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1 **Antibacterial, anti-adherent and cytotoxic activities of surfactin(s)**
2 **from a lipolytic strain *Bacillus safensis*F4**

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31

32 **Abstract**

33 The bacterial strain F4, isolated from olive oil-contaminated soil, has been found to produce
34 biosurfactants as confirmed by oil displacement test and the emulsification index results. The
35 identification of the strain F4, by 16S ribosomal RNA gene, showed a close similarity to
36 *Bacillus safensis*, therefore the strain has been termed *Bacillus safensis* F4. The Thin Layer
37 Chromatography (TLC) and the High Pressure LiquidChromatography-Mass Spectrometry
38 (HPLC-MS/MS) demonstrated that the biosurfactant had a lipopeptide structure and was
39 classified as surfactin. The present study showed also that the produced biosurfactant has an
40 important antibacterial activity against several pathogen strains as monitored with minimum
41 inhibitory concentration (MIC) micro-assays. In particular, it presented an interesting anti-
42 planktonic activity with a MIC of 6.25 mg mL⁻¹ and anti-adhesive activity which exceeded
43 80% against the biofilm-forming *Staphylococcus epidermidis* S61 strain. Moreover, the
44 produced lipopeptide showed an antitumor activity against T47D breast cancer cells and
45 B16F10 mouse melanoma cells with IC₅₀ of 0.66 mg mL⁻¹ and 1.17 mg mL⁻¹, respectively.
46 Thus, our results demonstrated that *Bacillus safensis* F4 biosurfactant exhibited a polyvalent
47 activity *via* a considerableantibiofilm and antitumoralpotencies.

48

49

50 **Keywords:**

51 Anti-adherent; anti-cancer; Biosurfactant; *Bacillussafensis* F4; Surfactin(s).

52

53 **Introduction**

54 Biosurfactants or bioemulsifiers are amphipathic surface-active molecules, which are
55 produced by micro-organisms, composed of hydrophobic (nonpolar) and hydrophilic (polar)
56 moieties. As a consequence, they have the ability to aggregate at interfaces between fluids with
57 different polarities such as oil/water or air/water, reduce the surface and interfacial tensions
58 and form emulsions (Sen et al. 2017). These compounds are characterized as glycolipids,
59 lipopeptides, lipopolysaccharides, fatty acids, phospholipids and neutral lipids (Bezza and
60 Chirwa 2016; Colla et al. 2010). Biosurfactants are produced by a wide variety of bacteria,
61 actinobacteria and fungi with different chemical structures. Some bacterial genera like
62 *Bacillus* and *Arthrobacter* are known with their production of lipopeptide biosurfactant (Sriram
63 et al. 2011). Some studies have described the biological activities of the biosurfactants
64 including antimicrobial, anti-adhesive and anti-biofilm properties (Silva et al. 2014, Gudiña et
65 al. 2010a). In fact, the bacterial infections and their biofilm formation abilities causing
66 resistance increase against drugs is getting a serious problem for human health. An urgent
67 need for solving this problem is based on the screening of novel drugs eradicating or
68 inhibiting biofilm formation. The adherence is the first step of the infectious process that
69 requires efficient antagonising molecules. Previous studies reported that based on their
70 amphiphilic structures, the biosurfactants reduce the surface tension and therefore affecting
71 the bacterial adherence (Janek et al. 2013). In this context, the lipopeptide biosurfactant
72 produced by *Bacillus subtilis* presented antibacterial, anti-adhesive and anti-biofilm activities
73 on uropathogenic bacteria (Moryl et al. 2015). Moreover, a glycolipid biosurfactant, presented
74 cytotoxic activities on cancer cell lines, was produced by a *Nocardia farcinica* strain (Christova
75 et al. 2015). The biosurfactants, which are selective in nature, act on the surface of liquids and
76 facilitate the action of certain enzymes such as lipases and/or esterases by reducing the surface

77 tension of liquids and/or improving the solubility of water immiscible substrates (Sekhon et
78 al. 2011, 2012).

79 Lipases are characterized by their ability to synthesize ester bonds in a non-aqueous media
80 (Ülker and Karaoglu 2012) and their production can be associated with several factors
81 including pH, temperature, carbon source and the presence of inducers such as oils and some
82 biosurfactants (Cherif et al. 2011; Colla et al. 2010).

83 Nowadays, biosurfactants take an important scientific interest with their interesting
84 proprieties such as the high biodegradability, lower toxicity, better environmental
85 compatibility, and important specific activity at extreme conditions of temperature, pH and
86 salinity (Sriram et al. 2011).

87 In this context, searching for novel biosurfactant producing strains with potential
88 biosurfactant production is required. For that, lipolytic strains could be a possible original
89 source of biosurfactant production (Sekhon et al. 2012). The present study describes the
90 biosurfactant production by a lipolytic strain *B. safensis* F4 and investigates its antibacterial,
91 anti-adhesive and antitumor activities.

92

93 **Materials and methods**

94 **Bacterial strains**

95 *B. safensis* F4, *B. subtilis*, *Staphylococcus aureus*, *Enterococcus faecium*, *Micrococcus*
96 *luteus*, *Agrobacterium tumefaciens*, *Salmonella enterica*, *Escherichia coli* and *Pseudomonas*
97 *savastanoi* were grown in LB (Luria-Bertani) medium. *S. epidermidis* S61, a biofilm-forming
98 bacterium isolated in our lab from the roof of an old house in Sfax, Tunisia (Jardak et al. 2017),
99 was grown in Tryptic Soy Broth (TSB) medium.

100

101 **Cell lines and cultures**

102 Breast cancer T47D and mouse melanoma B16F10 cell lines, obtained from the American
103 Type Culture Collection (ATCC), were grown in Dulbecco's modified Eagle's medium
104 (DMEM) supplemented with 10% foetal bovine serum, 50 IU/mL penicillin, 50 mg mL⁻¹
105 streptomycin at 37°C in a humidified 5% CO₂ atmosphere.

106 **Bacterial biosurfactant activity**

107 The oil displacement assay was performed according to Morikawa et al. (1993) using the Petri
108 plate (90 mm diameter) filled with 25 mL of distilled water then 10 µL of a crude oil was
109 added. 10 µL of a cell free culture supernatant was slowly placed on the center of the oil
110 surface. The diameter of the clear halo zone was measured after 30 seconds of incubation.

111 The determination of the emulsification index (E24) is carried out according to the following
112 equation (Cooper and Goldenberg 1987). E24 was measured using the cell free culture. Two
113 millilitres of a vegetable oil were added to an equal volume of cell free supernatant and
114 homogenized for 2 minutes at high speed. The height of emulsion layer was measured after
115 24h. All the experiments were done in triplicate.

116 **$E24 (\%) = (\text{Total height of the emulsified layer} / \text{Total height of the liquid layer}) \times 100$**

117

118 **Surface tension determination**

119 Surface tension of the 24 h culture broth supernatant was measured according to the De Nouy
120 methodology using a tensiometer TD1 (Lauda-Königshofen, Germany). The measurement
121 was performed in triplicate.

122 **Identification of bacterial strain**

123 Strain F4 was identified using the API 20E test *Enterobacteriaceae* (BioMérieux, France) and
124 by sequencing of the 16S rRNA gene. The genomic DNA of the strain F4 was extracted

125 following the protocol detailed by Wilson et al. (1987). The 16S ribosomal DNA of the strain
126 F4 was amplified by PCR (Polymerase chain reaction) using the universal bacterial primers
127 Fd1 and Rd1 (Fd1, 5'-AGAGTTTGATCCTGGCTCAG-3'; Rd1, 5'-AAGGAGG-
128 TGATCCAGCC-3'), and the following program: denaturation at 94°C for 30 sec, annealing
129 at 55°C for 45 sec and extension at 72°C for 1 min 45 sec for a total of 30 cycles.

130 The PCR products were purified with a Favor Prep GEL/ PCR Purification Kit
131 (FAVORGEN) and sequenced using the ABI PRISM, 3100. The obtained sequences were
132 compared with other bacterial sequences in the NCBI database using BLAST program. The
133 phylogenetic tree was constructed using the neighbour-joining method (Naruya and Nei 1987)
134 by MEGA 4.0.

135 **Bacterial biosurfactant production**

136 *B.safensis*F4 strain was retained as the best local strain producing biosurfactant. The strain was
137 incubated overnight at 30°C and 160 rpm in 250 mL shaking flasks with 100 mL of LB
138 medium. Two millilitres of culture were used as inoculum and were cultivated in 500 mL
139 shaking flasks containing 200 mL of the medium with 1% olive oil. The culture was
140 incubated for 24 h at 180 rpm and 30°C to allow maximum biosurfactant production. Cell-
141 free supernatant was obtained by centrifugation at 4°C during 20 min at 4000xg (ROTANTA
142 460 RF, Hettich). The obtained supernatant was treated by acidification to pH 2.0 using a 3M
143 HCl solution and incubated overnight at 4°C. Then, the acidified supernatant was extracted
144 with ethyl acetate and concentrated with a rotary evaporator (Gargouri et al. 2016).

145 **Thin layer chromatography (TLC)**

146 The extracted biosurfactant in ethyl acetate was analysed by TLC. The sample dissolved in
147 methanol was spotted on silica gel TLC plate (TLC Silica gel 60 F₂₅₄, Merck Darmstadt,
148 Germany). The plate was developed with a mobile phase of chloroform/methanol/water

149 respectively in the ratio of 65:25:4 (v/v/v). The dried plate was sprayed with a solution of
150 0.25% ninhydrin in acetone and then, incubated at 105°C for 5 min (Janek et al. 2010).

151 **Biosurfactant purification and identification**

152 The extracted biosurfactant in ethyl acetate was fractioned using solid-phase extraction (SPE)
153 (Alajlani et al. 2016). C₁₈ Phenomenex strata-X column (silica gel, 10 g) was conditioned by
154 the elution of 3 volumes of acetonitrile. The sample was deposited on the surface of the silica
155 and drawn through the solvent. For the mobile phase, the HPLC (High Pressure Liquid
156 Chromatography) grade acetonitrile (100% - 3 volume column) was used in first step, then a
157 binary mixture of HPLC grade dichloromethane/ methanol (v/v - 3 volume column) was used.
158 The obtained eluates were collected and dried under vacuum. Finally, the acetonitrile fraction
159 was retained.

160 Two microliters of acetonitrile fraction diluted at 5 mgmL⁻¹ in methanol, were injected in a
161 Dionex Ultimate 3000 UHPLC-HESI HRMS Q-Exactive focus system (Thermo Scientific)
162 connected to Xcalibur software. The chromatographic separation was conducted followed the
163 protocol of Girard et al. (2017) with slight modifications. The Hypersil GOLD C₁₈ column
164 (150 mm × 2.1 mm) with 1.9 µm particle size (Thermo Scientific) and constant flow rate of
165 0.5 mL min⁻¹. The column oven was set to 50°C. The water (eluent A) and acetonitrile (eluent
166 B) containing both 0.1% formic acid, were used as mobile phases. A gradient profile was
167 applied, starting with 5% of B and kept constant for 1 min. The percentage of B was linearly
168 increased to 100% in 15 min, and was kept at 100% for 9 min and returned to initial
169 conditions over 1 min. Four minutes of equilibration were followed, giving a total operating
170 time of 30 min. The instrument has been run in the full scan mode with a range of 100 to
171 1500m/z equipped with an electrospray interface (ESI). The polarity of the electrospray
172 interface was continuously switched between positive and negative polarity. The LB medium

173 was used as a control subjected to extraction with ethyl acetate. The common peaks between
174 the chromatographs of the samples and the medium were not retained.

175 **Determination of minimum inhibitory concentration (MIC)**

176 The minimum inhibitory concentration (MIC) for the lipopeptide, produced by *B.safensis* F4,
177 was defined as the lowest concentration that inhibited the growth of microorganisms after 24
178 h. The test was performed against several human and plant pathogenic strains (*S.aureus*,
179 *E.faecium*, *M. luteus*, *A. tumefaciens*, *S. enterica*, *E. coli* and *P. savastanoi*) and *B. subtilis*.

180 The choice of these strains is justified since we tried to maximize our chance for finding
181 interesting molecules that could be applied to fight against human or plant bacterial
182 infections. The biosurfactant anti-planktonic activity against *S. epidermidis* S61 was
183 performed with the same test. Each bacterium was grown in LB medium overnight at 30°C.
184 Bacterial cultures were then adjusted to an optical density of 0.6 at a wavelength of 600
185 nm. The crude biosurfactant was dissolved in Dimethylsulfoxide (DMSO) and then filtered.
186 Serial dilutions were made to yield volumes of 100 µL per well with final concentrations
187 ranging from 0.0125 to 25 mg mL⁻¹ in LB medium. Twenty microliters of bacterium overnight
188 culture, with appropriate OD, were added to each well and a final volume of 200 µL per well
189 was adjusted with medium. Wells containing just LB medium with inoculum and these
190 containing medium, inoculum and Ampicillin served as controls. The plate was then
191 incubated at 37°C for 24 h. Twenty microliters of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-
192 diphenyltetrazolium bromide) solution at 1 mg mL⁻¹ were added to each well.

193 The determination of the biosurfactant MIC was based on the MTT color change. In fact, the
194 viable bacteria were detected by the change of yellow MTT color to purple. For that, the well
195 devoid of bacterial growth (yellow color) was retained as MIC, which was expressed in mg
196 mL⁻¹. The same test was carried out against Gram-positive and Gram-negative strains.

197 **Anti-adhesive activity**

198 The 96-well flat bottom plates were used for biofilm cultures (Mathur et al. 2006).
199 *S. epidermidis* S61, a biofilm-forming strain (Jardak et al. 2017), was grown overnight in TSB
200 medium at 30°C and diluted with fresh medium supplemented with 2.25% (w/v) glucose. One
201 hundred microliters of the bacterial culture dilution was added into each well to obtain a final
202 OD_{600 nm} of 0.1. Then, 100 µL of *B. safensis* F4 biosurfactant dissolved in TSB, containing 20%
203 (v/v) of DMSO, at various concentrations, were added into wells to reach final concentrations
204 of 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5 and 10 mg mL⁻¹. Wells containing only TSB
205 medium supplemented with, 2.25% glucose and 20% (v/v) of DMSO, and bacterial
206 suspension were served as controls.
207 Plates were incubated for 24 h at 30° C under static conditions. After incubation, the wells
208 were emptied into a container by inverting the plates. Each well was gently washed twice with
209 250 µL of sterile phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl; 10 mM
210 Na₂HPO₄ ; 1.76 mM KH₂PO₄; pH 7.2) in order to remove the planktonic cells (Beenken et al.
211 2003) . After washing, plates were dried at 60°C for 60 min. Then, wells were stained with
212 150 µL of crystal violet (0.2%) prepared in 20% ethanol for 15 min at room
213 temperature (Vasudevan et al. 2003). After staining incubation, crystal violet was removed and
214 excess dye was washed three times with sterile water. Finally, 200 µL of glacial acetic acid
215 33% was added to each well and plates were incubated for 1 hour at room temperature. The
216 optical density (OD) was measured at 570 nm using a Varioskan microplate reader
217 (ThermoFisher).

218 The percentage of the adhesion inhibition was calculated by the following formula:

219
$$[(\text{OD (control*)} - \text{OD (treated strain)}) / \text{OD (control*)}] \times 100$$

220 *Control: untreated strain with the extract

221 The anti-adhesive activities of the crude biosurfactant and the acetonitrile fraction, against *S.*
222 *epidermidis* S61 were confirmed by microscopic observations using the OLYMPUS
223 fluorescent microscope BX50 equipped with a digital camera OLYMPUS DP70. The biofilms
224 were grown on glass pieces (\varnothing 10mm) placed in 24-well polystyrene plates treated with the
225 biosurfactant. Non-treated wells, containing TSB supplemented with 10% (v/v) of DMSO,
226 served as controls (Padmavathi and Pandian 2014). The biosurfactant was added at a final
227 concentration of 10 mg mL^{-1} in TSB with 10% (v/v) of DMSO. The bacterial inoculation was
228 adjusted to an $\text{OD}_{600\text{nm}}$ of 0.1. Plates were incubated at 30°C for 24 h. The wells were then
229 carefully emptied with pipetting and glass slides were washed with sterile PBS (1X) before
230 the treatment with $500 \mu\text{L}$ of acridine orange (0.1%, w/v, dissolved in PBS 1X). Visualization
231 was performed through a 40x objective using U-MWB2 filter with excitation at 460-490 nm
232 and emission at 520 nm.

233 **Cytotoxicity assays**

234 T47D breast cancer and B16F10 mouse melanoma cells were grown in 96-well plates
235 (Orange Scientific) until 40% confluence. The biosurfactant, was added at different
236 concentrations ($0.1, 1$ and 10 mg mL^{-1}) and incubated for 48 hours at 37°C in a humidified
237 atmosphere containing 5% CO_2 .

238 The cell viability was assessed using the MTT assay as previously described by Mosmann
239 (1983). After treatment, the medium was exchanged by a fresh one and $10 \mu\text{L}$ of MTT
240 solution (5 mg mL^{-1} in PBS) were added. After incubation for 4 hours, $100 \mu\text{L}$ of 10% SDS
241 (Sodium dodecyl sulfate) solution were added to each well to dissolve the formazan. The
242 optical density was measured at 570 nm using a Varioskan microplate reader (ThermoFisher).
243 The growth inhibition was expressed according to the following formula:

$$244 \quad (\%) \text{ cell survival} = (\text{AT}/\text{A0}) \times 100$$

245 A0: control absorbance; AT: treated cells absorbance.

246 **Statistical Analysis**

247 All experiments were done in triplicate. The obtained results are expressed as mean values with
248 the standard error. The statistical analyses were performed using Student's t-test to compare the
249 controls and treated samples at a significance level of 5%.

250

251 **Results and discussion**

252 **Selection of biosurfactant producing strain**

253 Morphological and biochemical tests showed that the rod-shaped strain F4 was motile, Gram-
254 positive, catalase-positive and oxidase-positive. Based on the phylogenetic analysis of the
255 16S rRNA gene sequences, the strain F4 was affiliated to the genus *Bacillus* with 99% of
256 similarity to *Bacillus safensis* FO-36b^T (AF234854) (Fig. 1) and was termed as *B. safensis*
257 F4. The 16S rRNA gene sequence, including 1378 nucleotides, was deposited in the GenBank
258 nucleotide database under the accession number MF927780.

259 In fact, *B. safensis* F4 was retained after laboratory screening of lipolytic strains for their ability
260 to produce biosurfactant during growth on olive oil. The oil displacement assay showed that
261 the selected strain presented the highest clear halo zone (about $21.08 \pm 1.46 \text{ cm}^2$). The
262 emulsification activity of the selected strain against sunflower oil was 74.99%. Previous
263 results showed that *Bacillus cereus* NK1 biosurfactant presented a clear halo zone of 2.95 cm^2
264 and 62% in the oil displacement test and emulsification activity against *n*-hexadecane,
265 respectively (Sriram et al. 2011). Ibrahim (2018) claimed that biosurfactant produced by
266 *Ochrobactrum anthropi* HM-1 culture showed a clear halo zone 38.5 cm^2 , while 33.17 cm^2
267 was presented by *Citrobacter freundii* HM-2 biosurfactant. The cell-free culture broths of

268 HM-1 and HM-2 strains successfully emulsified sunflower oil with approximately 70% and
269 60%, respectively.

270

271 **Surface tension determination**

272 Surface tension is a key parameter for the evaluation of biosurfactant production. In fact, a
273 microorganism is considered as a promising biosurfactant producer, if it could reduce the
274 surface tension to less than 40 mN m⁻¹ (Shete et al., 2006). The obtained results showed that
275 our biosurfactant is able to reduce surface tension until 30.73 mN m⁻¹ ± 0.48 which is lower
276 than results obtained by Ghazala et al. (2017) during the characterization of an anionic
277 lipopeptide produced by *Bacillus mojavensis* I4 where the surface tension of the culture
278 supernatant was 31.5 ± 0.8 mN m⁻¹. Moreover, our results are very close to those obtained by
279 Jemil et al., (2016) which showed that the best result in decreasing surface tension was
280 observed with *Bacillus methylotrophicus* DCS1 strain (31 mN m⁻¹). Likewise, other study
281 showed that biosurfactants produced by *O. anthropi* HM-1 and *C. freundii* HM-2 were able to
282 reduce surface tension until 30.8 ± 0.6 and 32.5 ± 1.3 mN m⁻¹, respectively (Ibrahim
283 2018). While, compared to surface tensions of some chemical surfactants studied by Ghazala
284 et al. (2017), *B. safensis* F4 cell free broth showed lower surface tension than SDS
285 (34.8 ± 1.3 mN m⁻¹) and Triton X-100 (32 ± 0.9 mN m⁻¹).

286 **Characterization of *B. safensis* F4 biosurfactant**

287 TLC analysis showed that *B. safensis* F4 biosurfactant is a lipopeptide. The relative front (R_f)
288 value was 0.56 (Fig. 2) which confirmed that the biosurfactant extract is a lipopeptide as
289 reported by similar previous studies (Fernandes et al. 2007).

290 In order to identify our biosurfactant, the acetonitrile fraction was collected, and then
291 analysed by LC-MS (Liquid chromatography–mass spectrometry) (Fig. 3). The details of the

292 obtained masses have been identified according to previous reported studies. Results showed
293 the presence of two surfactinderivates ($M+H^+=1022.6668$ and $1008.6513/ M-H+=1020.6579$
294 and 1006.6436 , respectively) at retention time of 15.98 min with the presence of adducts
295 ($M+Na$) (Table 2). The presence of surfactin was confirmed by the positive and negative
296 ionizationmode (Jasim et al. 2016). At the same retention time of 15.98 min, the two
297 compounds were identified as Leu/Ile-7, C_{14} surfactin and Leu/Ile-7, C_{13} surfactin with
298 different masses of 1021.66 m/z and 1007.65 m/z (Price et al. 2007). Another peak at 9.20
299 min has been depicted ($M-H^+ = 329.2328$), which could correspond to pinellic acid.
300 According to literature, pinellic acid is mainly known with its anti-allergic (Arulselvan et al.
301 2016) and anti-inflammatory (Nagai et al. 2004) activities.

302 **Determination of minimum inhibitory concentration (MIC)**

303 Das et al. (2008) reported that some types of biosurfactants produced by many *Bacillus* species
304 present antimicrobial activity against many bacteria including pathogenic strains.

305 Our lipopeptide showed limited activity against Gram-negative bacteria compared to that
306 obtained against the Gram-positive tested strains. The tested biosurfactant has a MIC of 0.78
307 $mg mL^{-1}$ against *B. subtilis* and 1.56 $mg mL^{-1}$ against *S. aureus*, *E. faecium* and *M. luteus*.
308 However, it presented a MIC value of 3.125 $mg mL^{-1}$ against *A. tumefaciens*, *S. enterica*, *E.*
309 *coli* and 1.56 $mg mL^{-1}$ against *P. savastanoi* (Table 1). Moreover, Singh and Cameotra (2004)
310 reported that the lipopeptide produced by *B. subtilis* C1 was found to be active against several
311 Gram-positive bacteria.

312 In another study, biosurfactant produced by the *Lactobacillus paracasei* ssp. *paracasei* A20
313 showed significant antimicrobial activities against pathogenic *E. coli*, *S. aureus* with
314 MIC higher values ranging between 25 and 50 $mg mL^{-1}$ comparing with our results (Gudiña et
315 al. 2010 b). Likewise, a high level of growth inhibition was observed against different

316 pathogens with a biosurfactant produced by *Lactobacillus helveticus* at a concentration of 25
317 mg mL⁻¹ (Sharma and Saharan 2016). Furthermore, many lipopeptides produced by *Bacillus*
318 *licheniformis*, (Yakimov et al. 2007; Fiechter 1992) and *B. subtilis* (Vollenbroich et al. 1997)
319 were known by their important antimicrobial activities. In other studies, the crude
320 biosurfactant produced by *Lactobacillus jensenii* presented approximately 100% activity
321 against *E. coli*, and *S. aureus* with a MIC of 50 mg mL⁻¹ which are higher than our MIC values
322 (Sambanthamoorthy et al. 2014).

323 Concerning the anti-planktonic activity, the crude biosurfactant and the acetonitrile fraction
324 were tested against *S. epidermidis* S61. Results showed that the crude biosurfactant and
325 acetonitrile fraction effectively inhibited its growth with MIC of 12.5 mg mL⁻¹ and 6.25 mg
326 mL⁻¹, respectively. However, biosurfactant produced by *L. helveticus* showed a high
327 percentage of growth inhibition (98.4%) against *S. epidermidis* with a concentration of 25 mg
328 mL⁻¹ (Sharma and Saharan 2016).

329 **Anti-adhesive activity**

330 The ability of the crude biosurfactant and the acetonitrile fraction to inhibit the early biofilm
331 formation at various concentrations was carried out against *S. epidermidis* S61. According to
332 the Figure 4, the crude biosurfactant and the tested fractions significantly ($P < 0.001$) inhibited
333 the biofilm formation with approximately the same percentages of 90% and 80% at the
334 concentrations of 10 and 5 mg mL⁻¹, respectively. However, at the concentration of 2.5 mg
335 mL⁻¹, the acetonitrile fraction, containing the surfactin, showed higher anti-adherence activity
336 with a percentage of inhibition of 64% against 53% of the crude biosurfactant. Comparing with
337 our results, the purified biosurfactant produced by *B. cereus* NK1 presented lower
338 percentages of biofilm inhibition of *S. epidermidis* at the rates of 33.55% and 26.46% at
339 concentrations of 10 and 5 mg mL⁻¹, respectively (Sriram et al. 2011). In similar studies, the

340 anti-adhesive activity of *B.methylotrophicus* DCS1 crudelipopeptide was evaluated against
341 different strains using biosurfactant pre-treated polystyrene surfaces. Results showed that the
342 highest anti-adhesive effect was observed against *C. albicans* with an inhibition percentage of
343 about 89.3% when biosurfactant was applied at a concentration of 1 mg mL^{-1} (Jemil et al.,
344 2017). In another study, the crude biosurfactant isolated from *L. paracasei ssp. paracasei* A20
345 inhibited the adherence of *S. epidermidis* at the concentration of 50 mg mL^{-1} with a
346 percentage of 72.9% (Gudiña et al. 2010 b) compared to 90 and 80% at 10 and 5 mg mL^{-1}
347 respectively of our present biosurfactant.

348 Moreover, biosurfactant produced by *L. helveticus* showed a potential anti adhesive
349 activity against *S.epidermidis* with a percentage of 85% which is similar to our results but at
350 higher concentration of 25 mg mL^{-1} (Sharma and Saharan 2016). Furthermore, biosurfactants
351 produced by *L. jensenii* and *L. rhamnosus* presented anti-adhesive and anti-biofilm activities
352 against the pathogen strains *A. baumannii*, *E. coli*, and *S. aureus* at concentrations ranging
353 between 25 and 50 mg mL^{-1} (Sambanthamoorthy et al. 2014).

354 The anti-adherence activity of the two extracts was confirmed by fluorescence microscopy.
355 The images of the acridine orange staining treated slides with extracts showed the reduction in
356 the biofilm covered surface compared to the control (Fig. 5). Lipopeptides are able to
357 decrease biofilm surface and interfacial tension (Zhao et al. 2017). Previous studies
358 demonstrated that biosurfactants had the ability to alter the surface characteristics of bacterial
359 cells and reduce their adhesive properties. In fact, the application of biosurfactant to a
360 substratum surface can decrease its hydrophobicity, interfere with the microbial adhesion and
361 microorganisms adsorption process (Rodrigues et al. 2006a).

362 **Cytotoxicity assays**

363 The cytotoxicity assay of the crude biosurfactant and the acetonitrile fraction was performed
364 against T47D breast cancer cells and B16F10 mouse melanoma cells.

365 Figures 6 showed that the crude biosurfactant and the acetonitrile fraction showed high
366 inhibition against T47D and B16F10 cells at 10 mg mL⁻¹ ($P < 0.001$). Furthermore, at the
367 concentration of 1 mg mL⁻¹, the acetonitrile fraction was more toxic against B16F10 cells
368 with a survival of 59.75% than T47D cells, whereas, at concentration of the 0.1 mg mL⁻¹, both
369 tested samples did not show any toxicity against both cell lines.

370 The acetonitrile fraction inhibited significantly cancer cell growth at almost all the tested
371 concentrations ($P < 0.01$). It presented an IC₅₀ of 1.17 mg mL⁻¹ and 0.66 mg mL⁻¹ against
372 B16F10 cells and T47D cells, respectively (Fig. 7).

373 These results can be correlated with the composition of the acetonitrile fraction, which mainly
374 consists of surfactin, belonging to lipopeptides. According to literature, lipopeptides can act
375 as antitumor agents (Rodrigues et al. 2006b).

376 Previous studies reported that a biosurfactant extracted from *Lactobacillus casei* showed anti-
377 proliferative potencies against an epithelial cell line with an IC₅₀ (The half-maximal inhibitory
378 concentration) ranging from 109.1±0.84 mg mL⁻¹ to 129.7±0.52 mg mL⁻¹ (Merghni et al.
379 2017) which are higher than the IC₅₀ values obtained by our biosurfactant.

380 Moreover, it was previously demonstrated that surfactin could disrupt the membrane structure
381 via two main mechanisms which are insertion into lipid bilayers, modification of membrane
382 permeabilization via channel formation or diffusion of ions across the membrane barrier and
383 membrane solubilization by a detergent-like mechanism (Deleu et al. 2013, Wu et al.
384 2017). Interestingly, Gudiña et al. (2016) reported that the surfactin anticancer activity is in
385 relation with its hydrophobic nature. In fact, the fatty acid moiety of surfactin strongly
386 interacts with the acyl chain of the phospholipids in order to penetrate the outer sheet of lipid

387 bilayer, while the peptide moiety interacts with the polar head group of the lipids in cancer
388 cells.

389 **Conclusion**

390 In the present study, the best producing biosurfactant strain has been screened and
391 selected. Termed *B. safensis*F4, it is a lipolytic bacterial strain that has the propriety to
392 produce surfactin with important surface-active properties. Crude and purified biosurfactant
393 showed important antibacterial activity under planktonic conditions, preventing also bacterial
394 adherence through inhibiting early stage biofilmformation. Interestingly, surfactin from
395 *Bacillus* sp. F4 has potent cytotoxic activity against cancer cell lines, T47D breast cancer cells
396 and B16F10 mouse melanoma cells. These findings make this studied surfactin a good
397 candidate for potential applications in preventing infectious diseases and treating cancer.

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401 **Conflict of interest**

402 The authors declare that they have no conflict of interest.

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