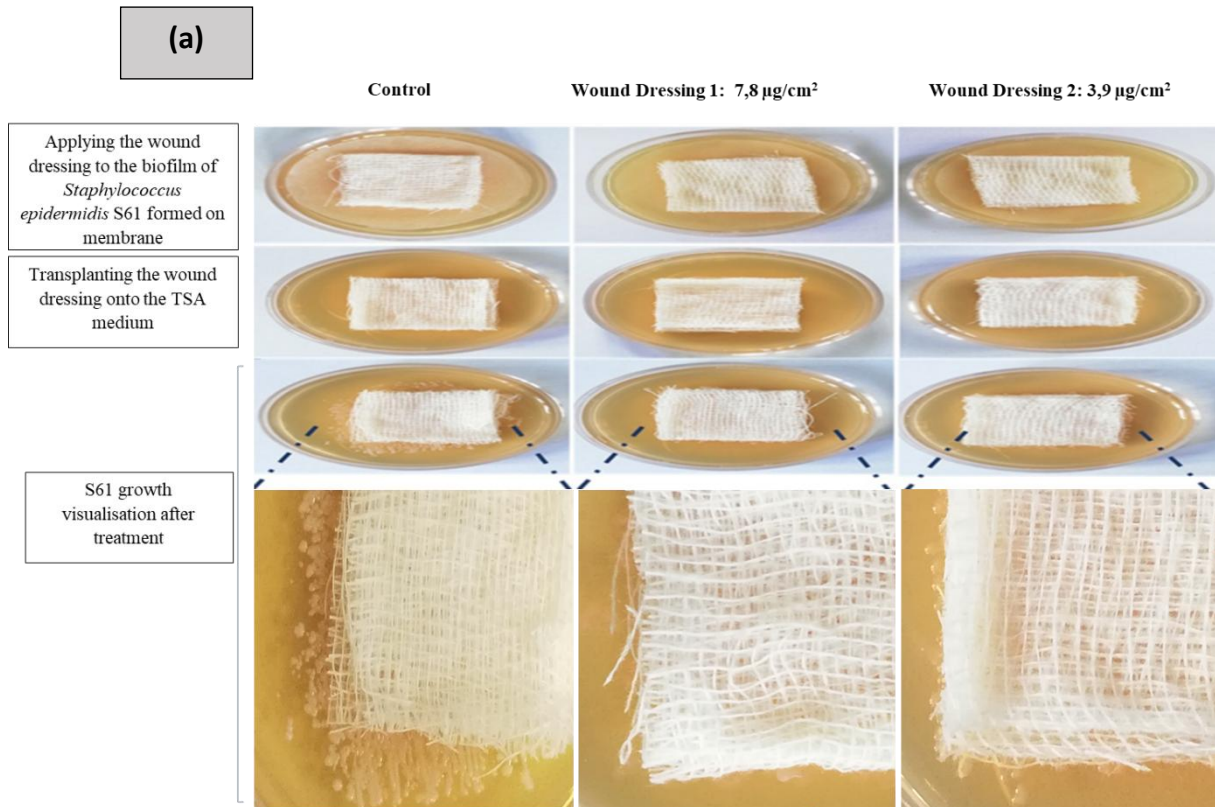
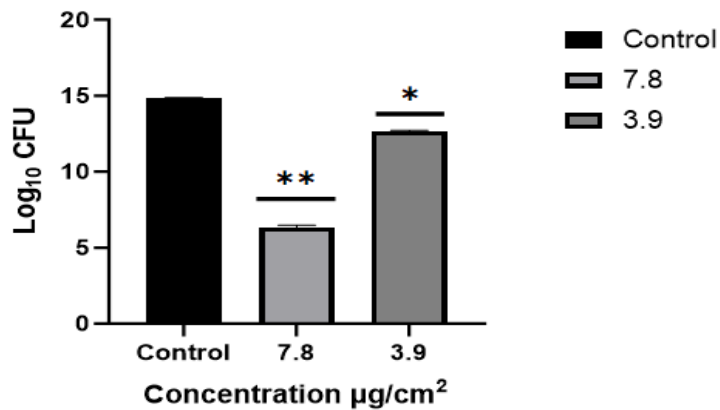


Fig. 9. Biofilm formation on different types of membranes. Representative images after crystal violet staining of biofilms formed by *Staphylococcus epidermidis* S61 on membranes: Images a, b and c show the biofilms formed on the filter paper for 24 h, 48 h and 72 h, respectively. Images d, e and f show the biofilms formed on the cellulose nitrate membrane for 24 h, 48 h and 72 h, respectively. Images g, h and i show the biofilms formed on the nylon membrane for 24 h, 48 h and 72 h, respectively.



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(b)



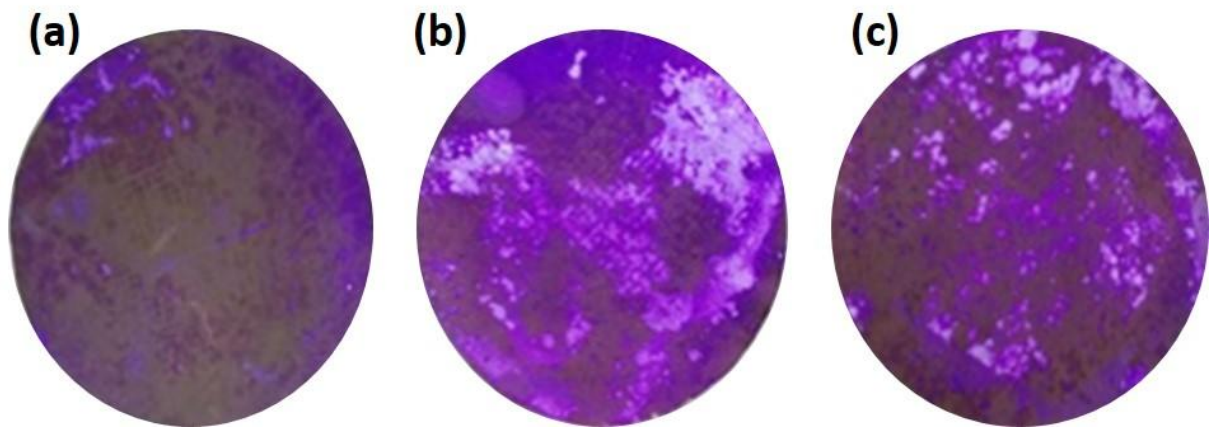
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6 **Fig. 10. Effect of dressings impregnated with surfactin-15F on the biofilm of**
 7 ***Staphylococcus epidermidis* S61. (a) The biofilms were grown on nylon membranes on**
 8 **TSA agar plates for 24 h and subjected to the action of the control dressing (without**
 9 **treatment) and the treatments with dressing 1 (7.8 µg/cm²) and dressing 2 (3.9 µg/cm²).**
 10 **(b) Biomass and viability of biofilms after treatment are assessed by colony forming**
 11 **units (CFU) on TSA plates. Results are presented as the mean ± standard deviation of**
 12 **three replicates. * p <0.05, and ** p <0.01 from control values.**

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19 **Fig. 11. Representative images of *Staphylococcus epidermidis* S61 biofilms formed on**
20 **nylon membranes after crystal violet staining. (a) biofilm remaining attached to a nylon**
21 **membrane following application of the control dressing. (b) and (c) biofilms remaining**
22 **on the membranes following the application of dressings with 7.8 and 3.9 µg/cm² of**
23 **surfactin-15F, respectively.**

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