

# Carotenogenesis of *Staphylococcus aureus*: new insights and impact on membrane biophysical properties

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24 **Abstract**

25 Staphyloxanthin (STX) is a saccharolipid derived from a carotenoid in  
26 *Staphylococcus aureus* involved in oxidative-stress tolerance and antimicrobial peptide  
27 resistance. In this work, a targeted metabolomics and biophysical study was carried out on  
28 native and knock-out *S. aureus* strains to investigate the biosynthetic pathways of STX and  
29 related carotenoids. Identification of 34 metabolites at different growth phases (8, 24 and  
30 48h), reveal shifts of carotenoid populations during progression towards stationary phase.  
31 Six of the carotenoids in the STX biosynthetic pathway and three menaquinones (Vitamin  
32 K<sub>2</sub>) were identified in the same chromatogram. Furthermore, other STX homologues with  
33 varying acyl chain structures reported herein for the first time, which reveal the extensive  
34 enzymatic activity of CrtO/CrtN. Fourier Transform infrared spectroscopy show that STX  
35 increases acyl chain order and shifts the cooperative melting of the membrane indicating a  
36 more rigid lipid bilayer. This study shows the diversity of carotenoids in *S. aureus*, and their  
37 influence on membrane biophysical properties.

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39 **Keywords:** *S. aureus*; staphyloxanthin; carotenogenesis; carotenoids; LC-MS<sup>n</sup>; FT-IR.

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41 **1. Introduction**

42 *S. aureus* is a Gram-positive bacterium naturally present in nasal passages and human  
43 skin. It is an opportunistic pathogen responsible for nosocomial and acquired infections,  
44 including pneumonia, osteomyelitis, meningitis, bacteremia and sepsis (Lowy, 1998; V.  
45 Recklinghausen, 2008; Tong et al., 2015). The main concern about this microorganism is the  
46 increasing number of resistant strains to different antibiotics (Oldfield and Feng, 2014). Thus,  
47 methicillin-resistant *S. aureus* (MRSA) strains pose a serious problems for health, limiting

48 the antimicrobial treatments as reported by the Center for Disease Control and Prevention  
49 (CDC), that relates MRSA with more than 323,000 hospitalized patients and 10,600  
50 estimated deaths in the US in 2017 (CDC, 2019). Thereby, an alternative to the use of  
51 conventional antibiotics against *S. aureus* has been to employ antimicrobial peptides, such  
52 as Daptomycin (Crass et al., 2019; Dhand and Sakoulas, 2014; Heiday et al., 2018;  
53 Steenbergen et al., 2005), which act by compromising bacterial membrane integrity. For such  
54 molecules, the antimicrobial activity has been shown to depend on how the microorganism  
55 modulates the physicochemical properties of its membrane, which include mechanical  
56 malleability and lateral diffusion, since they strongly influence the insertion of membrane  
57 active peptides. For this reason, antibiotic resistance in *S. aureus* strains has been associated  
58 with changes in the membrane composition (Kilelee et al., 2010; Mishra et al., 2009; Xue et  
59 al., 2019; Zhang et al., 2014).

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61 The number of studies focused on *S. aureus* membrane lipid composition has  
62 increased in recent years. Particularly, a great interest has been directed to a saccharolipid  
63 containing carotenoid, known as Staphyloxanthin (STX) (Braungardt and Singh, 2019;  
64 Perez-Lopez et al., 2019; Tiwari et al., 2018; Xue et al., 2019), a natural pigment with well-  
65 known antioxidant properties (Mishra et al., 2011; Tiwari et al., 2018; Zhang et al., 2018),  
66 responsible for the characteristic color of *S. aureus* (Kim and Lee, 2012; Marshall and  
67 Wilmoth, 1981a), and associated with tolerance to oxidative stress (Clauditz et al., 2006; Liu  
68 et al., 2005; Olivier et al., 2009). However, STX also plays an essential role on the regulation  
69 of membrane mechanical properties, and has been shown to hinder the permeability of the  
70 membrane to cationic antimicrobial peptides, increasing the virulence and bacterial fitness of  
71 *S. aureus* (Crass et al., 2019; Mishra et al., 2011; Vogeser and Zhang, 2018). In the first

72 comprehensive study on STX and other carotenoids of *S. aureus* S41, these pigments were  
73 isolated and their chemical structures determined (Marshall and Wilmoth, 1981a), leading  
74 to the identification of 17 triterpenic carotenoid compounds and proposal of a biosynthetic  
75 pathway (Marshall and Wilmoth, 1981b). However, it was not until 2005 that five of the six  
76 enzymes involved in the biosynthesis of STX in *S. aureus* were reported (Pelz et al., 2005).  
77 Recently, using *Escherichia coli* mutants (Kim and Lee, 2012), a sixth enzyme involved in  
78 the biosynthetic pathway of STX was reported (**Fig. 1a**). In these studies, open column  
79 chromatography (OCC) or thin-layer chromatography (TLC) were mostly used as the  
80 separation step prior to the mass spectrometric (MS) analysis of carotenoids. In addition to  
81 these carotenoids, other secondary metabolites exhibit structural similarities to STX related  
82 biproducts. These include menaquinones (MK) or vitamin K<sub>2</sub>, unsaturated polyisoprenes of  
83 2-methyl-1,4-naphthoquinones, involved in the aerobic or anaerobic respiration of *S. aureus*,  
84 thanks to the possible transfer of two electrons (Kurosu and Begari, 2010; Wakeman et al.,  
85 2012). Menaquinones are more non-polar compounds due to the lack of conjugated double  
86 bonds. *S. aureus* shows three types of menaquinones with different length of the aliphatic  
87 chain (**Fig. 2a**), MK (n=7, 8 and 9) (Marshall and Wilmoth, 1981a; Taylor' and Bavies, 1983;  
88 Wakeman et al., 2012). In this study, we have included the identification of these compounds  
89 to provide a more complete picture of carotenoid related metabolites present in the *S. aureus*  
90 membrane.

91 Previously, the fatty acids composition and headgroup composition of *S. aureus*  
92 phospholipids have been described as a mechanism for modulating the biophysical properties  
93 of the bacterial membrane, showing an influence on the pathogenicity and resistance to  
94 antimicrobial peptides (Hernández-Villa et al., 2018; Kilelee et al., 2010; Mishra and Bayer,  
95 2013; Sen et al., 2016; Zhang et al., 2014). Hence, biophysical studies on membrane stiffness

96 in *S. aureus* have been developed by fluorescence spectroscopy using the probe 1,6-diphenyl-  
97 1,3,5-hexatriene (DPH) (Mishra et al., 2011; Perez-Lopez et al., 2019; Sen et al., 2016;  
98 Tiwari et al., 2018). However, the insertion of fluorescent probes into the membrane is  
99 cumbersome, with a high risk of incorporation into the cell wall, therefore affecting the  
100 proper report of the membrane behavior. Instead, Fourier-transform infrared spectroscopy  
101 (FT-IR) provides a direct approach for studying the biophysical behavior *in vivo* of the lipids  
102 present in bacterial membranes (Ocampo et al., 2010; Schultz and Naumann, 1991). The CH<sub>2</sub>  
103 stretch vibration of phospholipids is a dominant signal in bacterial cells and reflects directly  
104 the physical properties of the lipids that compose the bilayer (Scherber et al., 2009). The  
105 thermotropic shift of the CH<sub>2</sub> stretch indicates cooperative changes in the lipid packing  
106 behavior of the membrane as the membrane moves from a tightly packed gel-like phase (L<sub>β</sub>)  
107 at low temperatures to a more mobile liquid-crystalline phase at high temperatures close to  
108 its growth temperature (Ocampo et al., 2010). *S. aureus* presents a reproducible cooperative  
109 shift in the CH<sub>2</sub> wavenumber around 15°C, suggesting that below 15°C the membrane of *S.*  
110 *aureus* has a significantly higher rigidity. Shifts in the wavenumber and this cooperative  
111 event are therefore used as indications of changes in the rigidity of the bilayer membrane  
112 (Ocampo et al., 2010).

113 In the present study, we conduct an in-depth characterization of the carotenoids of *S.*  
114 *aureus* at different stages of cell growth, in order to better understand their biosynthetic routes  
115 and their impact on Staphyloxanthin production and on membrane biophysical properties.  
116 For the identification of carotenoids and structurally related compounds, a robust  
117 methodology based on HPLC-DAD-MS/MS analysis was proposed. Complementary  
118 structural information was obtained from UV-visible absorbances and tandem-mass  
119 spectrometry-based detection systems. Further metabolites coverage was obtained applying

120 both APCI and ESI ionization sources. We present the versatility of a C18 column in the  
121 characterization of the carotenoids and STX-homologues compounds in wild type and mutant  
122 strains of *S. aureus*, complemented by the usefulness of a C30 column for the characterization  
123 of new Dehydro-STX-FA, through a simple and reliable method, without the need for  
124 previous TLC or OCC. In addition, we included analysis of the shift in the CH<sub>2</sub> stretch wave  
125 number by FTIR to determine the impact of these carotenoids on the biophysical properties  
126 of the bacterial membrane without the need of exogenous fluorescent probes.

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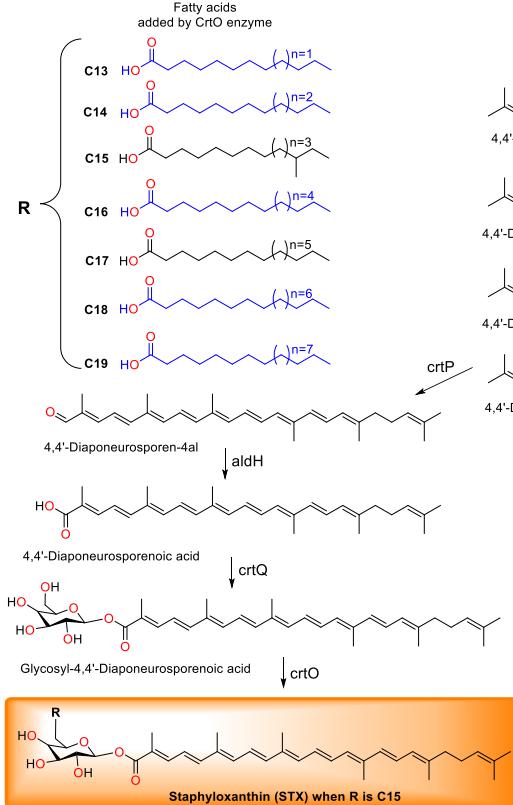
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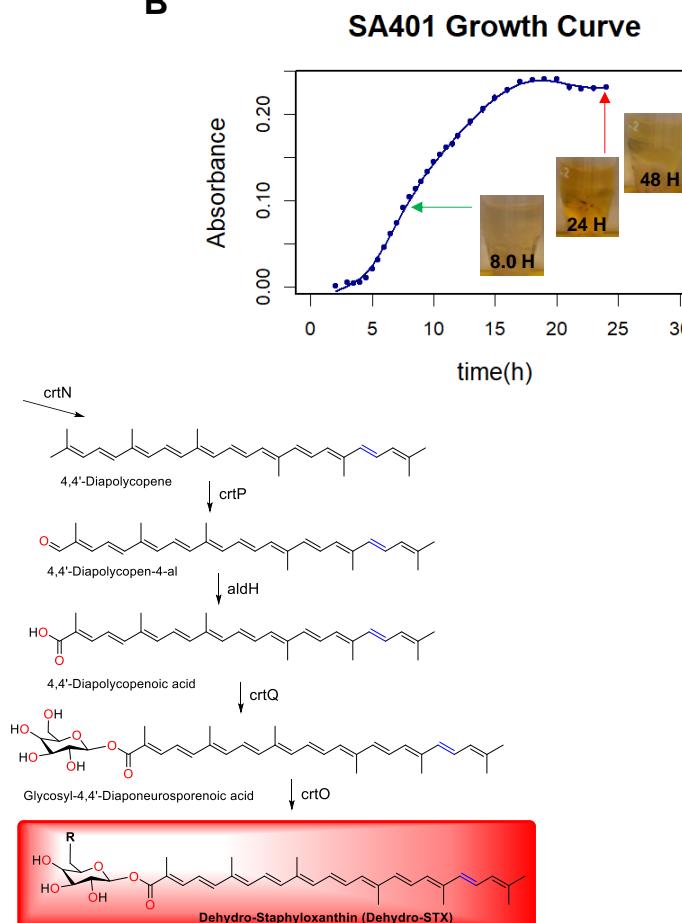
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A



B



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**Figure 1.** STX biosynthetic pathway and growth curve of *S. aureus*. (A) Main (right) and alternative (left) pathways including

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the variation of fatty acids, adapted from (Kim and Lee, 2012). (B) Growth curve for SA401 *S. aureus* strain for determinate the 146 exponential and stationary phases.

147        2.        **Experimental**

148        2.1 *Chemicals and reagents*

149            HPLC-grade methanol, acetonitrile, ethyl acetate, and methyl tert-butyl ether  
150            (MTBE) were purchased from Honeywell (Michigan, USA), J.T. Baker (Palo Alto, CA,  
151            USA) and VWR (Leuven, Belgium), respectively. HPLC-grade acetic acid and ammonium  
152            acetate were purchased from Fluka (St. Louis, MO, USA). Butylated hydroxytoluene (BHT)  
153            and NaCl ReagentPlus (>99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).  
154            Tryptone (OXOIO, Basigstoke, Hampshire, England), NaCl (ACS, J.T. Baker, USA), and a  
155            yeast extract (Dibico, México D.F., México) were used for LB medium preparation. HPLC-  
156            grade water was obtained from a water purification system Heal Force Smart-Mini (Shangai,  
157            China) and Milli-Q system (Millipore, Billerica, MA, USA).

158        2.2 *Bacterial growth conditions*

159            Two clinical methicillin-susceptible *Staphylococcus aureus* strains were used. The  
160            strain denominated SA401 was provided by CIMIC (Centro de Investigaciones  
161            Microbiologicas of Universidad de los Andes, Bogotá D.C., Colombia) and a full description  
162            of its biophysical properties have been published (Perez-Lopez et al., 2019), whereas SA144  
163            strain was obtained from the Center for Molecular and Translational Human Infectious  
164            Diseases Research (Houston, USA). In addition, a crtM deletion mutant of SA144 (SA145)  
165            and a crtMN plasmid complement variant of SA145 (SA147) that recovered the carotenoid  
166            biosynthesis, were used (Mishra et al., 2011). Also, *S. aureus* subsp. aureus Rosenbach  
167            ATCC® 25923™ strain was analyzed as well. One colony of each *S. aureus* strain was grown  
168            overnight (37°C), under constant agitation (250 rpm), in 10 mL of LB medium containing  
169            (per liter) 10 g of Tryptone, 10 g of NaCl, and 5 g of the yeast extract. SA147 was grown on  
170            LB medium containing erythromycin 1ug/ml. Then, the cells were diluted (1:1000) in flasks

171 containing 150 ml of fresh LB medium and cultivated for 8, 24 or 48 hours. Subsequently,  
172 the cells were harvested by centrifuging for 10 min at 8500 rpm at 4 °C (Thermo Scientific,  
173 USA), and the pellet was frozen (-80°C) and lyophilized for almost 24 hours (LABCONCO,  
174 Kansas City, MO, USA).

175 *2.3 Cellular growth curves*

176 After growing, optical densities (OD) measures were obtained from the main culture  
177 of *S. aureus* strain SA401, which was inoculated with 1:1000 of overnight culture and  
178 incubated at 37°C until full stationary phase (24 h) was reached (A600=0.20-0.25). Optical  
179 densities were measured in triplicates at 600nm with a NanoDrop 2000 UV-Vis  
180 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), every 30 minutes for the first  
181 12 hours and every hour during the next 12 hours. Once the full stationary phase was reached,  
182 the 48 hours of growth was considered the late stationary phase.

183 *2.4 Carotenoids extraction*

184 The extraction of carotenoids was achieved using a modified version of the Marshall  
185 method (Marshall and Wilmoth, 1981a). Briefly, 100 mg of lyophilized cells were accurately  
186 weighed in a falcon tube containing 10 glass beads, dissolved in 2.0 mL of methanol  
187 containing BHT (0.1%, w/v) and vortex-mixed for 5 min. After centrifugation at 8500 rpm  
188 for 10 min, the supernatant containing the pigments was gently aspirated with a glass Pasteur  
189 pipette and the extraction was repeated twice with 1.0 mL of MeOH each time. All  
190 methanolic phases containing the carotenoids were combined, successively shaken with ethyl  
191 acetate and 1.7M NaCl (1:3 v/v), and centrifuged again at 8500 rpm for 5 min. Successively,  
192 the upper organic phase was carefully drawn, dried with anhydrous Na<sub>2</sub>SO<sub>4</sub>, decanted into  
193 an amber glass tube, and finally dried with nitrogen gas. The extracts are removed from the

194 triglycerides (TAGs) that affect the ionization of carotenoids in LC-MS, using low  
195 temperatures (-20) (Mariutti and Mercadante, 2018; Marshall and Wilmoth, 1981a).

196 *2.5 HPLC-DAD-APCI-MS/MS analysis*

197 The extracts obtained were resuspended and analyzed by high-performance liquid  
198 chromatography with diode array detection and atmospheric pressure chemical ionization  
199 tandem mass (HPLC-DAD-APCI-MS/MS), using an Agilent 1100 series liquid  
200 chromatograph equipped with a binary pump, online degasser, and autosampler (Santa Clara,  
201 CA, USA) coupled to an Ion Trap Mass Spectrometer through APCI operated in positive and  
202 negative ionization mode (Agilent ion trap 6320, Agilent Technologies, Santa Clara, CA,  
203 USA). The instrument was controlled by LC ChemStation 3D Software Rev. B.04.03  
204 (Agilent Technologies, Santa Clara, CA, USA). All extracts were dissolved (10-40 mg mL<sup>-1</sup>)  
205 in pure MeOH or ACN (0.1% acetic acid) and filtered using 0.45 µm nylon filters prior to  
206 analysis. The RP-HPLC separation was carried out at room temperature with 10 to 20 µL  
207 injection volume on a Zorbax SB-C18 column (150 mm x 4.6 mm i.d., 3.5 µm particle size,  
208 Agilent Technologies, Santa Clara, CA, USA), and a YMC-C30 reversed-phase column (150  
209 × 4.6 mm i.d., 3 µm particle size; YMC Europe, Schermbeck, Germany). A precolumn YMC-  
210 C30 (10 × 4 mm, 5 µm particle size) was used for the analysis. The mobile phases used were  
211 ammonium acetate 400 mg/L in a solvent mixture of methanol: methyl tert-butyl ether: water  
212 (80:18:2 v/v/v for solution A and 8:90:2 v/v/v for solution B). The elution gradient in C18  
213 column at a constant flow rate of 300 µL/min was as follows: 5% B in the first 3 minutes,  
214 after from 5% to 13% B in 9 minutes, from 13% to 100% B in 7 minutes and an isocratic  
215 hold at 100% B for 4 minutes. Final reconditioning from 100% to 5% solution B in 2 minutes  
216 and then maintained isocratically for 9 minutes (Hrvolová et al., 2016; Schex et al., 2018).  
217 For C30 column the elution gradient was: 5% B in the first 3 minutes, after from 5% to 13%

218 B in 9 minutes, from 13% to 25% B in 7 minutes, from 25% to 100% B in 4 minutes and an  
219 isocratic hold at 100% B for 2 minutes. Final reconditioning from 100% to 5% solution B in  
220 2 minutes and then maintained isocratically for 9 minutes at a constant flow rate of 500  
221  $\mu$ L/min. The DAD recorded at 230, 330, 460, and 490 nm, although spectra from 190 to 700  
222 nm were also obtained (peak width 0.1 min (2 s), slit 4 nm).

223 The APCI source was operated with the following parameters: drying temperature,  
224 350 °C; vaporizer temperature, 400 °C; drying gas flow rate, 7 L/min; capillary voltage, -3.6  
225 kV; nebulizer gas pressure, 45 psi; corona current, 4000 nA. Full scan spectra were obtained  
226 in the range from m/z 150 to 1200 (Hrvolová et al., 2016; Novotny et al., 2005). Untargeted  
227 and targeted MS/MS data dependent-scans were carried out, fragmenting the two highest  
228 precursor ions (10000 counts threshold; 1 V Fragmentor amplitude).

229 *2.6 HPLC-DAD-ESI-MS/MS analysis*

230 In order to widen the range of detectable carotenoids and menaquinones observed  
231 with the APCI source, the extracts were also analyzed by HPLC-DAD-MS/MS with  
232 electrospray ionization (ESI) using a ultra-high-performance liquid chromatographer Dionex  
233 UltiMate 3000 equipped with a binary pump, online degasser, autosampler, and a  
234 thermostated column compartment coupled with an LCQ Fleet™ Ion Trap Mass  
235 Spectrometer through ESI source operated in positive mode (Thermo Scientific, San Jose,  
236 CA, USA). Raw metabolite data were acquired and processed using the Xcalibur 3.0 software  
237 (Thermo Scientific, San Jose, CA, USA). The RP-HPLC separation was carried out at 30°C  
238 with 10  $\mu$ L injection volume, using the same gradients described in section 2.5. Diode-array  
239 detection was performed over the entire UV-vis range (190 - 800 nm), and the characteristic  
240 absorbances of the carotenoids were extracted between 230-550 nm.

241 MS operating conditions were previously optimized using flow injection analysis of  
242 a 10-ppm solution of the carotenoid extract in ACN (0.1% acetic acid). The ESI source was  
243 operated with the following parameters: ionization voltage 5.5 kV, capillary temperature of  
244 330°C, sheath gas flow rate of 9 arbitrary units, auxiliary gas flow rate of 2 arbitrary units.  
245 The ion trap was set to operate in full scan ( $m/z$  65-1200 mass range), and data-dependent  
246 MS/MS (30% collision energy) mode to obtain the corresponding fragment ions with an  
247 isolation width of 3  $m/z$ .

248 *2.7 FTIR measurements*

249 FTIR analysis was performed according to a previously described method (Ocampo  
250 et al., 2010), with some modifications. Briefly, *S. aureus* strains were grown in the same  
251 conditions described in section 2.2. Cells were measured from inoculations (1:1000)  
252 overnight culture and incubated for 18 hours. Subsequently, cells were washed with 30 mL  
253 phosphate-buffered saline (PBS) solution and centrifugated. Cell pellet was smeared onto Ge  
254 windows and placed within an adapted-built Peltier temperature controller inside of the FTIR  
255 chamber (IRTracer-100, Shimadzu, Japan). Temperature was ramped between 5 °C and 50  
256 °C, performing a scan from 4000 to 400  $\text{cm}^{-1}$ , with a resolution of 1  $\text{cm}^{-1}$  and 80  
257 spectrograms for each temperature point. Analysis was carried out for data between 2860 and  
258 2840  $\text{cm}^{-1}$  where the  $\text{CH}_2$  symmetric stretch vibration is centered, enabling to characterize  
259 the thermotropic chain melting behavior of native bacterial membranes from *S. aureus*. For  
260 every temperature, data was fitting to a polynomial function using R (RStudio, 2020). With  
261 the same program, pick position in the spectrograms and derivates were determined.

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265        **3. Results**

266        *3.1.- Establishing *S. aureus* growth phases and carotenoids extraction*

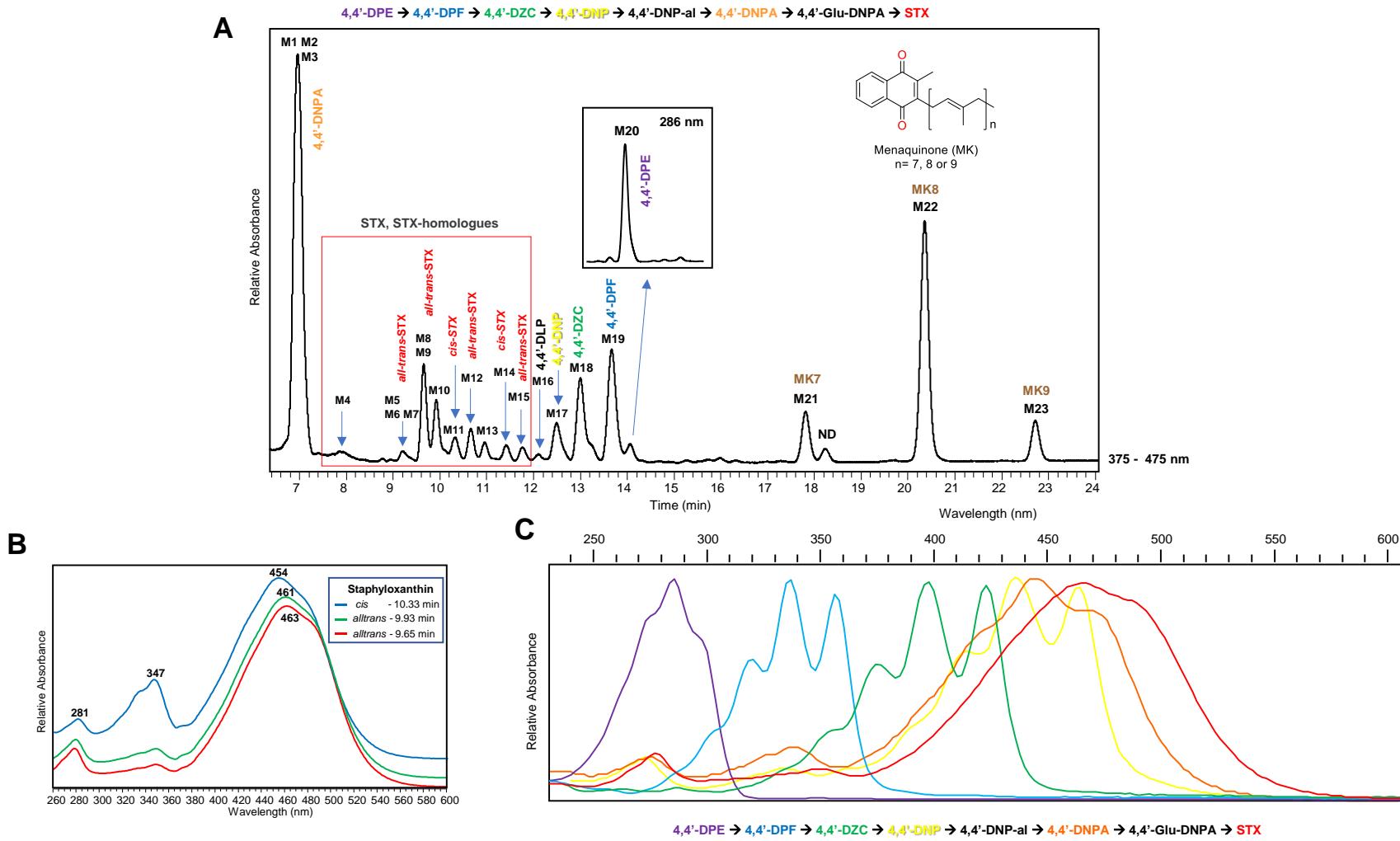
267        In order to monitor the progression of carotenoid species in *S. aureus* during STX  
268        biosynthesis at different growth stages, a bacterial growth curve of SA401 strain (wild type)  
269        was first measured to determine the exponential (8 hours), full stationary (24 hours) and late  
270        stationary (48 hours) growth stages, as depicted in Fig. 1b. This information is useful to  
271        understand the metabolic change in the different growth stages, observed by comparing  
272        chromatographic profiles of these phases, as discussed in the end of Section 3.2.

273        For the extraction of carotenoids, several factors such as light exposure and  
274        temperature were carefully controlled to avoid degradation. Reported methods frequently  
275        involve the use of different solvents, such as methanol, acetone, and ethyl acetate (Kim and  
276        Lee, 2012; Marshall and Wilmoth, 1981a). In this work, a modified version of the Marshall  
277        method was used, changing incubation with MeOH heated in a water bath at 55°C (Marshall  
278        and Wilmoth, 1981a) by a maceration step using vortexing with glass beads for 5 minutes  
279        (Hewelt-Belka et al., 2014), to improve the cell lysis (Hartz et al., 2018; Ye et al., 2006), and  
280        prevent thermal degradation of carotenoids. The resulting crude extracts showed an orange  
281        color of high intensity (24 hours), indicating the presence of carotenoid derivatives.  
282        Carotenoid extracts from SA401 strain were obtained at different growth stages for 8, 24,  
283        and 48 hours (**Fig. 1b**), whereas ATCC, SA144, SA145 and SA147 strains were all grown  
284        for 24 hours before extraction. Experiments were carried out in triplicate, following the same  
285        extraction procedure.

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290 **Figure 2.** HPLC-DAD analysis. (A) Chromatogram extracted in the UV-visible region (375-475 nm and 286 nm). (B) Absorbances of  
291 *cis* and *trans* STX. (C) Bathochromic shift in the absorbances of six carotenoids involved in STX biosynthetic pathway.

292 3.2.- *Carotenoids profiling by HPLC-DAD-(APCI/ ESI)-MS/MS analysis*

293 Different stationary phases, buffer solutions and gradients were tested to optimize  
294 chromatographic resolution before HPLC-DAD-MS/MS analysis (Chu et al., 2011; Kim and  
295 Lee, 2012; Mijts et al., 2005; Pelz et al., 2005). Although C30 columns are commonly used  
296 for carotenoids analysis due to the higher resolution capacity for these large molecular size  
297 terpenoids, improved chromatographic separation for a larger number of target metabolites  
298 involved in STX biosynthesis were found with a C18 column, employing a typical mobile  
299 phase for carotenoids analysis based on MTBE:MeOH:Water with ammonium acetate  
300 (Amorim-Carrilho et al., 2014; Hrvolová et al., 2016; Novotny et al., 2005; Schex et al.,  
301 2018), as described in the experimental section. Nevertheless, both C18 and C30 stationary  
302 phases showed complementary information in the metabolomic analysis. Selecting specific  
303 wave lengths for xanthophylls, carotenes, and menaquinones in the UV-visible region (230 -  
304 520 nm), a total of 34 metabolites were detected in the extracts of *S. aureus* after 8 h and 24  
305 h of growth (**Fig. 2a**). The identity of the detected metabolites could be confidently assigned  
306 by comparing UV-visible absorbances and MS/MS spectral information obtained using both  
307 APCI and ESI ionization sources. Table 1 summarizes the tentatively identified metabolites  
308 (M1-M34), including their retention time, ionization mode as well as MS and UV-visible  
309 spectral information. The analyzed *S. aureus* extracts allowed the identification of a large  
310 family of carotenoids attached to a saccharolipid residue, including STX (M3-M5, M8, M10,  
311 and M11), STX-homologues (M6, M7, M9, M12-M15, M24, M27, and M34), as well as  
312 Dehydro-STX (M26, M28 and M32) and Dehydro-STX-homologues (M29, M30, M31, and  
313 M33). In addition, biosynthetic precursors, such as hydrocarbon carotenes (M16-M20) and  
314 carotenoid acids (M1 and M2), as well as the three terpenoid derivatives such as  
315 menaquinones (M21-M23) were also characterized (see Table 1).

316 The MS fragmentation pattern of carotenoid acids, such as 4,4'-diaponeurosporenoic  
317 acid (4,4'-DNPA) and 4,4'-diapolycoenoic acid (4,4'-DLPA), is mainly characterized by the  
318 loss of HCOOH (-46 amu) or CO<sub>2</sub> (-44 amu), in positive and negative ionization mode,  
319 respectively. STX is a glycosylated 4,4'-DNPA bounded to a C15 fatty acid (FA), which can  
320 be represented as FA-Glu-4,4'-DNPA. The MS/MS fragmentation of STX and other  
321 identified biosynthetic homologues is characterized by the loss of the fatty acid-glucose (FA-  
322 Glu) residue. Thus, STX and STX-homologues show the characteristic *m/z* 431 [M-FA-Glu-  
323 H]<sup>-</sup> product ions in negative ionization mode, whereas Dehydro-STX and Dehydro-STX  
324 homologues exhibit *m/z* 429 as major product ion. The different fatty acids (FA) attached to  
325 the STX core (Glu-4,4'-DNPA) or Dehydro-STX core (Glu-4,4'-DLPA) could also be  
326 confirmed by minor product ions in the MS/MS spectra. Free FA were also found in the first  
327 minutes of the chromatographic separation (3-4.5 min) at C18 column.

328 LC-MS<sup>n</sup> analysis of carotenoid extracts with an APCI source allowed the  
329 identification of the thirty metabolites involved in the carotenoid biosynthetic pathway in *S.*  
330 *aureus*. The carotenoid acids 4,4'-DLPA (M1) at *m/z* 429 [C<sub>30</sub>H<sub>38</sub>O<sub>2</sub>-H]<sup>-</sup> (in SA147) (Kim  
331 and Lee, 2012) and 4,4'-DNPA (M2) at *m/z* 431 [C<sub>30</sub>H<sub>40</sub>O<sub>2</sub>-H]<sup>-</sup> presented characteristic loss  
332 of CO<sub>2</sub> in APCI(-) mode. The metabolite 4,4'-DNPA was also detected at *m/z* 433  
333 [C<sub>30</sub>H<sub>40</sub>O<sub>2</sub>+H]<sup>+</sup> in APCI(+), showing MS/MS spectrum peaks at *m/z* 415 [M+H-18]<sup>+</sup> and *m/z*  
334 387 [M+H-46]<sup>+</sup>, which indicate the loss of H<sub>2</sub>O and HCOOH, respectively (Figure S1) (Kim  
335 and Lee, 2012; Marshall and Wilmoth, 1981a; Pelz et al., 2005). Similar ions were observed  
336 in ESI (+). In addition, this carotenoid acid presented absorbances at (420 nm), 446 nm, and  
337 472 nm, similar to those reported in methanol (Marshall and Wilmoth, 1981a).

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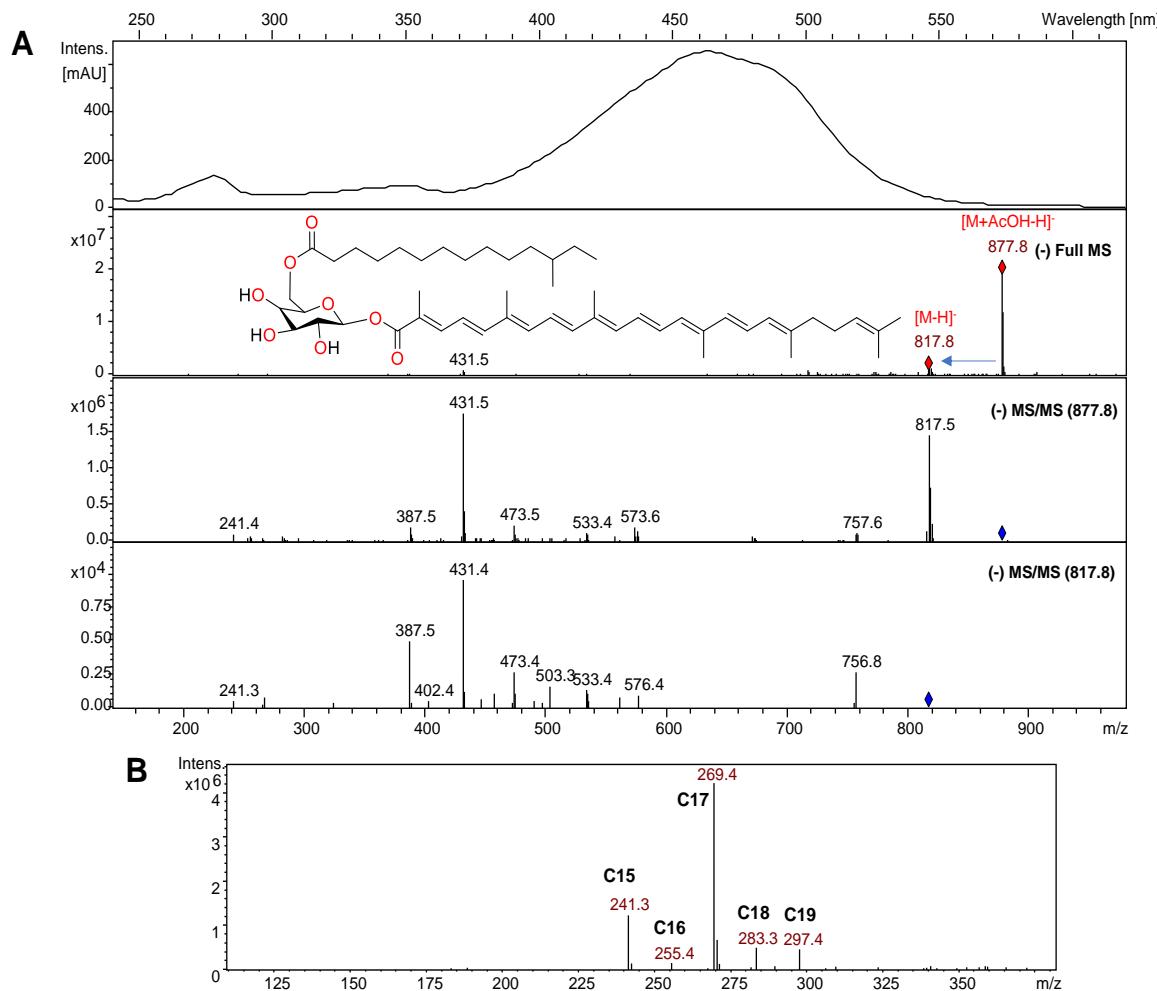
339 In the case of STX and STX-homologues, two major ions were observed in the mass  
340 spectrum in APCI (-). STX isomers (M3, M4, M5, M8, M10 and M11) exhibited  $m/z$  817.8  
341  $[\text{M}-\text{H}]^-$  (Kim and Lee, 2012) and  $m/z$  877.8  $[\text{M}+\text{AcOH}-\text{H}]^-$ , as deprotonated molecular ions  
342 and the STX adduct with acetic acid (**Fig. 3a**), respectively. Acetic acid from the mobile  
343 phase is presumably attached to the hydroxyl groups of the glucose (Amorim-Carrilho et al.,  
344 2014; Hrvolová et al., 2016; Novotny et al., 2005; Schex et al., 2018). Different lipid chains,  
345 ranging from C13 to C20, attached to the STX core (Glu-4,4'-DNPA) evidenced the broad  
346 variety of the STX homologues in this biosynthetic pathway. Thus, STX-homologues such  
347 as STX-C13 (M6, M7) at  $m/z$  849.7, STX-C14 (M24) at  $m/z$  863.7, STX-C16 (M27) at  $m/z$   
348 891.7, STX-C17 (M9, M12, M13, M25) at  $m/z$  905.6, STX-C18 (M14) at  $m/z$  919.7, STX-  
349 C19 (M15) at  $m/z$  933.8, and STX-C20 (M34) at  $m/z$  947.6 were assigned by the  $[\text{M}+\text{AcOH}-$   
350  $\text{H}]^-$  adduct ion, see table 1 and figure S2. Besides, MS/MS analysis showed characteristic  $m/z$   
351 431 and  $m/z$  387, corresponding to the loss the fatty acid-glucose (FA-Glu) residue, which  
352 demonstrate the structural similarity of STX-homologues with 4,4'-DNPA. In addition, free  
353 FAs observed in the first minutes of the chromatogram confirm the wide diversity of these  
354 molecules bonded to the STX core, as previously stated (**Fig. 3b**). STX was found in ESI(+)  
355 as the ions previously reported in the literature (Marshall and Wilmoth, 1981a; Pelz et al.,  
356 2005). Thus, the molecular ion at  $m/z$  818.3  $[\text{C}_{51}\text{H}_{78}\text{O}_8]^+$  and the sodium adduct at  $m/z$  840.4  
357  $[\text{C}_{51}\text{H}_{78}\text{O}_8+\text{Na}]^+$ , addition typical fragments associated to STX were observed (Table 1).  
358 Both 4,4'-DNPA and STX lose a toluene molecule ( $\text{M}+\text{H}-92$ ) and ( $\text{M}-386-92$ ) respectively,  
359 generating a fragment at  $m/z$  340, characteristic of carotenoids (Amorim-Carrilho et al.,  
360 2014). Additionally, the  $m/z$  749  $[\text{M}-69]^+$  ion was generated by the loss of an isopentenyl  
361 fragment (Figure S3). Similarly, these ions were observed in MS/MS experiments performed  
362 on  $m/z$  819  $[\text{M}+\text{H}]^+$  by flow injection analysis of the crude carotenoid extract (data not

363 shown). Furthermore, different types of visible absorbances were observed for STX and  
364 STX-homologues peaks, detected between 9.21 and 11.76 minutes, which allowed the  
365 classification as *cis/trans* isomers (**Fig. 2a**). Thus, the peaks at 10.33 and 11.41 minutes  
366 presented absorption in visible region (453-454 nm) and an additional peak in the UV region  
367 (347 nm), typically called *cis* peak (**Fig. 2b**) (O'Neil and Schwartz, 1992), whereas the other  
368 peaks absorbed only in the visible region at longer wavelengths (461-463 nm), characteristic  
369 of all-*trans* isomers (O'Neil and Schwartz, 1992; Seel et al., 2020).

370 Four hydrocarbon carotenes, including 4,4'-diaponeurosporene (4,4'-DNP, M17),  
371 4,4'-diapo- $\zeta$ -carotene (4,4'-DZC, M18), 4,4'-diapophytofluene (4,4'-DPF, M19), and 4,4'-  
372 diapophytoene (4,4'-DPE, M20), were identified in APCI(+), showing pseudo-molecular ions  
373 at  $m/z$  403.5  $[\text{C}_{30}\text{H}_{42}+\text{H}]^+$ ,  $m/z$  405.5  $[\text{C}_{30}\text{H}_{44}+\text{H}]^+$ , 407.5  $[\text{C}_{30}\text{H}_{46}+\text{H}]^+$ , and 409.5  
374  $[\text{C}_{30}\text{H}_{48}+\text{H}]^+$ , respectively, (Figure S4) (Marshall and Wilmoth, 1981b; Pelz et al., 2005;  
375 Taylor' and Bovies, 1983). In addition, drastic changes in UV-visible absorbances for these  
376 metabolites allowed confirming their presence. Operating in ESI(+), only 4,4'-DNP was  
377 detected at  $m/z$  402.5  $[\text{C}_{30}\text{H}_{42}]^+$ , with MS/MS fragments at  $m/z$  387 and 310, which  
378 correspond to loss of methyl and toluene molecule, respectively (Kim and Lee, 2012;  
379 Marshall and Wilmoth, 1981a; Pelz et al., 2005). The carotene 4,4'-diapolyycopene (4,4'-DLP,  
380 M16) was identified in the SA147 strain at  $m/z$  401.5  $[\text{C}_{30}\text{H}_{40}+\text{H}]^+$  in APCI(+), whereas in  
381 ESI(+) this carotene was observed at  $m/z$  400.5  $[\text{C}_{30}\text{H}_{40}]^+$  and tandem mass analysis presented  
382 the same fragments of 4,4'-DNP (Table 1). The carotenoids 4,4'-DLP and 4,4'-DLPA have  
383 been previously reported (Kim and Lee, 2012). Another interesting aspect to highlight is the  
384 bathochromic shift in UV-visible spectra analysis of carotenoids involved in the STX  
385 biosynthesis. Interestingly, as the degree of unsaturation increases, wavelengths shift from  
386 the UV to visible region and the multiplicity of the bands is lost due to the greater conjugation

387 of the final molecules (4,4'-DNPA and STX) (**Fig. 2c**). Thus, the main six carotenoids  
388 involved in the STX biosynthetic pathway from *S. aureus* (4,4'-DPE, 4,4'-DPF, 4,4'-DZC,  
389 4,4'-DNP, 4,4'-DNPA y STX) are characterized herein.

390



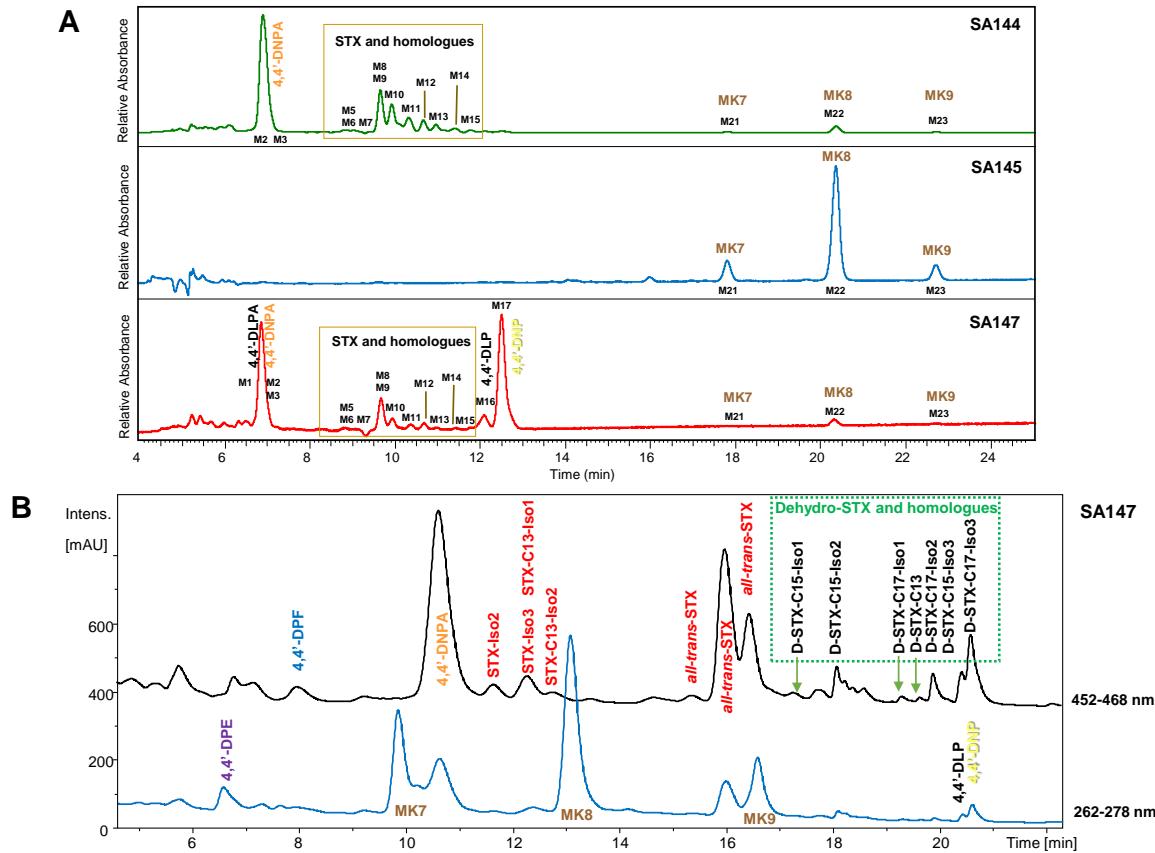
391 **Figure 3.** Spectrum of STX and free fatty acids. (A) UV-vis, Full MS and MS/MS spectra  
392 of STX. (B) Full MS spectra of fatty acids.

393

394

395

396



398 **Figure 4.** HPLC-MS analysis of *S. aureus* cells extracts in C18 and C30 column. (A)  
399 Chromatographic profile of SA144, SA145 and SA147 strains at 24 hours of culture in C18  
400 column. (B) Chromatogram extracted in the visible region (322-338 nm and 422-436 nm) in  
401 C30 column.

402

403 Three metabolites with the highest retention time were identified as menaquinones  
404 MK7 (M21), MK8 (M22), and MK9 (M23), with pseudo-molecular ions at  $m/z$  649.7  
405 [ $C_{46}H_{64}O_2+H$ ]<sup>+</sup>,  $m/z$  717.7 [ $C_{51}H_{72}O_2+H$ ]<sup>+</sup>, and  $m/z$  785.8 [ $C_{56}H_{80}O_2+H$ ]<sup>+</sup>, respectively, as  
406 observed in APCI(+) and in ESI(+) mode. As can be observed in table 1 and figure S5,  
407 menaquinones showed characteristic fragmentation ions at  $m/z$  227 and  $m/z$  187, and  
408 absorbances at (247 nm, 269 nm, and 329 nm), in accordance with data reported in literature

409 for *S. aureus* (Marshall and Wilmoth, 1981a; Taylor' and Bavies, 1983; Wakeman et al.,  
410 2012). The presence of 4,4'-DNPA, STX and STX-homologues (STX-C13, STX-17, STX-  
411 C18 and STX-C19) was observed in the ATCC strain, in addition to the menaquinones MK7,  
412 MK8, and MK9.

413

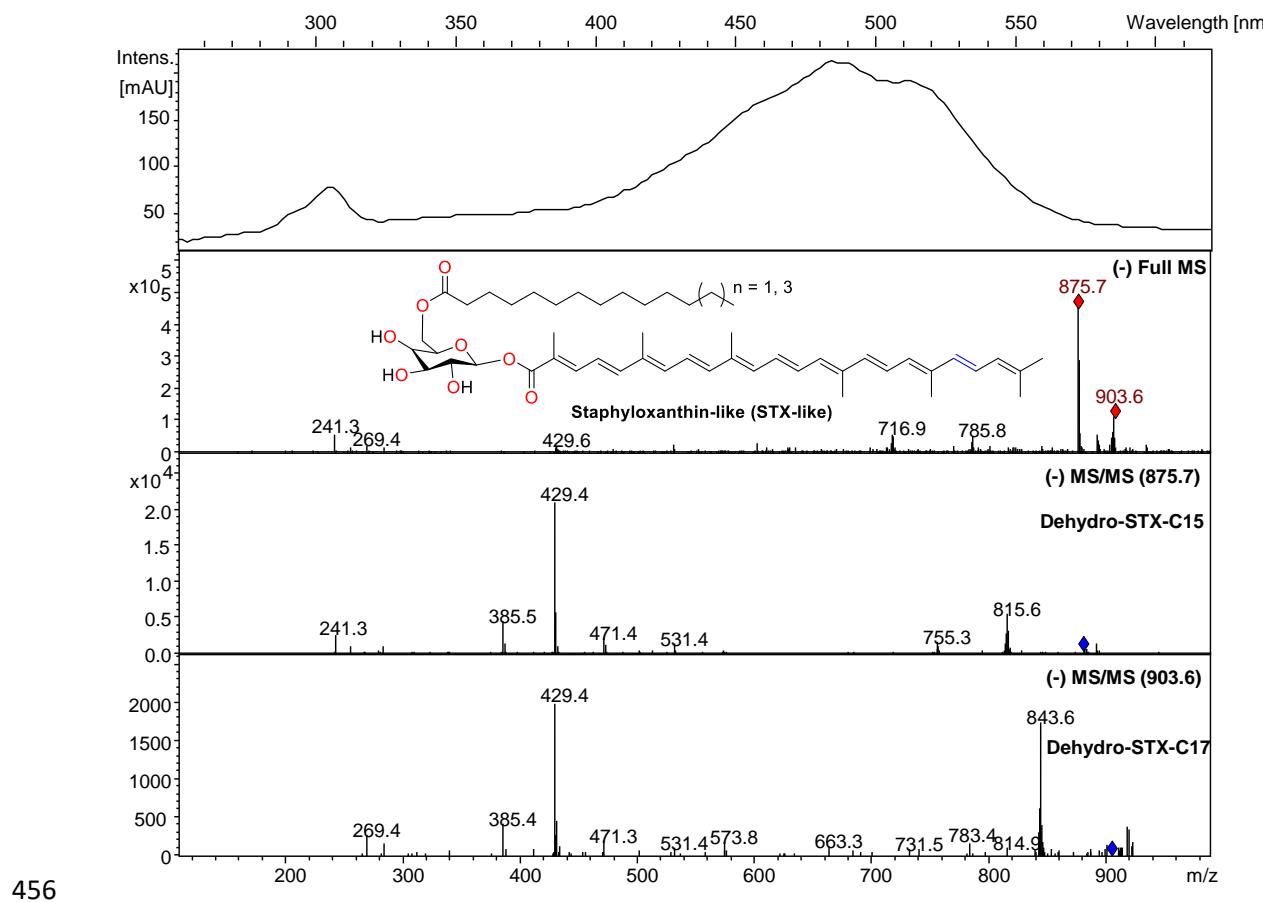
414 The chromatographic elution obtained on the C18 column was according to the  
415 increasing polarity of the carotenoids, and provided a good resolution of *cis/trans* carotenoid  
416 isomers, typically reported for a C30 column (Amorim-Carrilho et al., 2014; Saha et al.,  
417 2019). Nevertheless, although STX is expected to elute before 4,4'-DNPA for its sugar  
418 moiety the final elution observed is according to the degree of polarity, similar to previous  
419 reports (Kim and Lee, 2012). The C18 chromatographic profiles of SA144, SA145, and  
420 SA147 strains extracts obtained after 24-hour growth were comparatively evaluated. While  
421 SA144 strain presented a similar carotenoid profile to SA401, SA145 only showed the  
422 characteristic menaquinone (M24-M26), as expected for the inhibition of carotenoids  
423 biosynthesis in this strain. In turn, SA147 presented an increase in the peak areas between 11  
424 and 15 minutes, attributable to the reactivation of *S. aureus* carotenoid synthesis. (**Fig. 4a**).  
425 On the other hand, employing the C30 column, a similar resolution for *cis/trans* isomers in  
426 STX peaks was obtained compared to C18 column; however, carotenes 4,4'-DNP, 4,4'-DPF,  
427 4,4'-DPE and menaquinones (MK7, MK8, MK9) are more broadly distributed throughout the  
428 chromatogram, due to the increasing resolution capacity of C30 stationary phase for  
429 isoprenoid derivates (Amorim-Carrilho et al., 2014; Saha et al., 2019). Nonetheless, the  
430 menaquinones mentioned above coelute with 4,4'-DNPA, STX or STX-homologues,  
431 therefore making more difficult the characterization (**Fig. 4b**).

432                   Alternatively, the C30 column allowed the identification of new molecules in strain  
433                   SA147, tentatively assigned as Dehydro-STX-C15, Dehydro-STX-C13 and Dehydro-STX-  
434                   C17 (**Fig. 4b**), in addition to allow the confirmation of the carotenoid acid 4,4'-DLPA and  
435                   hydrocarbon carotene 4,4'-DLP. Dehydro-STX-C13 (M30), Dehydro-STX (M26, M28 and  
436                   M32), and Dehydro-STX-C17 (M29, M31 and M33) show  $[M+AcOH-H]^-$  adduct ions at  $m/z$   
437                   848.0,  $m/z$  875.7, and  $m/z$  903.6, similarly to the adducts observed for STX and STX-  
438                   homologues. MS/MS fragmentation in produced  $m/z$  429 and  $m/z$  385, related to the loss of  
439                   CO<sub>2</sub> (-44 amu) and typical masses of FA (**Fig. 5b**). In addition, Dehydro-STX and its  
440                   homologues exhibited similar absorbances ((455), 483, 512 nm) to those reported for 4,4'-  
441                   DLPA (Kim and Lee, 2012), a structurally related carotenoid (**Fig. 2**). Therefore, difference  
442                   in two atomic mass units between fragments at  $m/z$  431 (generated from STX-C13, STX and  
443                   STX-C17) and  $m/z$  429 (generated from Dehydro-STX-C13, Dehydro-STX and Dehydro-  
444                   STX-C17) was attributed to an additional unsaturation in the carotenoid moiety. Thus,  
445                   dehydro-STX homologues share structural similarity to their biosynthetic precursors 4,4'-  
446                   DLP and 4,4'-DLPA also characterized in this work, as part of the alternative Dehydro-STX  
447                   biosynthetic pathway illustrated in Fig. 1 (right side).

448

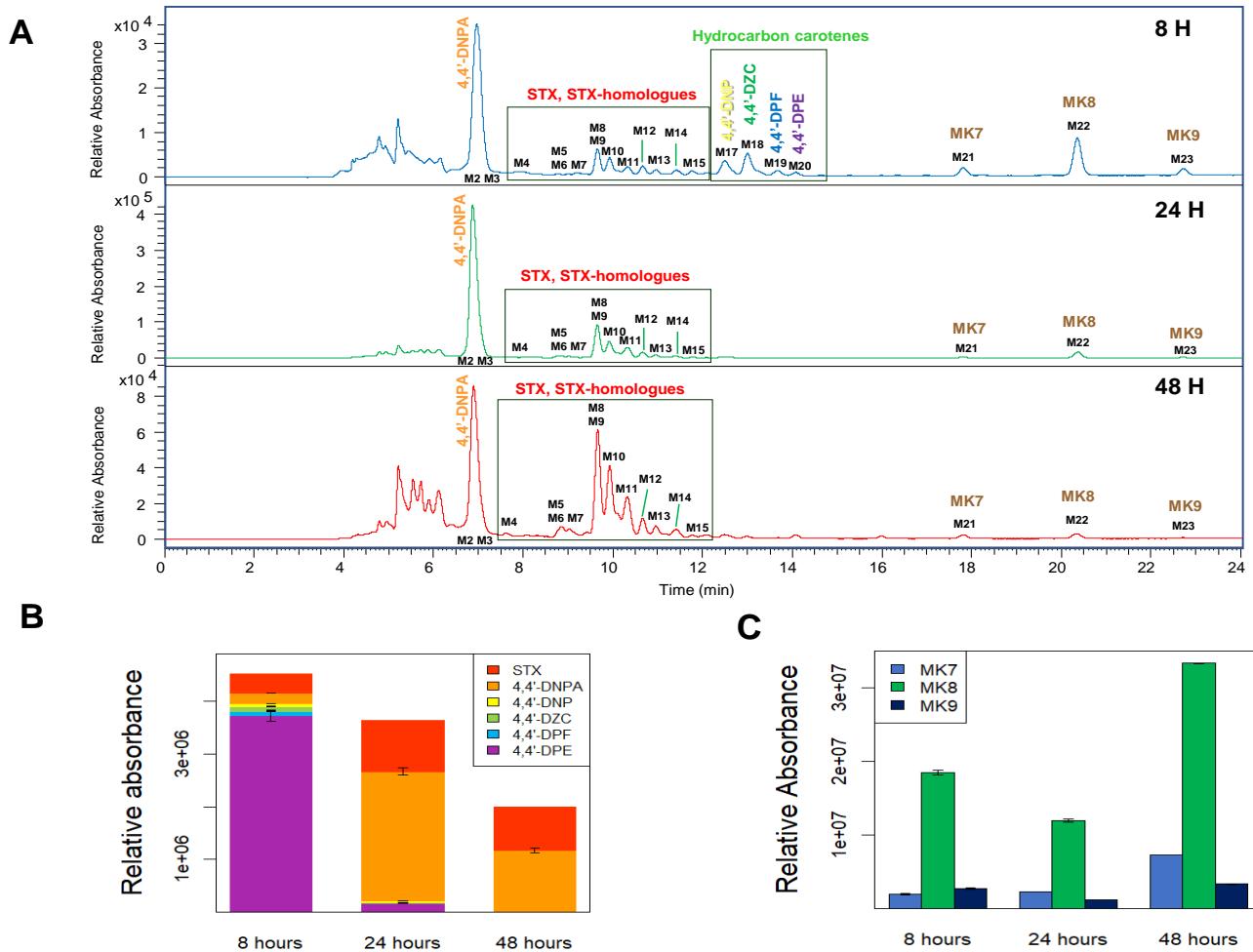
449                   Kinetic studies in *S. aureus* were performed by comparing the carotenoids profiles of  
450                   SA401 *S. aureus* cell extracts obtained at different cell growth phases (8, 24 and 48 h), as  
451                   illustrated in Fig. 6. Between exponential (8h) and stationary (24h) phases, a carotenes to  
452                   xanthophylls interconversion can be clearly observed in the chromatographic profile (**Fig. 6**),  
453                   which show the decrease or disappearance of carotenes: 4,4'-DPE, 4,4'-DPF, 4,4'-DZC, 4,4'-  
454                   DNP, whereas the xanthophylls 4,4'-DNPA and STX (including STX-homologues) increase.

455



468 that in the extreme phases of growth *S. aureus* prioritizes the synthesis of molecules that help  
469 it survive, since menaquinones are associated with cellular respiration, while carotenes and  
470 xanthophylls are secondary metabolites that have no direct function in survival unless there  
471 is stress external to the cells.

472



473 **Fig. 6.** Carotenoids and menaquinones analysis from *S. aureus* cells at 8, 24, and 48 hours.  
474 (A) Comparative chromatograms of carotenoids at three growing times. (B) Metabolic  
475 changes in carotenes and xanthophylls composition at three growing times. (C) Metabolic  
476 changes in menaquinones composition at three growing times.

477

478 3.3. - *Membrane biophysical properties assessment*

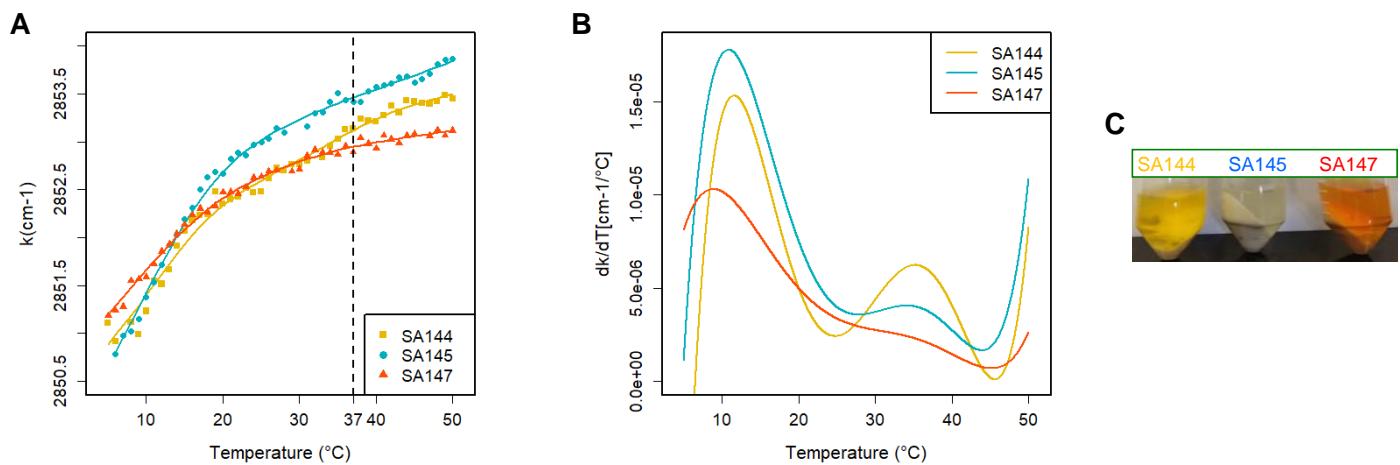
479 To understand the influence of carotenoids on the biophysical behavior of *S. aureus*  
480 membranes *in vivo*, wild type (SA144), knock-out (SA145), and regenerated (SA147) strains  
481 were comparatively evaluated by FTIR. Thus, the CH<sub>2</sub> symmetric stretch band was analyzed  
482 between 2860 to 2840 cm<sup>-1</sup> as a function of temperature changes from 5 to 50 °C, as shown  
483 in **Fig. 7**. Increasing wavenumber values can be related to changes in membrane lipid packing  
484 since the CH<sub>2</sub> stretch indicates the level of trans/gauch isomerization in the acyl chains. A  
485 lower wavenumber indicates a high number of trans isomers that are associated to a straight  
486 acyl chain, which result in higher lipid packing. An increase in the wavenumber indicates an  
487 increase in gauch rotomeres related to a more disordered acyl chain in the phospholipid  
488 components of the membrane and an increase in lipid spacing. Lower temperatures favor the  
489 all trans configuration in lipids. Pure saturated phospholipids are characterized by  
490 cooperative first-order chain melting events that occur at specific melting temperatures.  
491 These are well documented transitions from a more tightly packed gel phase (L<sub>β</sub>) to a liquid  
492 crystalline phase (L<sub>α</sub>). In pure phospholipid species such as 1,2-dipalmitoyl-sn-glycero-3-  
493 phosphocholine (DPPC) with T<sub>m</sub> = 41 °C, 1,2-dimyristoyl-sn-glycero-3-phosphocholine  
494 (DMPC) at 24 °C, and several saturated Phosphatidyl Glycerol (PG) lipids characteristic of  
495 *S. aureus*. Although the more complex composition of bacterial membranes reduces the  
496 cooperativity of these melting events. thermotropic transition of the CH<sub>2</sub> stretch vibration  
497 have been reported for *S. aureus* around 15 °C involving the cooperative melting of PG lipids  
498 contained in the plasma membrane of *S. aureus* (Ocampo et al., 2010; Scherber et al., 2009;  
499 Schultz and Naumann, 1991). Figure 7a shows cooperative melting events for SA144,  
500 SA145, and SA147 indicated by thermotropic shifts in the CH<sub>2</sub> stretch vibration. The absence  
501 of carotenoid synthesis in strain SA145 results in an overall increase in the CH<sub>2</sub> stretch

502 wavenumber for temperatures above the phase transition temperature, and in particular at the  
503 growth temperature of *S. aureus* (Fig. 7b). This is direct indication of decreased chain order  
504 in the phospholipid acyl chains in the absence of carotenoids, and is consistent with previous  
505 studies using DPH and Laurdan (Perez-Lopez et al., 2019). When carotenoid synthesis is  
506 reestablished (SA147), the wavenumber drops. Indicating increased lipid packing. The  
507 results support the importance of carotenoids as regulators of lipid packing in *S. aureus*  
508 membranes (Mishra et al., 2011; Perez-Lopez et al., 2019; Sen et al., 2016; Tiwari et al.,  
509 2018).

510 The first derivative of the measurements in FTIR (**Fig. 7b**) indicate thermal events  
511 where the slope of the thermotropic curve is accentuated (See Fig 7a). These cooperative  
512 events reflect a change in the gauch/trans rotomer ratio for the acyl chain, indicating a clear  
513 change in the phospholipid packing behavior. The different packing levels of the membrane  
514 of *S. aureus* have been correlated to changes in the resistance of the membrane to  
515 antimicrobial agents (Bali et al., 2009; Mishra et al., 2011; Ocampo et al., 2010; Perez-Lopez  
516 et al., 2019). Strain SA144 shows two cooperative melting events appearing at around 10.5  
517 and 33.0 °C. Strain SA145 exhibits a more accentuated change in the transition at around  
518 11°C indicating that carotenoids tend to smooth the difference between the gel-like phase  
519 and the liquid-crystalline phase. This has been observed in model lipid systems in the  
520 presence of carotenoid extracts from *S. aureus* with the use of fluorescent probes and is  
521 confirmed here *in vivo* by FTIR (Perez-Lopez et al., 2019). This effect on the cooperativity  
522 of the chain melting event is similar to that observed in the presence of cholesterol in model  
523 systems and live cells (Bali et al., 2009). As carotenoid synthesis is reestablished (SA147)  
524 we observe a reduction in the cooperativity of the chain melting event (Fig. 7b SA147). In  
525 conclusion, Fig. 7 clearly indicates that carotenoids are regulators of membrane lipid packing

526 in the *in vivo* system. at the growth temperature, and it is interesting to note that the small  
527 transition event that occurs around 35°C in the SA144 strain, also reported for the SA401  
528 strain in a previous study (Ocampo et al., 2010), vanishes for SA145 and SA147. This small  
529 transition at a high temperature close to the growth temperature must be investigated further  
530 to be identified.

531



532 **Fig. 7.** (A) Thermotropic phase behavior of the CH<sub>2</sub> asymmetric stretch for *S. aureus* *in vivo*  
533 characteristic of the acyl chains in the membrane phospholipids in *S. aureus* for the native  
534 (SA144), CrtN knockout (SA145), and CrtN knockout incorporated with a plasmid  
535 containing CrtN (SA147) strains. (B) First derivative of the data obtained in (a) used to  
536 indicate the position of the main melting event and the cooperativity of the transition. (C)  
537 Different colors observed for extracts of SA144, SA145 and SA147.

538 **Table 1.** Carotenoids and menaquinones identified in *S. aureus*.

Metabolite number	Ret. time (min)	Identified compound	Abbreviation	Molecular formula	Ionization	Molecular ion (m/z)	MS/MS product ions (m/z) in ESI	MS/MS product ions (m/z) in APCI	UV-Vis max wavelength (nm)
M1	6.65	4,4'-diapolyisoprenoic acid	4,4'-DLPA	C <sub>30</sub> H <sub>38</sub> O <sub>2</sub>	APCI (-)	[M+H] <sup>+</sup> /429.4 [M-H] <sup>-</sup>	ND	385, 279, 227	446, 474, 500
M2	6.86	4,4'-diaponeurosporenoic acid	4,4'-DNPA	C <sub>30</sub> H <sub>40</sub> O <sub>2</sub>	ESI (+) / APCI (-)	433.3 [M+H] <sup>+</sup> /431.7 [M-H] <sup>-</sup>	415, 387, 363, 340, 309, 288, 274, 267.	387, 267, 229	(420), 447, 472
M3	7.00	Staphyloxanthin-Isomer1	STX-Iso1	C <sub>51</sub> H <sub>78</sub> O <sub>8</sub>	APCI (-)	877.7 [M+AcOH-H] <sup>-</sup>	ND	818 (M <sup>+</sup> ), 758, 473, 431, 387, 255.	465 (488)
M4	7.60	Staphyloxanthin-Isomer2	STX-Iso2	C <sub>51</sub> H <sub>78</sub> O <sub>8</sub>	APCI (-)	877.7 [M+AcOH-H] <sup>-</sup>	ND	818 (M <sup>+</sup> ), 758, 473, 431, 387, 255.	465 (488)
M5	8.80	Staphyloxanthin-Isomer3	STX-Iso3	C <sub>51</sub> H <sub>78</sub> O <sub>8</sub>	APCI (-)	877.7 [M+AcOH-H] <sup>-</sup>	ND	818 (M <sup>+</sup> ), 758, 473, 431, 387, 255.	465 (488)
M6	8.80	Tridecanoyl-glucosyl-4,4'-4,4'-diaponeurosporenoic acid-Isomer1	STX-C13-Iso1	C <sub>49</sub> H <sub>74</sub> O <sub>8</sub>	APCI (-)	849.7 [M+AcOH-H] <sup>-</sup>	ND	789,6 (M-H <sup>-</sup> ); 531, 471, 431, 387.	463 (488)
M7	9.15	Tridecanoyl-glucosyl-4,4'-4,4'-diaponeurosporenoic acid-Isomer1	STX-C13-Iso2	C <sub>49</sub> H <sub>74</sub> O <sub>8</sub>	APCI (-)	849.7 [M+AcOH-H] <sup>-</sup>	ND	789,6 (M-H <sup>-</sup> ); 531, 471, 431, 387.	463 (488)
M8	9.65	Staphyloxanthin-Isomer4	STX-Iso4	C <sub>51</sub> H <sub>78</sub> O <sub>8</sub>	ESI (+) / APCI (-)	818.3 [M] <sup>+</sup> ; 841.4 [M+Na] <sup>+</sup> / 877.7 [M+AcOH-H] <sup>-</sup>	M <sup>2</sup> [818.3] => 800,749, 726, 432, 415, 387, 340. M <sup>2</sup> [841.4] => 823, 735, 456, 410.	818 (M <sup>+</sup> ), 758, 473, 431, 387, 255.	463, (488)
M9	9.65	Heptadecanoyl-glucosyl-4,4'-diaponeurosporenoic acid-Isomer1	STX-C17-Iso1	C <sub>53</sub> H <sub>82</sub> O <sub>8</sub>	APCI (-)	905.6 [M+AcOH-H] <sup>-</sup>	ND	845,4 (M-H <sup>-</sup> ); 431; 387; 269,5.	463 (488)
M10	9.93	Staphyloxanthin-Isomer5	STX-Iso5	C <sub>51</sub> H <sub>78</sub> O <sub>8</sub>	ESI (+) / APCI (-)	818.3 [M] <sup>+</sup> ; 841.4 [M+Na] <sup>+</sup> / 877.7 [M+AcOH-H] <sup>-</sup>	M <sup>2</sup> [818.3] => 800,749, 726, 432, 415, 387, 340. M <sup>2</sup> [841.4] => 823, 735, 456, 410.	818 (M <sup>+</sup> ), 758, 473, 431, 387, 255.	463, (488)
M11	10.33	Staphyloxanthin-Isomer6	STX-Iso6	C <sub>51</sub> H <sub>78</sub> O <sub>8</sub>	ESI (+) / APCI (-)	818.3 [M] <sup>+</sup> ; 841.4 [M+Na] <sup>+</sup> / 877.7 [M+AcOH-H] <sup>-</sup>	M <sup>2</sup> [818.3] => 800,749, 726, 432, 415, 387, 340. M <sup>2</sup> [841.4] => 823, 735, 456, 410.	818 (M <sup>+</sup> ), 758, 473, 431, 387, 255.	463, (488)
M12	10.66	Heptadecanoyl-glucosyl-4,4'-diaponeurosporenoic acid-Isomer2	STX-C17-Iso2	C <sub>53</sub> H <sub>82</sub> O <sub>8</sub>	APCI (-)	905.6 [M+AcOH-H] <sup>-</sup>	ND	831,4 (M-H <sup>-</sup> ); 431; 387; 311; 255.	463 (488)
M13	10.97	Heptadecanoyl-glucosyl-4,4'-diaponeurosporenoic acid-Isomer3	STX-C17-Iso3	C <sub>53</sub> H <sub>82</sub> O <sub>8</sub>	APCI (-)	905.6 [M+AcOH-H] <sup>-</sup>	ND	831,4 (M-H <sup>-</sup> ); 431; 387; 311; 255.	463 (488)
M14	11.76	Octadecanoyl-glucosyl-4,4'-diaponeurosporenoic acid	STX-C18	C <sub>54</sub> H <sub>84</sub> O <sub>8</sub>	APCI (-)	919.8 [M+AcOH-H] <sup>-</sup>	ND	859,6 (M-H <sup>-</sup> ); 431; 387; 283.	463 (488)
M15	11.41	Nonadecanoyl-glucosyl-4,4'-diaponeurosporenoic acid	STX-C19	C <sub>55</sub> H <sub>86</sub> O <sub>8</sub>	APCI (-)	933.9 [M+AcOH-H] <sup>-</sup>	ND	873,5 (M-H <sup>-</sup> ); 431; 387; 297.	463 (488)
M16	12.11	4,4'-Diapolyisopocene	4,4'-DLP	C <sub>30</sub> H <sub>40</sub>	ESI (+) / APCI (+)	400.3 [M+H] <sup>+</sup> / 401.5 [M+H] <sup>+</sup>	385, 357, 308	ND	441, 465, 496
M17	12.29	4,4'-Diaponeurosporene	4,4'-DNP	C <sub>30</sub> H <sub>42</sub>	ESI (+) / APCI (+)	402.4 [M+H] <sup>+</sup> / 403.5 [M+H] <sup>+</sup>	388, 334, 310, 242.	386, 356, 346, 327, 291, 267, 187, 148.	412; 435; 464
M18	13.00	4,4'-Diapo- $\zeta$ -carotene	4,4'-DZC	C <sub>30</sub> H <sub>44</sub>	APCI (+)	405.5 [M+H] <sup>+</sup>	ND	386, 362, 336, 295, 225, 173, 157.	382; 399; 423

M19	13.66	4,4'-Diapophytofluene	4,4'-DPF	C <sub>30</sub> H <sub>46</sub>	APCI (+)	407.5 [M+H] <sup>+</sup>	ND	388, 349, 336, 323, 295, 225, 173, 159.	330; 347; 366
M20	14.07	4,4'-Diapophytoene	4,4'-DPE	C <sub>30</sub> H <sub>48</sub>	APCI (+)	409.5 [M+H] <sup>+</sup>	ND	392, 368, 354, 326, 299, 286, 215, 159.	275; 286; 297
M21	17.82	Menaquinone 7	MK7	C <sub>46</sub> H <sub>64</sub> O <sub>2</sub>	ESI (+) / APCI (+)	649.4 [M+H] <sup>+</sup> / 649.7 [M+H] <sup>+</sup>	592, 567, 442, 291, 265, 225, 187.	632, 594, 568, 464, 227, 187.	247, 269, 329
M22	20.36	Menaquinone 8	MK8	C <sub>51</sub> H <sub>72</sub> O <sub>2</sub>	ESI (+) / APCI (+)	717.4 [M+H] <sup>+</sup> / 717.7 [M+H] <sup>+</sup>	699, 635, 593, 531, 291, 265, 227, 225.	700, 649, 636, 532, 500, 403, 227.	247, 269, 329
M23	22.72	Menaquinone 9	MK9	C <sub>56</sub> H <sub>80</sub> O <sub>2</sub>	ESI (+) / APCI (+)	785.4 [M+H] <sup>+</sup> / 785.8 [M+H] <sup>+</sup>	767, 717, 649, 633, 599, 581, 525, 291, 265, 225.	744, 730, 718, 704, 636, 600, 568, 500, 227.	247, 269, 329
M24*	14.76*	Tetradecanoyl-glucosyl-4,4'-diaponeurosporenoic acid	STX-C14	C <sub>50</sub> H <sub>76</sub> O <sub>8</sub>	APCI (-)	863.6 [M+AcOH-H] <sup>-</sup>	ND	803.4 (M-H <sup>-</sup> ), 531, 471, 431, 387.	430, 460, 483
M25*	15.30*	Heptadecanoyl-glucosyl-4,4'-diaponeurosporenoic acid-Isomer4	STX-C17-Iso4	C <sub>53</sub> H <sub>82</sub> O <sub>8</sub>	APCI (-)	905.6 [M+AcOH-H] <sup>-</sup>	ND	845.5 (M-H <sup>-</sup> ), 603.	466 (488)
M26*	17.35*	Pentadecanoyl-glucosyl-4,4'-diapoly copenoic acid-Isomer1	Dehydro-STX-C15-Iso1	C <sub>51</sub> H <sub>76</sub> O <sub>8</sub>	APCI (-)	875.7 [M+AcOH-H] <sup>-</sup>	ND	815.6 (M-H <sup>-</sup> ); 429; 385; 283; 255; 241.	455, 483, 512
M27*	17.70*	Hexadecanoyl-glucosyl-4,4'-diaponeurosporenoic acid	STX-C16	C <sub>52</sub> H <sub>80</sub> O <sub>8</sub>	APCI (-)	891.7 [M+AcOH-H] <sup>-</sup>	ND	831.6 (M-H <sup>-</sup> ); 431; 387; 255.3.	430, 460 (488)
M28*	18.57*	Pentadecanoyl-glucosyl-4,4'-diapoly copenoic acid-Isomer2	Dehydro-STX-C15-Iso2	C <sub>51</sub> H <sub>76</sub> O <sub>8</sub>	APCI (-)	875.7 [M+AcOH-H] <sup>-</sup>	ND	815.6 (M-H <sup>-</sup> ); 429; 385; 283; 255; 241.	455, 483, 512
M29*	19.37*	Heptadecanoyl-glucosyl-4,4'-diapoly copenoic acid-Isomer1	Dehydro-STX-C17-Iso1	C <sub>53</sub> H <sub>80</sub> O <sub>8</sub>	APCI (-)	903.6 [M+AcOH-H] <sup>-</sup>	ND	843.6 (M-H <sup>-</sup> ); 429; 387.5; 283, 269.	455, 483, 512
M30*	19.44*	Tridecanoyl-glucosyl-4,4'-diapoly copenoic acid	Dehydro-STX-C13	C <sub>49</sub> H <sub>72</sub> O <sub>8</sub>	APCI (-)	847.6 [M+AcOH-H] <sup>-</sup>	ND	787.6 (M-H <sup>-</sup> ); 531, 471, 431, 387.	455, 483, 512
M31*	19.60*	Heptadecanoyl-glucosyl-4,4'-diapoly copenoic acid-Isomer2	Dehydro-STX-C17-Iso2	C <sub>53</sub> H <sub>80</sub> O <sub>8</sub>	APCI (-)	903.6 [M+AcOH-H] <sup>-</sup>	ND	843.6 (M-H <sup>-</sup> ); 429; 387.5; 283, 269.	455, 483, 512
M32*	19.86*	Pentadecanoyl-glucosyl-4,4'-diapoly copenoic acid-Isomer3	Dehydro-STX-C15-Iso3	C <sub>51</sub> H <sub>76</sub> O <sub>8</sub>	APCI (-)	875.7 [M+AcOH-H] <sup>-</sup>	ND	815.6 (M-H <sup>-</sup> ); 429; 385; 283; 255; 241.	455, 483, 512
M33*	20.55*	Heptadecanoyl-glucosyl-4,4'-diapoly copenoic acid-Isomer3	Dehydro-STX-C17-Iso3	C <sub>53</sub> H <sub>80</sub> O <sub>8</sub>	APCI (-)	903.8 [M+AcOH-H] <sup>-</sup>	ND	843.6 (M-H <sup>-</sup> ); 429; 387.5; 283, 269.	455, 483, 512
M34**	21.15**	Eicosanoyl-glucosyl-4,4'-diaponeurosporenoic acid	STX-C20	C <sub>56</sub> H <sub>88</sub> O <sub>8</sub>	APCI (-)	947.7 [M+AcOH-H] <sup>-</sup>	ND	887.6 (M-H <sup>-</sup> ); 431; 387; 311.	ND

\*Compounds present only Strain SA147 and retention time in C30 column

Figure 4b

\*\*Compound observed in Strain SA401, retention time in C30 column.

540        **4. Discussion**

541            The profiling analysis of *S. aureus* extracts obtained at different growth times led to  
542            the identification of six carotenoids belonging to the STX biosynthetic pathway. The six  
543            carotenoids (4,4'-DPE, 4,4'-DPF, 4,4'-DZC, 4,4'-DNP, 4,4'-DNPA and STX (including STX-  
544            homologues)) could be identified in the bacteria at 8 hours of cell culture. The relative  
545            abundance of these precursor compounds at 8 hours compared with 24 or 48 hours reflects  
546            the reactivation of the STX biosynthesis route, which was shown to be downregulated in the  
547            early exponential phase (Perez-Lopez et al., 2019). In fact, the precursor 4,4'-DPE exhibited  
548            the highest abundance, (**Fig. 6b**) in accordance to the results reported by Wieland *et al.*  
549            (Wieland et al., 1994), who found a 50% lower concentration of STX compared with 4,4'-  
550            DNP after 12 hours of growth. The lower presence of xanthophylls at the initial growth stage  
551            is in line with proteomic studies of *S. aureus*, which also observed significant differences in  
552            protein expression levels between resuscitating and freezing survived cells (Suo et al., 2018).  
553            The metabolic change between the relative abundances of metabolites observed in the  
554            exponential state (8h) and those observed in the stationary states (24 h and 48 h), suggests  
555            that carotenoid biosynthesis reached the highest level of xanthophyll production late in the  
556            late exponential phase (Fig. 6b), reaching maturation of STX synthesis in the early stationary  
557            phase. The greater similarity in the proportion of metabolites when comparing the phase  
558            stationary profiles suggests that after reaching stationary phase *S. aureus* has a stable level  
559            of STX and 4,4'-DNPA. It is interesting to note that carotenoid acid 4,4'-DNPA remains  
560            stable for all growth states. Lower abundance of xanthophylls in general can be observed at  
561            the latter growth phase (48h) compared to the 24h cell culture (**Fig. 6b**), which can be  
562            explained by the depletion of nutrients in the LB culture medium (not renewed) in late  
563            stationary phase. These results are in good agreement with that reported by Wieland *et al.* in

564 *S. aureus*, which indicate a greater amount of STX at 24 hours compared to 36 hours of cell  
565 growth (Wieland et al., 1994). Also, the observed increase in menaquinones content at 48  
566 hours might be due to a stress for lack of nutrients (medium LB) on the microbiological  
567 system (**Fig. 6c**), that led to an increase in the production of these molecules associated with  
568 the respiration of the bacteria (Kurosu and Begari, 2010; Wakeman et al., 2012).

569 The reported structural diversity in fatty acid chains for *S. aureus* grown in LB broth,  
570 show a high concentration (77.2%) of branched-chain fatty acids (BCFAs), whereas straight-  
571 chain fatty acids (SCFAs) account for 22.8% (Sen et al., 2016), ranging from a C15 to C20  
572 chain length. In this line, our characterization reveals the presence of a wide diversity of  
573 STX-analogues bonded to C13, C14, C16, C18, C19 and C20 fatty acid chains.  
574 Demonstrating that the acylation of glycosyl-4,4-diaponeurosporenoate mediated by CrtO  
575 enzyme is not exclusive to C15 and C17 fatty acids, as initially reported (Marshall and  
576 Wilmoth, 1981a), thus, C13 to C20 fatty acid chains that have been previously reported on  
577 the SA401 strain (Perez-Lopez et al., 2019) and other wild type *S. aureus* strains (Braungardt  
578 and Singh, 2019; Sen et al., 2016) were observed bonded to Glu-4,4'-DNPA core. The broad  
579 specificity of the acyltransferase CrtO was also reported in *E. coli* mutants with the formation  
580 of STX analogues derivatives that include C14 and C16 fatty acids and other fatty acids of  
581 different lengths of not yet characterized (Kim and Lee, 2012). Furthermore, the presence of  
582 a Staphyloxanthin derivate with three additional units of sugars, a molecule associated with  
583 microdomains generated by *S. aureus*, was recently indicated (García-Fernández et al.,  
584 2017), confirming the diversity of homologues in the synthesis respect to this carotenoid with  
585 saccharolipid nature. STX analogues with such broad range of fatty acid chains, has not been  
586 reported in *S. aureus* grown in LB media. We believe that STX-analogues have higher

587 proportion of BCFAs, according to previous reports (Perez-Lopez et al., 2019; Sen et al.,  
588 2016; Tiwari et al., 2018).

589 In this regard, previous reports indicate that CrtN knock-out strains harboring a  
590 plasmid containing CrtN, generated carotenoid extracts of red color, associated with the  
591 presence of additional alternative metabolites (Umeno et al., 2005). Thus, carotenoid extracts  
592 in wild type *S. aureus* strains are orange color, whereas *S. aureus* strains where CrtN has  
593 been reincorporated through a plasmid are reddish, with products which are alternative to the  
594 main carotenoids biosynthetic pathway (Kim and Lee, 2012). The regenerated strain SA147  
595 presents additional desaturase activity that converts 4,4'-DNP into the red-colored 4,4'-DLP  
596 carotene, as observed in the extract of these cells (**Fig. 7c**), as well as 4,4'-DLPA due to the  
597 oxidation of the 4,4'-DLP. Similar visible region absorptions and characteristic *m/z* values  
598 suggest that this strain has the capacity to generate three compounds, here denominated  
599 Dehydro-STX and its homologues, through the alternate route indicated in the right part of  
600 figure 1a. The characterization of Dehydro-STX, Dehydro-STX-C13, and Dehydro-STX-  
601 C17 described herein pose a valuable contribution to the work previously reported (Kim and  
602 Lee, 2012). In addition, the presence of STX homologues in the ATCC strain indicates that  
603 variation in fatty acid chains is not exclusive to the other strains studied. However, the C15  
604 fatty acid chain is still predominant in proportion to the other FAs in all strains.

605 In light of the results, it is worthy to highlight that the profiling methodology proposed  
606 in this work made it possible to characterize STX and Dehydro-STX with their respective  
607 homologues, by analyzing the extract instead of fractions obtained by TLC or OCC  
608 separations as reported in previous works (Kim and Lee, 2012; Marshall and Wilmoth,  
609 1981a; Pelz et al., 2005). Thus, the profiling approach proposed in this work avoids  
610 overlooking minor compounds. Another remarkable analytical aspect is the *cis/trans* isomers

611 resolution capacity shown by the C18 column, comparable to C30 columns, commonly used  
612 for carotenoids separation and for resolution of geometric isomers. These results add to the  
613 previously reported efficiency of C18 columns for the analysis of xanthophylls (Amorim-  
614 Carrilho et al., 2014; Saha et al., 2019). Besides, the resolution capacity demonstrated by the  
615 C18 could be explained as the greater interaction between this stationary phase and the  
616 analyte, that depends on the combination of hydrophobicity and dispersion forces, while  
617 the C30 relies exclusively on the hydrophobicity of the interaction with the carotenoids (Saha  
618 et al., 2019). This was also corroborated by the change in the order of elution of the carotenes,  
619 from the most non-polar to the most polar 4,4'-DPE, 4,4'-DPF, 4,4'-DNP, in accordance with  
620 the more hydrophobic character of the initial mobile phase. Also, the broad distribution of  
621 metabolites throughout the chromatogram showed by triacontyl (C30) stationary phase  
622 generates characterization issues, due to coelution of xanthophylls and menaquinones, as  
623 described in the results section. However, an outstanding aspect of the C30 column was its  
624 resolution capacity in the xanthophylls of the SA147 strain, as it allowed the characterization  
625 of the new Dehydro-STX include their homologues and allowed the completion of the  
626 alternate biosynthetic pathway described here.

627 Decrease in the membrane fluidity of *S. aureus* has been associated with the increase  
628 of STX content in the bacteria (Mishra et al., 2011; Perez-Lopez et al., 2019; Sen et al., 2016),  
629 hence the interest in the study of this secondary metabolite. However, many of these reports  
630 about the membrane biophysical behavior of *S. aureus* do not consider the intermediate  
631 species that may be present in the crude extract, mainly characterized by UV-vis  
632 spectrophotometry. In addition, previous studies on STX biosynthesis using mutant strains  
633 of *S. aureus*, *S. carnosus*, and *E. coli* (Kim and Lee, 2012; Pelz et al., 2005), showed the  
634 coexistence of intermediate species in the carotenoid extract. Thus, the results reported here

635 on two wild-type strains of *S. aureus* (SA401 and SA144) and one mutant (SA147) allow us  
636 to confirm that this coexistence of two major species (STX or STX-homologues and 4,4'-  
637 DNPA) observes at 24- and 48- hour of growth is proper to the bacterium. This aspect should  
638 be considered when proposing model compositions to study biophysical aspects of *S. aureus*  
639 membranes, including studies related to the activity of antimicrobial peptides. Since in  
640 different reports, the inhibition of antimicrobial peptide activity is only attributed to the  
641 presence of STX and not to other carotenoid metabolites (Mishra et al., 2011; Sen et al.,  
642 2016). According to the above, we hypothesize that the precursor carotenoid 4,4'-DNPA is  
643 an intrinsic component of the *S. aureus* membrane, which is likely to play an important role  
644 in regulating membrane stiffness. This species does not contain the additional sugar group or  
645 acyl chain that is present in STX and should be treated as a free fatty acid with a highly rigid  
646 chain group inserted in the membrane.

647 Finally, FTIR data on *S. aureus* cell *in vivo* show two distinct results. First, the level  
648 of acyl chain order, measured as the proportion of gauche/trans rotometer in the acyl chains  
649 of the phospholipids, increases significantly in the presence of carotenoids in the high  
650 temperature range, which includes the growth temperature. This is consistent with previous  
651 studies using indirect methods for measuring acyl chain order and headgroup spacing using  
652 extrinsic fluorescent probes such as DPH and Laurdan (Perez-Lopez et al., 2019).  
653 Additionally, the FTIR results show that the cooperativity of the main melting event around  
654 11°C does not present a significant shift in temperature when carotenoids are present.  
655 However, the level of cooperativity of this transition event, measured as the intensity of the  
656 first derivative of the thermograms, is greatly reduced when carotenoids are present. This  
657 behavior is consistent with that observed for the incorporation of cholesterol into mammalian  
658 cells, where the presence of cholesterol increases chain order in the liquid-crystalline phase,

659 inducing the formation of a liquid-ordered phase (Bali et al., 2009; Gousset et al., 2002;  
660 Mannock et al., 2006). In addition, cholesterol has been shown to reduce the cooperativity of  
661 the gel to liquid-crystalline phase. This effect is related to the rigid and planar ring structure  
662 of the cholesterol molecule which forces the acyl chain of neighboring lipids in the liquid-  
663 crystalline phase to increase the proportion of trans rotamers (Bali et al., 2009; Mannock et  
664 al., 2006; Potrich et al., 2009). The rigid and extended structure of the tripenoids  
665 characteristic of STX in *S. aureus* appear to serve a similar function (Mishra et al., 2011).  
666 Recent, publications have presented evidence to indicate that STX homologues is involved  
667 in the formation of lipid domains (García-Fernández et al., 2017), and that these STX-  
668 enriched lipid domains play a role in methicillin resistance. This propensity to form lipid  
669 domains is likely related to the biophysical properties of the molecule and needs to be studied  
670 further to elucidate the mechanism by which these lipid domains are formed.

671

672 **5. Conclusions**

673

674 We employed a suitable LC-MS<sup>n</sup> method for the analysis of the carotenoids present  
675 in *S. aureus* with minimum sample preparation of the extract. The joint spectral information  
676 allows the simultaneous analysis of carotenes, xanthophylls, and menaquinones from *S.*  
677 *aureus*. The tentative identification of 34 carotenoids and menaquinones produced by the  
678 microorganism was achieved and it is clear that STX is not the main component, even when  
679 the carotenoid composition is stabilized in the stationary phase, although it is responsible for  
680 the characteristic color of the bacteria. Also, the use of the ion trap (IT)-MS in this method  
681 allowed the complete identification of characteristic patterns of fragmentation of carotenoids,  
682 including new unreported molecules in knockout *S. aureus* strain incorporated with a CrtN

683 containing plasmid. Results based on growth times show the sequential progression of  
684 metabolite precursors during late exponential phase (8 hours) leading towards a mature  
685 carotenoid profile of end products which includes 4,4'-DNPA and STX as the main  
686 components in the stationary phase (24 and 48 hour). These results reveal that in the interest  
687 of studying these most carotenoids of this microorganism, it is best to carry out its culture  
688 and extraction at 8 hours. Eventually, this method could lead to performing quantitative  
689 analysis of carotenoids in *S. aureus* and other microorganisms to identify intermediate  
690 species in different biosynthesis routes. In addition, the characterization of the melting  
691 temperatures in the fatty acid chains was achieved using FTIR and associated with increase  
692 acyl chain order in the presence of carotenoids and changes in the cooperativity of the  
693 membrane melting events.

694

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702

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