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1 Membrane phospholipid composition of *Pseudomonas aeruginosa* grown in a cystic fibrosis
2 mucus-mimicking medium

3

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13

14 Keywords: *Pseudomonas aeruginosa*, CF sputum, membrane lipids, antibiotic resistance,
15 culture medium, mass spectrometry

16

17 Abbreviations: CF, cystic fibrosis; MHB, Mueller Hilton broth; SCFM, synthetic cystic fibrosis
18 medium; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; SCFM-PC, DOPC complemented
19 SCFM; LB, lysogeny broth; ASM, artificial sputum medium; PE, phosphatidylethanolamines;
20 PC, phosphatidylcholines; PG, phosphatidylglycerols; CL, cardiolipins; LC-MS, liquid
21 chromatography - mass spectrometry; MS/MS, tandem mass spectrometry; MIC, minimal
22 inhibitory concentration; ESI-MS, electrospray ionisation-mass spectrometry; PAK, *P.*
23 *aeruginosa* strain PAK; CFU, colony forming units; OD600, optical density at 600 nm; AFM,
24 atomic force microscopy; (n), number of replicates.

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28 **ABSTRACT**

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29 **Background:** *Pseudomonas aeruginosa* is a bacterium able to induce serious pulmonary
 30 infections in cystic fibrosis (CF) patients. This bacterium is very often antibiotic resistant,
 31 partly because of its membrane impermeability, which is linked to the membrane lipid
 32 composition. This work aims to study the membrane phospholipids of *P. aeruginosa* grown
 33 in CF sputum-like media.

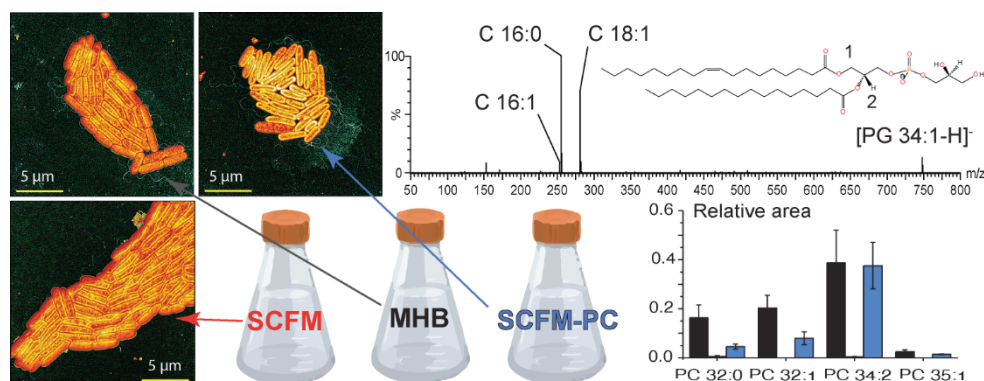
34 **Methods:** Three media were used: Mueller Hilton broth (MHB), synthetic cystic fibrosis
 35 medium (SCFM) and 1,2- dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) complemented SCFM
 36 (SCFM-PC). Lipids were extracted and LC-MS/MS analyses were performed. Growth curves,
 37 atomic force microscopy images and minimal inhibitory concentration determination were
 38 performed in order to compare the growth and potentially link lipid modifications to
 39 antibiotic resistance.

40 **Results:** Semi-quantification showed phospholipid quantity variation depending on the
 41 growth medium. Phosphatidylcholines were detected in traces in SCFM. MS/MS experiments
 42 showed an increase of phospholipids derived from DOPC in SCFM-PC. We observed no
 43 influence of the medium on the bacterial growth and a minor influence on the bacterial
 44 shape. MIC values were generally higher in SCFM and SCFM-PC than in MHB.

45 **Conclusions:** We defined a CF sputum-like media which can be used for the membrane lipid
 46 extraction of *P. aeruginosa*. We also showed that the growth medium does have an
 47 influence on its membrane lipid composition and antibiotic resistance, especially for SCFM-
 48 PC in which *P. aeruginosa* uses DOPC, in order to make its own membrane.

49 **General significance:** Our results show that considerable caution must be taken when
 50 choosing a medium for lipid identification and antibiotic testing —especially for
 51 phospholipids-enriched media.

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55 1. Introduction

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56 *Pseudomonas aeruginosa* is an opportunistic pathogen bacterium which is a major
57 public health concern for its ability to survive on healthcare equipments [1]. This leads to
58 nosocomial infections which are very difficult to eradicate. Due to its high pathogenicity for
59 immunocompromised patients, such as cystic fibrosis (CF) patients [2,3], *P. aeruginosa* is one
60 of the deadliest bacteria in hospitals. Moreover, *P. aeruginosa* can easily develop many
61 mechanisms of resistance against antibiotics [4], leading to most antibiotics becoming
62 useless. For these reasons, *P. aeruginosa* was set priority number one for the research and
63 development of new antibiotics and treatments, by the world health organization (WHO) [5].

64 In order to develop new antibiotics, new targets and strategies are required. One of
65 the current new drug strategies involves targeting membrane phospholipids (e.g.
66 cardiolipins [6]). Such new drugs could, by themselves, allow membrane lysis or could be
67 used in synergy to increase the effectiveness of antibiotics [7,8]. Previous analyses of the
68 membrane lipid composition of *P. aeruginosa* were performed in culture media such as
69 minimal glucose medium (MGM) [9,10], Muller-Hilton broth (MHB) [11,12] or lysogeny broth
70 (LB) [13]. The membrane lipid composition mainly consisted of four lipid chemical classes:
71 phosphatidylethanolamines (PE), phosphatidylcholines (PC), phosphatidylglycérols (PG) [13]
72 and cardiolipins (CL) [12]. Moreover, these phospholipids were described to be constituted
73 of saturated, unsaturated or cyclopropylated, fatty acyl chains ranging from C14 to C18 [10].

74 Since *P. aeruginosa* is known to survive in diverse environments, by adapting its
75 metabolism according to the available energy resources [14], an influence of the culture
76 medium on the membrane lipids should be expected. Due to the high pathogenicity of *P.*
77 *aeruginosa* in cases of CF lungs chronic infections, the CF lung sputum was chemically
78 analysed with the purpose to reproduce it in laboratories [15]. This sputum was
79 characterized by specific amino acid concentrations, the presence of DNA, lipids and mucin
80 and a high viscosity [16]. Thus, several media mimicking the CF sputum were described in
81 the literature [15–17]. The synthetic cystic fibrosis medium (SCFM) [15], first described,
82 contained amino acids, salts and sugars at adjusted concentrations, but lacked DNA, lipids
83 and mucin. This medium was then improved, as SCFM2, by the addition of DNA, lipids and
84 mucin [16].

85 The Artificial Sputum medium (ASM) contained amino acids, mucin, DNA and egg yolk as a
86 phospholipid nutrient [17]. However, most of these media are not compatible with
87 membrane lipid extraction, as the high viscosity of such media [16,17] does not allow
88 enough bacterial growth for exploitable quantities of lipid material. For this specific reason,
89 this work uses the commercial MHB as a reference medium and the SCFM one [15]. By
90 adding phosphatidylcholine to SCFM (SCFM-PC), we also analysed the impact of this choline
91 nutrient source on the *P. aeruginosa* lipid composition. 1,2-dioleoyl-sn-glycero-3-
92 phosphocholine (DOPC) was chosen in order to simulate the presence of lipids from
93 eukaryote cells, since DOPC is widely used for eukaryote cell modelling [18].

94 Herein, to our knowledge, this work reported, for the first time, the membrane
95 phospholipid composition of *P. aeruginosa* grown in CF sputum-mimicking media —*e.g.*
96 SCFM and SCFM-PC. The membrane lipids were identified and semi-quantified using liquid
97 chromatography - mass spectrometry (LC-MS). Tandem mass spectrometry (MS/MS)
98 experiments were performed to determine the fatty acyl composition of phospholipids. With
99 the aim to link membrane lipid modifications and antibiotic resistance, the minimal
100 inhibitory concentration (MIC) of four antibiotics was determined as well.

101

102 **2. Material and methods**

103 *2.1 Chemicals*

104 All SCFM components, chemicals required for the membrane lipid extraction and
105 antibiotics were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). 1,2-
106 Dioleoyl-sn-glycero-3-phosphocholine (DOPC), and phospholipids internal standards (PC
107 17:0/17:0, 0.5 µg; PE 17:0/17:0, 2.5 µg; PG 17:0/17:0, 1 µg; LPC 17:0, 1 µg; LPE 13:0, 2.5 µg;
108 CL 14:0/14:0/14:0/14:0, 5 µg) were purchased from Avanti polar lipids (AL, USA). MHB
109 media, brand Difco, was purchased from Fischer Scientific (UK). Milli Q grade water was
110 obtained from an UltraClear TWF system (Evoqua water technologies, Germany).

111

112 Methanol, chloroform, acetonitrile and isopropanol were purchased from Fischer Scientific
113 (UK), ammonium formate from Sigma-Aldrich (Saint Quentin Fallavier, France), formic acid
114 from Merck (Germany). Except methanol and chloroform which were HPLC grade, all
115 solvents and buffers used were LC-MS grade.

116

117 2.2. SCFM and SCFM-PC media preparation

118 The SCFM medium was first described by Palmer *et al.* [15]. In order to prepare this
119 chemically defined medium more easily by hand, the number of individual stock solutions
120 was reduced by mixing some of the individual stock solutions together (*e.g.* from 19
121 individual amino acids stock solutions to 6 individual or mixed amino acids stock solutions).
122 Moreover, part of the medium was autoclaved.

123 The stock solutions were prepared from powder, using either autoclaved Milli-Q
124 grade water or NaOH solutions (concentrations will be detailed later) and stocked in the dark
125 at 4°C. The bottles used for the stock solutions were autoclaved as well.

126 A first stock solution was prepared by mixing the following thirteen amino acids,
127 whose final concentrations were: 360 mM alanine, 60 mM arginine –HCl, 40 mM cysteine –
128 HCl, 300 mM glutamate –HCl, 240 mM glycine, 100 mM histidine –HCl, 420 mM lysine–HCl,
129 120 mM methionine, 140 mM ornithine –HCl, 340 mM proline, 280 mM serine, 200 mM
130 threonine and 220 mM valine. A second stock solution was prepared by mixing isoleucine
131 and phenylalanine at 110 mM and 50 mM, respectively. Leucine was stocked at 100 mM.
132 Aspartate was stocked at 103 mM in 0.5 M NaOH. Tyrosine was stocked at 100 mM in 1 M
133 NaOH. Tryptophan was stocked at 130 mM in 0.2 M NaOH.

134 The following salts were mixed into a single stock solution at these concentrations:
135 23 mM NH₄Cl, 153 mM KCl, 511 mM NaCl, 13 mM NaH₂PO₄, 13 mM Na₂HPO₄, 3 mM
136 K₂SO₄, 4 mM NaNO₃. Morpholinepropanesulfonic acid (MOPS) was stocked at 100 mM. The
137 mixed salts and the MOPS stock solution were then autoclave sterilised for storage purpose.

138 CaCl₂ and MgCl₂ were mixed as a stock solution at 877 mM and 303 mM
139 respectively. FeSO₄ was stocked at 4 mM. D-glucose was stocked at 1 M. Sodium L-lactate
140 was stocked at 10% w/w. CaCl₂/MgCl₂, D-glucose and Sodium L-lactate stock solutions were
141 filter sterilised through a 0.22- μ m-pore-size filter. Since the FeSO₄ stock solution could
142 neither be autoclaved (precipitation) nor filter sterilised (filter blockage), the FeSO₄ powder
143 was diluted into an autoclave sterilised bottle using autoclave sterilised Milli-Q water. Fresh
144 FeSO₄ stock solution was systematically prepared to prevent precipitation during the
145 storage. The full protocol for the preparation of the stock solutions for SCFM and SCFM-PC
146 medium is given in the Supplementary Information S1.

147 For 1 L of SCFM, the following volumes of stock solutions were added: 100 mL mixed
148 salts, 25 mL MOPS, 805 mL Milli-Q water, 10 mL mixed amino acids (first solution), 10 mL
149 isoleucine and phenylalanine (second solution), 16 mL leucine, 8 mL aspartate, 8 mL
150 tyrosine, 0.1 mL tryptophan. The solution was then autoclave sterilised. After cooling to
151 room temperature, the pH was adjusted to 6.5 using HCl 33% w/w. Finally, the following
152 volumes of stock solutions were added: 2 mL CaCl₂/MgCl₂, 1 mL FeSO₄, 3 mL D-glucose,
153 10.5 mL L-lactate.

154 The SCFM-PC medium preparation was based on the SCFM 2 developed by Turner *et*
155 *al.* [16]. SCFM 2 is a modification of SCFM in which DOPC (PC 18:1 (9Z) / 18:1 (9Z)), DNA and
156 mucin are added. However, in our study, we had to develop SCFM-PC in which only DOPC
157 was added to SCFM. Indeed, when DNA and mucin are used, the extraction of membrane
158 lipids cannot be performed due to the high viscosity of the medium (unpublished work). The
159 SCFM-PC medium was prepared as the following: aliquots of DOPC stock solutions were
160 prepared in order to make different volumes of SCFM-PC with a final DOPC concentration of
161 100 μ g/mL, (aliquots of 10 mg, or 33 mg, to respectively prepare 100 mL, or 330 mL, of
162 SCFM-PC). The stock solutions of DOPC (250 mg/mL) were prepared in chloroform and were
163 stored under argon, in the dark and at -20 °C. The DOPC stock solution was slowly added to
164 the fresh SCFM medium by making it run along the inside of the Erlenmeyer flask—in order
165 to evaporate the chloroform as much as possible. Then, the solution was stirred until neither
166 traces nor lipid droplets could be seen anymore. Finally, in order to make sure that no traces

167 of chloroform were left, the SCFM-PC medium was left to rest 30 min under a biosafety
168 cabinet.

169 The full protocol for the preparation of SCFM and SCFM-PC medium are given in the
170 Supplementary Information S1. To verify that the autoclave sterilisation had not degraded
171 the amino acids (at the concentration of the medium and without adding sugars), SCFM
172 medium was checked using electrospray ionisation-mass spectrometry (ESI-MS) before and
173 after sterilisation (for MS parameters, see Section 2.6). No difference was observed
174 suggesting that no degradation occurred. Growth curves of PAK into the autoclave sterilised
175 or filtered sterilised media were performed as well and showed no impact of the sterilisation
176 method on the growth of *P. aeruginosa*. MS spectra and growth curves are given in S2.

177

178 2.3. Bacterial strain, growth conditions and MICs determination

179 The *P. aeruginosa* strain PAK (PAK), used in this study, was obtained from Dr.
180 Lhousseine Touqui (Institut Pasteur, Paris, France). The *P. aeruginosa* PAK strain is known for
181 its high virulence, a high expression of pili and flagella, glycosylations and pathogenicity
182 islands [19] and is commonly used in medical studies. PAK was stocked in Muller-Hinton
183 broth (MHB)/glycerol (70/30) at -80°C . For growth kinetics, PAK was pre-cultured from this
184 stock solution, overnight at 37°C in MHB, SCFM or SCFM-PC. This pre-culture was used to
185 inoculate 100 mL of MHB, SCFM or SCFM-PC (in a 250 mL Erlenmeyer flask) with $5 \cdot 10^5$ colony
186 forming units (CFU)/mL. The culture was incubated at 37°C for 24 h on a rotary shaker (140
187 rpm). Every hour for 8 h and at a final point of 24 h, OD_{600} (optical density at 600 nm with
188 Cary 100 Bio spectrophotometer, Varian) was monitored and 100 μL of culture were
189 collected for CFU numeration. For the MICs determination of carbenicillin, gentamicin,
190 ciprofloxacin, and colistin, the broth dilution method, in 96-well plates, was performed
191 according to Wiegand *et al.* [20], using an inoculum at $\text{OD}_{600} = 0.001$. All these experiments
192 were performed in biological triplicates for MHB, SCFM and SCFM-PC.

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197 *2.4. Atomic force microscopy (AFM) imaging*

198 All glass substrates (glass cover for haemocytometer, 20x26mm, 0.4 mm thick,
199 Hirschmann) were cut in 4 parts, cleaned with ethanol and dried. Using a 6-well plates, one
200 glass substrate was placed into each well and covered with 1 mL of MHB, SCFM or SCFM-PC.
201 A PAK preculture in either MHB, SCFM or SCFM-PC media (see Section 2.3) was used to
202 inoculate each well at 10^7 CFU/mL. In order to have enough attached bacteria, but with as
203 low as possible phenotype changes, the plate was incubated for 6 h at 37 °C, without any
204 mixing, and protected from light. A bacterial monolayer and irreversible attachment of the
205 biofilm could be achieved using this method. Then, the culture medium was removed using a
206 Pasteur pipette. Each glass substrate was rinsed twice using water baths, carefully drained,
207 then dried overnight.

208 AFM imaging was performed with a Nanoscope 8 Multimode microscope (Bruker
209 Nano Surfaces, Santa Barbara, CA, USA) equipped with a 100 μ m piezoelectric scanner.
210 Imaging was achieved in the air either using the contact mode (for bacteria length
211 measurements) or using the PeakForce® mode (for image presentation). In the contact
212 mode, the cantilevers used were characterized by a low spring constant of about 0.06 N/m
213 and were equipped with classical pyramidal Silicon Nitride tip. All the measurements were
214 performed with the feedback loop on. For the PeakForce® mode imaging, we used a soft
215 Silicon Nitride cantilevers (spring constant of about 0.35 N/m) with sharp Silicon Nitride tip.
216 Images were obtained with a PeakForce Tapping frequency of 2kHz with the auto-amplitude
217 on. Average force applied during scanning was 5 nN.

218

219 *2.5. Membrane lipid extraction*

220 The membrane lipid extraction of PAK was performed as described by Deschamps *et*
221 *al.* [12]. Briefly, from a pre-culture overnight (see Section 2.3), PAK was grown for 7 h in

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222 MHB, SCFM or SCFM-PC (5.10^5 CFU/mL for initial inoculum) and a pellet was collected by
223 centrifugation.

224 Then, alginate lysis was performed using alginate lyase in PBS (0.15 mg/mL), followed by
225 sonication, centrifugation and ultracentrifugation. Finally, lipids were extracted using a
226 modified version of the Bligh and Dyer protocol [21]. In the present work, the liquid-liquid
227 extraction was modified with the addition of internal standards as followed:

228 For a pellet of 20 mg, 500 μ L of milli-Q water was added to resuspend the pellet and
229 then vortex-mixed for 5 min. 1.2 mL of methanol was added and then vortex-mixed for 15
230 min, to degrade proteolipids. Then, the mixture was centrifuged ($4000 \times g$, 10 min, 22 °C) and
231 the liquid phase recovered. After adding 0.6 mL of chloroform spiked with phospholipid
232 standards (PC 17:0/17:0, 0.5 μ g; PE 17:0/17:0, 2.5 μ g; PG 17:0/17:0, 1 μ g; CL
233 14:0/14:0/14:0/14:0, 5 μ g), the solution was vortex mixed for 15 min and sonicated for 15
234 min. 0.6 mL of chloroform was added, then the solution was vortex mixed for another 15
235 min. 0.62 mL of 1 M ammonium acetate was added and the solution was vortex-mixed for
236 15 min. Finally, the mixture was left to rest for 30 min. One milliliter of the organic phase
237 was recovered and 0.94 mL of chloroform was added to the aqueous phase for a second
238 liquid-liquid extraction. After vortex mixing for 15 min, the mixture was left to rest again for
239 30 min. The aqueous phase was removed and organic phases were pooled together and
240 evaporated under argon. Lipid extracts were conserved under argon at -20 °C. For other
241 samples pellet masses, solvent volumes and standard masses were adjusted proportionally.
242 This extraction was performed in biological triplicate.

243

244 *2.6. Liquid chromatography - mass spectrometry (LC-MS) analysis*

245 The sample preparation for LC-MS analyses, the MS and LC parameters—including LC
246 gradient— were as described by Deschamps *et al.* [12]. Briefly, the dried lipids extracts were
247 re-constituted into 50 μ L of chloroform/methanol (2/1, v/v) and 1/5 diluted into
248 isopropanol/ acetonitrile/water (4/3/1, v/v).

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251 The LC-MS experiments were performed using an UHPLC system (Dionex Ultimate 3000
252 RSLC, Thermo Scientific, San Jose, CA, USA) coupled to a hybrid quadrupole time-of-flight
253 with an ion mobility cell instrument (Q-IMS-TOF, SYNAPT G2 HDMS, Waters MS
254 Technologies, Manchester, UK) equipped with an electrospray ionisation source. LC-MS data
255 were acquired in triplicate in positive and negative ion modes.

256 The column used was a 1.0 × 100 mm, Acquity UPLC CSH C18 1.7 µm column (Waters,
257 Manchester, UK) equipped with a 0.2 µm prefilter. Mobile phase A consisted of
258 acetonitrile/aqueous 10 mM ammonium formate (60/40) + 0.1% formic acid and mobile
259 phase B consisted of isopropanol/acetonitrile/aqueous 10 mM ammonium formate (88/8/4)
260 + 0.1% formic acid. Data acquisition was performed using MassLynx 4.1 (Waters,
261 Manchester, UK).

262 Data processing, automatic identification and integration of peaks were performed
263 using UNIFI 1.8.2.169 (Waters, Manchester, UK). Relative areas were calculated by dividing
264 the area of the identified phospholipids by the area of the standard from the corresponding
265 class of phospholipids (*e.g.* $relative\ area = \frac{Area\ of\ PE\ 34:1}{Area\ of\ PE\ 17:0/17:0}$). Areas were automatically

266 calculated, from the extracted ion chromatograms, using the monoisotopic peak of $[M+H]^+$
267 or $[M-H]^-$ ions, for positive or negative ionisation respectively. The monoisotopic peak was
268 used, instead of the sum of all isotopes, since not all isotopes could be observed for
269 phospholipids at small concentrations (*e.g.* CL 67:3). We also selected only protonated or
270 deprotonated species, instead of the sum of all detected adducts (*i.e.* $[M+Na]^+$, $[M+NH_4]^+$,
271 $[M+CH_3COO]^-$) for similar reasons. The standard deviation and relative standard deviation of
272 the relative area (the number of replicates (n) in which the phospholipids were identified
273 were; $7 \leq n \leq 9$ for PE, PC and PG, $3 \leq n \leq 6$ for CL) were calculated to plot error bars and to check
274 the repeatability of the measurements. Anova tests and Turkey mean comparisons were
275 performed using OriginPro 2016 (64-bit) b9.3.226.

276 MS/MS experiments were performed in biological triplicate, using the parameters
277 previously described [12].

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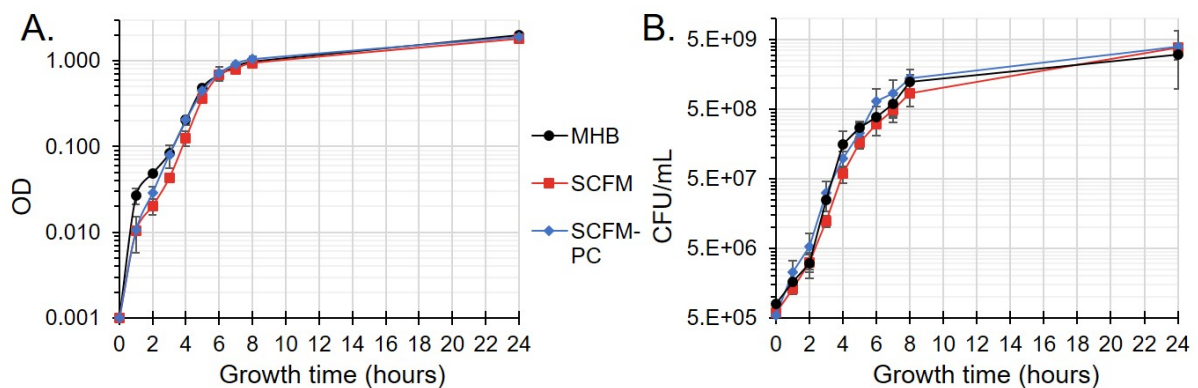
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279 MS/MS experiments were performed in negative ion mode using a collision energy ramp
 280 from 25 eV to 45 eV. For PE (*i.e.* PE 32:1, PE 34:1, PE 36:2) and PG species (*i.e.* PG 32:1, PG
 281 34:1, PG 36:2), the ion selected in the quadrupole was the $[M-H]^-$, whereas for PC species
 282 (*i.e.* PC 32:1, PC 34:1, PC 36:2), the ion selected was the $[M+HCO_2]^-$. MS/MS spectra were
 283 processed using MassLynx 4.1 (Waters).

285 3. Results

286 3.1. Impact of the medium composition on the growth and MIC determination

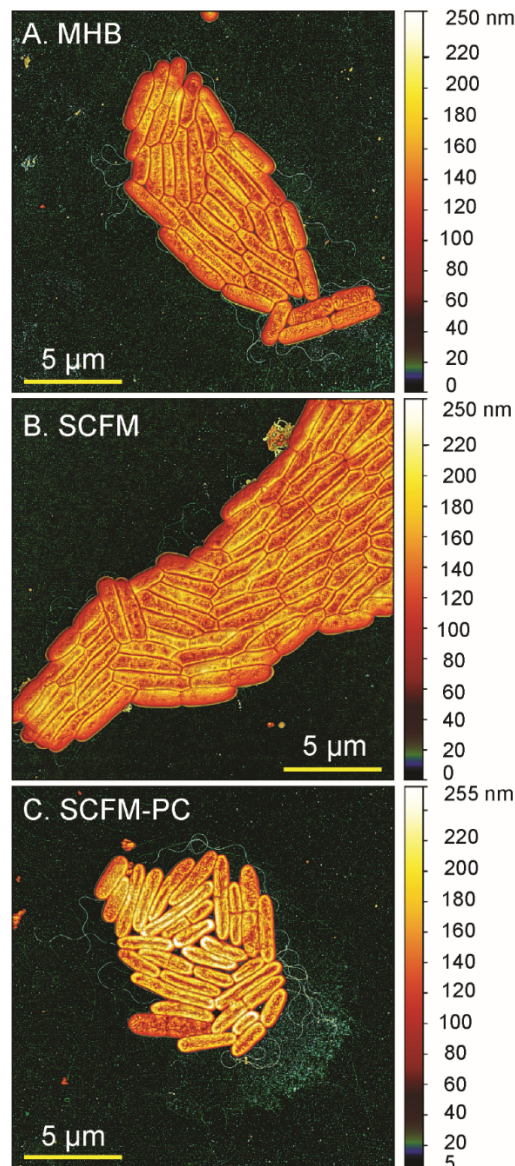
287 The growth curves of PAK grown in MHB, SCFM or SCFM-PC are given in Fig. 1.
 288 Measurements of OD₆₀₀ (Fig. 1.A) or CFU/ mL (Fig. 1.B) as a function of time showed a similar
 289 growth of PAK in the three media. This observation included no lag phase time, an
 290 exponential phase time of approximately 8 h and a doubling time of 43, 42 or 44 minutes in
 291 MHB, SCFM or SCFM-PC, respectively. As a matter of fact, using these growth curves, the
 292 growth time for the lipid extraction (7h) was chosen as it corresponds to the end of the
 293 exponential phase. Thus, exploitable quantities of bacteria could be collected without risking
 294 a lack of nutrients.



297 **Fig. 1. Growth curves of PAK strain in MHB, SCFM and SCFM-PC.** black circles for MHB, red squares for SCFM, blue
 298 diamonds for SCFM-PC. **A.** optical density (OD) plotted against time. **B.** Colony Forming Unit (CFU)/ mL plotted against time.
 299 Error bars: standard deviation.

300

301 AFM images (Fig. 2. A, B, C) show similar bacterial shapes for all media. For MHB and
302 SCFM, the average lengths ($2.8 \mu\text{m} \pm 0.7 \mu\text{m}$) of PAK were equivalent. However, when PAK
303 was grown in SCFM-PC, the average length was a little smaller ($2.6 \mu\text{m} \pm 0.6 \mu\text{m}$), but as a
304 counterpart, the height was higher (Fig.2.C), showing that PAK seemed to spread less on the
305 surface than in the other media. Interestingly, as contrary to the other two media, colonies
306 of PAK grown in SCFM-PC showed bacteria were not glued to each other, probably due to
307 the presence of DOPC in the growth medium.



308

309 **Fig. 1. AFM images of PAK young biofilms grown on glass substrates.** Images are shown as topographic images overlaid
 310 with a local contrast image (Kernel size: 2; blending depth: 2; weight: 1). Classical filters as flattening and 3-points levelling
 311 were applied to the topographic image using the Gwyddion software (<http://www.gwyddion.net>). **A.** PAK grown in MHB; **B.**
 312 PAK grown in SCFM; **C.** PAK grown in SCFM-PC.

313 The MIC values of four antibiotics (carbenicillin, gentamicin, ciprofloxacin, colistin) for
 314 PAK grown in MHB, SCFM or SCFM-PC are given in Table 1. Susceptibility (S) or resistance (R)
 315 to the antibiotic were given according to the norm Eucast SFM 2019. In fact, a two-fold MIC
 316 difference cannot be considered significant, since the experiment error is of one dilution (for
 317 the protocol, see 2.4). Consequently, the influence of the medium on the MIC values could
 318 significantly be noted for carbenicillin, gentamicin and colistin, but not for ciprofloxacin. The
 319 MIC of gentamicin and colistin were higher when grown in SCFM or SCFM-PC rather than
 320 into MHB. Moreover, a switch from susceptibility to resistance could be observed for colistin
 321 and gentamicin, depending on the medium of growth. Surprisingly, the MIC of carbenicillin
 322 was much smaller when PAK was grown in SCFM rather than in MHB, but was identical
 323 between MHB and SCFM-PC media.

324

325 **Table 1. Minimal inhibitory concentrations (MIC) for PAK strain.** (S) for susceptibility of PAK to the antibiotic,
 326 (R) for resistance. Both (S) and (R) are given according to the norm Eucast SFM 2019 —apart for carbenicillin for
 327 which reference values was not given in the norm.

medium/ antibiotic	carbenicillin	gentamicin	ciprofloxacin	colistin
	(MIC in µg/mL)			
MHB	64-128	0.5 (S)	0.125 (S)	0.125-0.25 (S)
SCFM	16	4-8 (R)	0.125 (S)	2-4 (R)
SCFM-PC	64-128	4-8 (R)	0.25 (S)	2 (R)

328

329 **3.2 Comparison of the membrane phospholipids composition**

330 *3.2.1 Semi-quantification*

331 The membrane lipids of PAK were identified using a home-made retention time-*m/z*
 332 library, which was previously described for PAK grown in MHB [12]. Lipid annotation was

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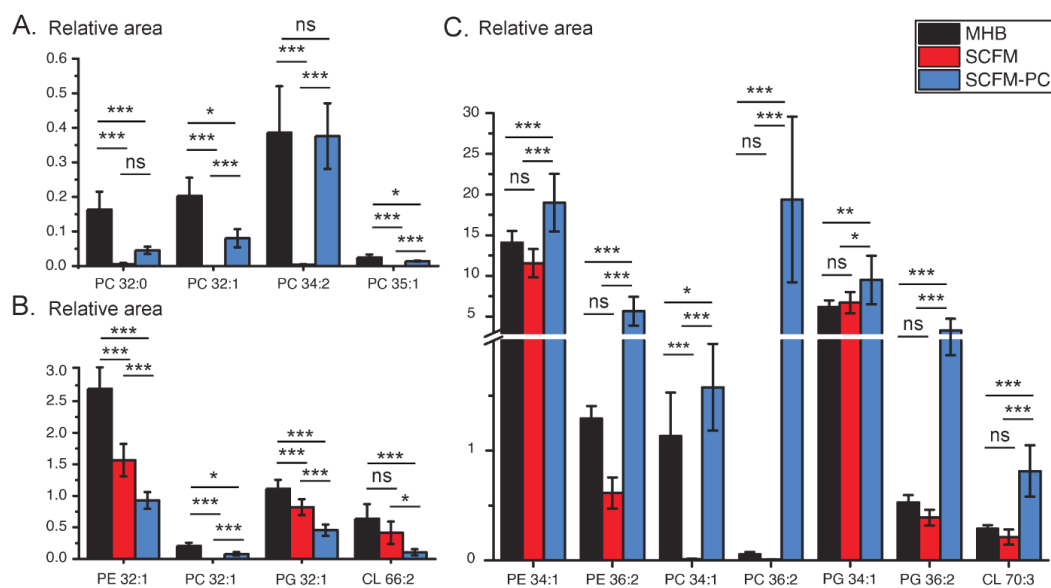
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333 based on the nomenclature of Liebisch *et al.* [22]. Forty-two lipids were identified. Twenty
 334 phospholipids were identified using positive ionisation: 7 phosphatidylcholines (PC) and 13
 335 cardiolipins (CL).

336 Twenty-two were identified using negative ion mode: 13 phosphatidylethanolamines (PE)
 337 and 9 phosphatidylglycerols (PG). Please note that PE and CL could be observed in both
 338 positive and negative ion modes. However, using our analytical conditions (especially the LC
 339 mobile phase with buffers), a higher signal was observed in positive ionisation mode for CL
 340 and in negative ionisation mode for PE.

341 When PAK grown in SCFM or SCFM-PC, no new phospholipids were detected.
 342 Therefore, a semi-quantitative approach was used in order to highlight the influence of the
 343 medium. Please note that, while PC 34:0, PE 34:0 and PG 34:0 were detected as traces in the
 344 membrane of PAK, the relative area of these phospholipids could not be determined.
 345 Actually, using our chromatographic conditions, these phospholipids could not be separated
 346 from the standards (*i.e.* PC 17:0/17:0, PE 17:0/17:0, PG 17:0/17:0), therefore, the PC 34:0, PE
 347 34:0 and PG 34:0 produced by PAK were excluded from the study.

348 Fig. 3.A., 3.B. and 3.C. show relative areas of some depictive examples of the
 349 membrane phospholipids extracted from PAK grown in MHB, SCFM or SCFM-PC media. The
 350 relative areas for all the identified phospholipids are given in S2 (Fig S2.4 to S2.7).



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352 **Fig. 2. Relative areas of phospholipids extracted from PAK.** $Relative\ area = \frac{PAK\ PL\ area}{PL\ standard\ area}$. Black bars: PAK
 353 grown in MHB, red bars: PAK grown in SCFM, blue bars: PAK grown in SCFM-PC. Error bars: standard deviation ($7 \leq n \leq 9$ for
 354 PE, PC and PG, $3 \leq n \leq 6$ for CL). P-values are reported —between MHB and SCFM, SCFM and SCFM-PC and MHB and SCFM-PC
 355 — as: * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, ns for non-significant. **A.** PC less or equally produced in SCFM-PC than
 356 MHB and mostly not produced in SCFM; **B.** phospholipids more produced in MHB than SCFM or SCFM-PC; **C.** phospholipids
 357 more produced in SCFM-PC than SCFM or MHB.

358 Depending on the medium of growth, three trends of relative areas could be observed. As
 359 shown in Fig. 3.A., apart from PC 34:1 and PC 36:2 (Fig. 3.C.), PC were significantly less
 360 produced for PAK grown in SCFM rather than MHB or SCFM-PC. As shown in Fig. 3.B. and S2
 361 (Fig S2.4 to S2.7), most phospholipids (23) were more produced in MHB than SCFM or SCFM-
 362 PC. As shown in Fig. 3.C., phospholipids containing oleic acid (C18:1) were greatly over-
 363 produced in SCFM-PC, especially for PE 34:1, PC 34:1, PG 34:1 and PE 36:2, PC 36:2, PG 36:2.
 364 Caution must be taken regarding the high relative area of PC 36:2 extracted from PAK grown
 365 in SCFM-PC. Indeed, this may be due to i) direct incorporation of DOPC inside the bacteria
 366 membrane —without any modifications, ii) contamination of our samples with the culture
 367 medium DOPC or iii) both of i) and ii). However, it is clear from the over-production of PE
 368 36:2, PG 36:2 and phospholipids containing oleic acid, that DOPC is at least recycled by the
 369 bacteria to produce phospholipids. Then, the presence of DOPC directly incorporated into
 370 the bacteria membrane would be a very likely hypothesis.

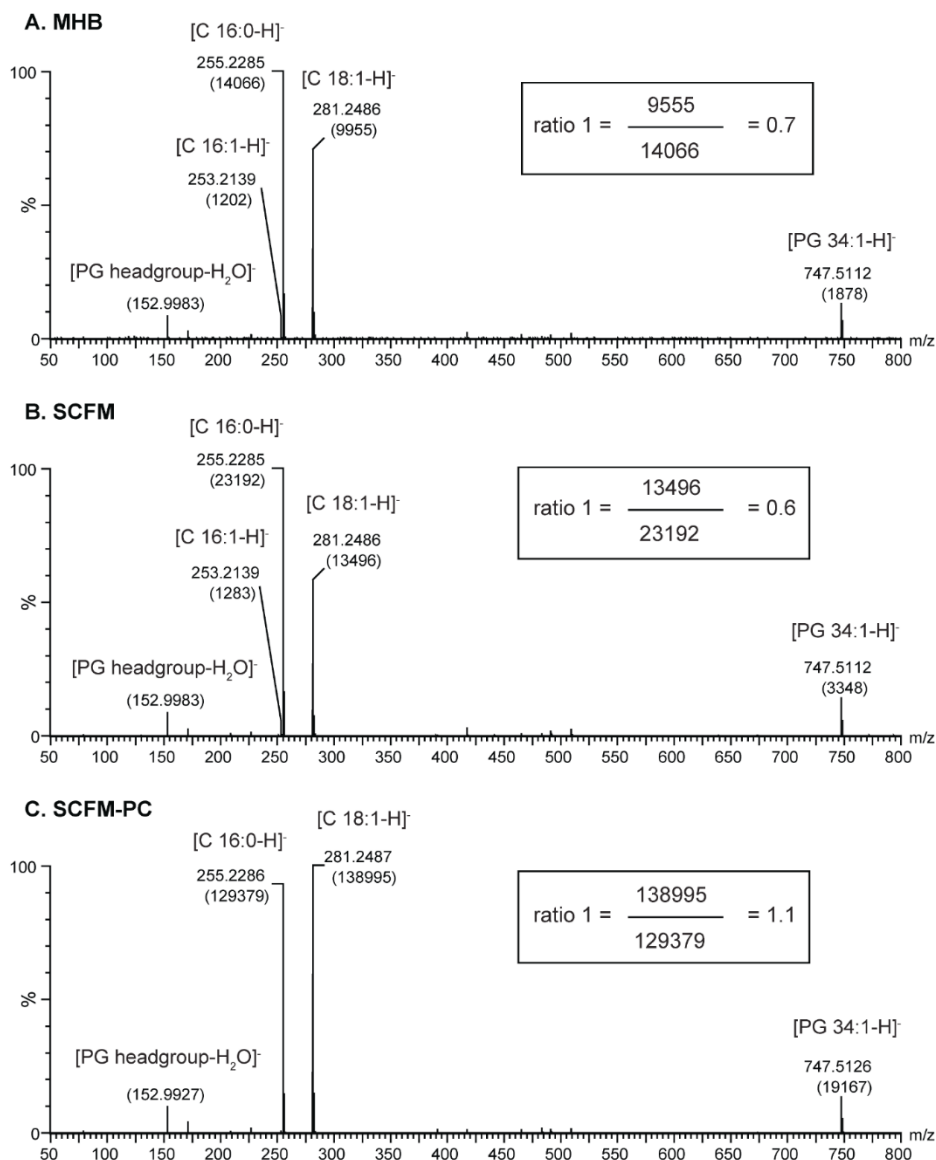
371

372 3.2.2 Fatty acid composition

373 In order to evaluate the influence of the growth medium on the fatty acid
 374 composition, MS/MS experiments were performed, on the most intense phospholipids, in
 375 biological triplicates and for each medium. Fragment identification was performed using
 376 phospholipid fragments described in the literature [23,24]. S3 gives all the fatty acid
 377 fragments of interest in negative ion mode —carboxylate anions R_1COO^- — for PC/PE/P
 378 32:1, 34:1 and 36:2. Please note that for SCFM medium, PC species could not be detected in
 379 negative ion mode, since they were only produced as traces in this medium (see 3.2.1) and
 380 PC are known to not ionize well in negative ion mode, even with the $[M+HCO_2]^-$ adduct.

381 As previously described by *Hsu et al.* [23], the loss of the fatty acid in the sn-2
382 position is favoured. Hence, in order to compare the proportion of the two sn-1/2 position
383 isomers (*e.g.* PG 16:0/16:1 and PG 16:1/16:0) between the three media, the following ratio
384 was calculated $ratio\ 1 = \frac{Intensity\ fatty\ acid\ sn-1}{Intensity\ fatty\ acid\ sn-2}$. When several isomers of fatty acyl chain
385 length (*e.g.* PE 16:0/16:1 and PE 14:0/18:1) were observed, the following ratio was
386 calculated:

387 $ratio\ 2 = \frac{Intensity\ fatty\ acid\ sn-2\ of\ isomer\ 2}{Intensity\ fatty\ acid\ sn-2\ of\ isomer\ 1}$. The ratio 2 was calculated using only the sn-2
388 fatty acid since the sn-1 fatty acid of the second isomer (less abundant) was not detected for
389 all phospholipids. Fig 4. shows the MS/MS spectra obtained for PG 34:1 in the three media
390 (A. MHB, B. SCFM, C. SCFM-PC) and illustrates the determination of the ratio 1. The sn-2
391 fatty acid of isomer 2 (C16:1) was also given to illustrate the abundance difference between
392 isomer 1 and isomer 2. The sn-1 fatty acid of isomer 2 (C18:0) was not detected. All
393 calculated ratios means, standard deviation and relative standard deviation, are given in S3.



394

395 **Fig. 4 MSMS Spectra of [PG 34:1-H]⁻ and determination of the ratio 1.** Ratio 1 allows to compare the proportion of the two
 396 sn-1/2 position isomers $\left(ratio\ 1 = \frac{Intensity\ fatty\ acid\ sn-1}{Intensity\ fatty\ acid\ sn-2} \right)$ between the three media. Bracket values: ion
 397 intensities. **A.** PAK grown in MHB; **B.** PAK grown in SCFM; **C.** PAK grown in SCFM-PC.

398 Table 2 shows the major isomers identified for PC 32:1, PE 32:1, PG 32:1, PC 34:1, PE
 399 34:1 and PG 34:1. The major isomer was identical in MHB and SCFM media. However, when
 400 compared to MHB and SCFM, the SCFM-PC medium shows different isomers for two
 401 phospholipids: PC 32:1 and PG 34:1. In SCFM-PC, the major isomer was PC 14:0/18:1 for PC
 402 32:1 (PC 16:0/16:1 in MHB), and PG 16:0/18:1 for PG 34:1 (PG 18:1/16:0 in MHB or SCFM).
 403 Regarding sn-1/2 position isomers, for PG 32:1, PC 34:1/ PE 34:1/ PG 34:1, the respective

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404 16:1/16:0 and 16:0/18:1 isomers were significantly more produced in SCFM-PC than in SCFM
405 or in MHB (ratio values in S3).

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407 **Table 2. Fatty acid composition of the major isomer for six phospholipids, depending on the medium of**
408 **growth.**

	PC 32:1	PE 32:1	PG 32:1	PC 34:1	PE 34:1	PG 34:1
MHB	16:0/16:1	16:0/16:1	16:0/16:1	18:1/16:0	18:1/16:0	18:1/16:0
SCFM	nd	16:0/16:1	16:0/16:1	nd	18:1/16:0	18:1/16:0
SCFM-PC	14:0/18:1	16:0/16:1	16:0/16:1*	18:1/16:0*	18:1/16:0*	16:0/18:1

nd: not detected; * for PG 32:1, PC 34:1 and PE 34:1, the respective 16:1/16:0, 16:0/18:1 and 16:0/18:1 isomers were significantly more produced in SCFM-PC than in SCFM or in MHB.

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410 Regarding PC/PE/PG 36:2 (see S3), the major isomers were PC/PE/PG 18:1/18:1 for all
411 media. However, whereas no other isomer could be observed for PC 36:2 in any media,
412 other isomers were observed for PE 36:2 and PG 36:2. For PE 36:2, PE 19:1/17:1 was
413 detected, in trace level, in MHB (ratio 2 = 0.07 ± 0.02), SCFM (ratio 2 = 0.05 ± 0.02) and
414 SCFM-PC (ratio 2 = 0.01 ± 0.01). For PG 36:2, PG 20:1/16:1 was observed in MHB (ratio 2 =
415 0.55 ± 0.09), SCFM (ratio 2 = 0.52 ± 0.19) and in trace level in SCFM-PC (ratio 2 = 0.04 ± 0.01).

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419 **4. Discussion**

420 To our knowledge, this is the first study to analyse the membrane lipid composition
421 of *P. aeruginosa* in CF sputum-like media (*e.g.* SCFM and SCFM-PC) and compare it with a
422 classical medium. The three following media were used: the commercial MHB —as a

423 reference medium, SCFM [15] and a modified version of SCFM by the addition of a
424 phosphatidylcholine (SCFM-PC).

425 When studying the influence of the culture medium on the production of molecules
426 by bacteria, checking that the bacterial growth is not affected by the culture medium is
427 crucial. Here, growth curves (see 3.1) showed that the growth of PAK was identical when
428 grown in MHB, SCFM or SCFM-PC. As detailed in the protocol of the membrane lipid
429 extraction (see 2.5), the culture was stopped at the same time for every medium (end of the
430 exponential phase, see 3.1). Therefore, the following discussion could be made assuming
431 that the membrane lipid composition modifications were not linked to a difference in the
432 bacterium growth phase. This should be noted as a recent work by Le Sénéchal *et al.* [13]
433 showed that the phospholipid content of *P. aeruginosa* PAO1 was closely related to the
434 bacterial growth.

435 Before going further into the discussion, one should note that a plausible criticism of our
436 methodology is that *P. aeruginosa* was grown in planktonic conditions instead of biofilm
437 conditions, which should better reproduce the CF sputum growth conditions. However, since
438 laboratory biofilm are grown on abiotic sterile surfaces, the adherence of the biofilm is likely
439 to differ from the one in CF sputum, leading to possible lipid modifications. Moreover, many
440 studies on clinical *P. aeruginosa* were performed in shaken flasks since even the sampling of
441 the bacterium would be enough to alter its metabolic phenotype [25]. Despite these
442 limitations, we believe that SCFM-PC is a good compromise medium for rigorous analysis of
443 the membrane lipids of *P. aeruginosa*.

444 The main focus of this study was to investigate the influence of the medium on the
445 phospholipids composition of the membrane. Using LC-MS in qualitative manner, as a first
446 approach, we could not observe any influence of the medium on the type of identified
447 phospholipids.

448 Therefore, a semi-quantification approach (comparison of relative intensities, using internal
449 standards) was used (see 3.2.1). The most remarkable result was that most phospholipids
450 were less produced into SCFM, compared to MHB. Knowing that lipids could be found in CF-
451 sputum [26], SCFM-PC was made, by adding DOPC to SCFM, in order to avoid the lack of PC

452 species. When PAK was grown in SCFM-PC, we could see the increase of the phospholipids
453 with the longest fatty acyl chains (see Fig. 3.C). This increase might counterbalance the
454 shortage of other lipids. However, for SCFM, we haven't identified any new phospholipids
455 nor increase of other phospholipids. Ornithine-lipids, which were described to be produced
456 by *P. aeruginosa* when grown in phosphate limiting conditions [27], were not detected. Two
457 hypothesis can be assumed regarding the decrease of phospholipids of PAK grown in SCFM:
458 i) novel phospholipids species were produced, but were neither extracted using our
459 extraction conditions (see 2.6), nor detected using our analytical conditions (see 2.7), ii) the
460 lipid-protein ratio, which is supposed to be around 1/1 (w/w) for bacteria [28], was modified.
461 The hypothesis that PAK grown in SCFM could be smaller than grown in MHB —leading to an
462 overall lighter weight of phospholipids— was also considered. However, as shown by the
463 AFM images in Fig. 2. (see 3.1), no significant influence of the medium on size nor on the
464 shape of PAK was observed, consequently this hypothesis was rejected.

465 MS/MS experiments were performed, as well, in order to evaluate the influence of
466 the growth medium on the fatty acid composition of phospholipids (see 3.2.2). The most
467 remarkable observation was that the fatty acid C18:1 was strikingly more abundant in
468 phospholipids produced by PAK grown in SCFM-PC than SCFM or MHB. This resulted into an
469 inversion of the most abundant isomer for PC 32:1 and PG 34:1 (from MHB or SCFM to
470 SCFM-PC). Indeed, for PC 32:1, both PC 14:0/18:1 and PC 16:0/16:1 were detected into MHB
471 and SCFM-PC, but for SCFM-PC the major isomer was PC 14:0/18:1, whereas for MHB, the
472 major isomer was PC 16:0/16:1. Also, for PG 34:1, the major isomer was PG 16:0/18:1 in the
473 case of SCFM-PC, whereas for MHB or SCFM, the major isomer was PG 18:1/16:0. Moreover,
474 isomers containing C 18:1 in sn-2 position were favoured when PAK was grown in SCFM-PC.
475 Regarding PG 32:1, PC 34:1 and PE 34:1, the respective 16:1/16:0, 16:0/18:1 and 16:0/18:1
476 isomers were significantly more produced in SCFM-PC than in SCFM or in MHB.

477 The most likely explanation for these observations would be that *P. aeruginosa* would take
478 DOPC from the SCFM-PC, and modify it, in order to produce its own phospholipids, as
479 previously described for polyunsaturated fatty acids [29]. As a matter of fact, this hypothesis
480 can also be supported by the overproduction of PE 36:2 and PG 36:2 (see Fig. 3.C. in part

481 3.2.1) in SCFM-PC. Indeed, in most bacteria, PC are synthesised from PE, while PG are
482 synthesised using another pathway [30]. However, in the case of *P. aeruginosa*, it was
483 demonstrated that PC are synthesised from a phosphatidic acid (PA) and a choline [31].
484 Therefore, PC, PE and PG are synthesised from three different pathways which uses PA as a
485 common precursor. Thus, PA 36:2, recycled from DOPC, is likely used to synthesise PE 36:2
486 and PG 36:2. Consequently, these modifications would include: i) change of the polar head
487 group and ii) replacement of the sn-1 fatty acid. Since adding a phospholipid to the growth
488 medium had an influence on the membrane phospholipids of *P. aeruginosa*, we should note
489 that caution must be taken when choosing this phospholipid, in regards to the aim of the
490 study.

491 Finally, as described in 3.4.1, the other less abundant isomer of PE 36:2 (*i.e.* PE 19:1/17:1)
492 and PG 36:2 (*i.e.* PG 20:1/16:1) were produced for MHB and SCFM, but only in traces for
493 SCFM-PC. Taking into account these observations and the inversion of the major isomer
494 previously described, one should note that caution must be taken when analysing the fatty
495 acid composition of phospholipids. Our results show that for an identical fatty acyl chain
496 length, the fatty acid composition might not be the same for different polar heads (*e.g.* PC
497 32:1, PE 32:1 and PG 32:1).

498 In a second approach, the MIC values of four antibiotics were determined (see 3.1)
499 with the aim to link membrane lipids modifications and antibiotic resistance. Indeed, the
500 antibiotic susceptibility of *P. aeruginosa* is related to its ability to guarantee membrane
501 homeostasis —maintenance of the membrane physical properties [32]. This homeostasis can
502 happen by modifying phospholipids in different ways. The stability of the membrane and,
503 accordingly, the decrease of the permeability, can be enhanced by increasing the fatty acyl
504 chain length [33]. Conversely, the fluidity and permeability can be increased by introducing a
505 double bond or a cyclopropyl (more stable to acidic conditions than a double bond), or a
506 terminally branched methyl on the fatty acyl chain [34].

507 The ratio of negatively charged phospholipids (CL and PG) and neutral (PE) or positively
508 charged phospholipids (PC) can also be modified in order to make the membrane less
509 sensitive to pH or to the medium ion composition [35]. The replacement of PE by PC was

510 also described to enhance the membrane stability due to the ability of PC to form bilayers
511 more easily than PE [33].

512 As described in 3.4.1., PAK was found to produce less PC species when grown in SCFM than
513 into MHB. MIC values of carbenicillin were also seemingly smaller when PAK was grown in
514 SCFM ($MIC_{SCFM} = 16 \mu\text{g/mL}$) than in MHB ($MIC_{MHB} = 64\text{-}128 \mu\text{g/mL}$). Carbenicillin mechanism
515 of action consists in inhibiting the synthesis of the bacterial cell-wall peptidoglycan. Also,
516 since carbenicillin is an hydrophobic antibiotic ($\log P = 1.13$ [36]), we may assume that this
517 antibiotic can cross the lipid membrane instead of going through the porins. Hence, we can
518 make the hypothesis that PAK, when grown in SCFM, was more susceptible to carbenicillin
519 because the membrane cell was less stable due to the lack of PC species. Then, when DOPC
520 was added to the SCFM medium, the presence of PC in the membrane restore a
521 susceptibility to carbenicillin similar to the one in MHB ($MIC_{MHB} = MIC_{SCFM-PC}$).

522 Regarding the other antibiotics tested, the influence of the growth medium on the
523 MIC values was observed for colistin and gentamicin, but not for ciprofloxacin (see 3.1). It is
524 notable that, MIC values of gentamicin, colistin and ciprofloxacin in SCFM were in agreement
525 with those from Pusic *et al.* [37] (SCFM medium). We also observed an increase of MIC values
526 from a rich medium (MHB) to the chemically defined SCFM (see Table 1). This observation
527 was also described by Pusic *et al.* (from LB to SCFM), and for colistin between MHB and ASM,
528 by Diaz Iglesias *et al.* [38]. In SCFM-PC, the MIC values of colistin and gentamicin were equal
529 to the ones in SCFM. Therefore, the link between the membrane stability (decrease of the
530 membrane stability in SCFM but restored in SCFM-PC) and the MIC, described for
531 carbenicillin, might not be suitable for other antibiotics. The membrane permeability
532 changes, induced by the growth medium, may partly explain *P. aeruginosa* antibiotic
533 susceptibility modifications. Actually, it is rather a multi-factor issue since the nutrients
534 present in the growth medium can induce particular resistance mechanisms of *P. aeruginosa*
535 [26].

536 |

537 |

538 5. Conclusion

539 In this study, we reported the membrane lipid composition of *P. aeruginosa* grown
540 into CF sputum-mimicking media (SCFM and SCFM-PC). We showed that the growth medium
541 had an influence on the phospholipid quantities. We observed that PC species were
542 produced in trace level in SCFM. Implementing DOPC to SCFM (SCFM-PC medium), as a
543 choline nutrient, not only had an influence on the quantities of PC species, but also on the
544 length of the fatty acids parts of the phospholipids and phospholipid isomerism. Such
545 modifications may affect the membrane permeability. Actually, MIC variations of the
546 hydrophobic carbenicillin antibiotic support this hypothesis. These results suggest that the
547 membrane lipid composition should be taken into account for antibiotic susceptibility
548 testing. Since we showed that the growth medium can influence the membrane lipid
549 composition, caution must be taken when choosing a medium, especially for clinical studies.

550

551 Conflict of interest

552 The authors declare that they have neither conflict of interest nor competing financial
553 interest.

554

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559

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- 660

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661 **Supplementary Information S1: SCFM and SCFM-PC preparation**

662 **Stock solutions preparation for the SCFM medium**

663 **Part I: amino acids**

664 Solutions can be kept in the dark at 4 °C for less than two months.

665 **Amino acids stock solution to dilute into 100 mL of Mili-Q water**

amino acid	m (g)
Alanine	1.60
Arginine – HCl	0.63
Cysteine – HCl	0.32
Glutamate – HCl	2.75
Glycine	0.90
Histidine – HCl	0.96
Lysine – HCl	3.84
Methionine	0.90
Ornithine – HCl	1.18
Proline	1.96
Serine	1.47
Threonine	1.19
Valine	1.29

666

667 Note: a few minutes of mixing at 37°C can help the dissolution of the amino acids.

668 **Isoleucine and phenylalanine stock solution to dilute into 100 mL of Mili-Q water**

amino acid	m (g)
Isoleucine	1.44
Phenylalanine	0.83

669

670 **Leucine stock solution to dilute into 200 mL of Mili-Q water**

amino acid	m (g)
Leucine	2.62

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673 Using Mili-Q water, prepare the following NaOH solutions for the last 3 amino acids
674 solutions

C (mol/L)	V (L)	m (g)
1	0.10	4.00
0.5	0.10	2.00
0.2	0.10	0.80

675

676 Aspartate stock solution to dilute into 50 mL of NaOH 0.5 M

amino acid	m (g)
Aspartate	0.69

677

678 Tyrosine stock solution to dilute into 50 mL of NaOH 1 M

679

amino acid	m (g)
Tyrosine	0.91

680

681 Tryptophan stock solution to dilute into 50 mL of NaOH 0.2 M

682

amino acid	m (g)
Tryptophan	1.33

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Part II: salts and others

687

Solutions can be kept in the dark at 4 °C for less than two months.

688 **Mixed salts stock solution to dilute into 500 mL of Mili-Q water**

689 This solution can be autoclave sterilised for storage purpose.

Salt	m (g)
NH ₄ Cl	0.61
KCl	5.70
NaCl	14.92
NaH ₂ PO ₄	0.78
Na ₂ HPO ₄	0.72
K ₂ SO ₄	0.24
NaNO ₃	0.15

690

691 **Morpholinepropanesulfonic acid (MOPS) to dilute into 250 mL of Mili-Q water**

692 This solution can be autoclave sterilised for storage purpose.

Buffer	m (g)
MOPS	5.23

693

694 **CaCl₂/ MgCl₂ stock solution to dilute into 25 mL of Mili-Q water**

695 **Filter sterilise through a 0.22-µm-pore-size filter.**

696

Salt	m (g)
CaCl ₂	2.43
MgCl ₂	0.72

697

698 **FeSO₄ stock solution to dilute into an autoclave sterilised bottle using 100 mL of autoclave sterilised Mili-Q water**

700 **Regularly check for precipitation; prepare fresh stock solution in case of precipitation.**

701

Salt	m (g)
FeSO ₄	0.10

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702 **D-glucose stock solution to dilute to 50 mL using Mili-Q water**

703 **Filter sterilise through a 0.22- μ m-pore-size filter.**

704

Sugar	m (g)
D-glucose	9.01

705

706 **60 % (w/w) Sodium L-Lactate to dilute to 10 % (w/w) using Mili-Q water**

707 **Filter sterilise through a 0.22- μ m-pore-size filter.**

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SCFM preparation

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This medium needs to be prepared in sterile conditions.

711

Stock solution	V for 1 L of SCFM	V for 330 mL of SCFM
Mixed salts	100 mL	33 mL
MOPS	25 mL	8.25 mL
Mili-Q water	805 mL	265 mL
Mixed amino acids	10 mL	3.3 mL
Isoleucine + Phenylalanine	10 mL	3.3 mL
Leucine	16 mL	5.28 mL
Aspartate	8 mL	2.64 mL
Tyrosine	8 mL	2.64 mL
Tryptophan	0.1 mL	33 µL
Autoclave sterilise		
Wait for the medium to cool to room temperature		
Adjust pH to 6.5 using HCl 33 % w/w (approx. 600 µL for 330 mL)		
CaCl ₂ / MgCl ₂	2 mL	0.66 mL
FeSO ₄	1 mL	0.33 mL
D-glucose	3 mL	0.99 mL
Sodium L-lactate	10.5 mL	3.5 mL

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Before SCFM-PC preparation

717

718 **!\1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) should be Avanti polar Lipids quality.**

719 Make DOPC aliquots of 10mg or 33mg (depends on the required volume of SCFM-PC

720 required; in SCFM-PC, DOPC is at a final concentration of 100µg/mL), in chloroform, at

721 approximately 250 mg/mL.

722 Stock these aliquots in chloroform, under argon, at -20°C, in the dark.

723

724

SCFM-PC preparation

725

726 In order to add the aliquot of DOPC to SCFM (SCFM not older than 3 weeks):

727 **!\ Use an erlenmeyer or a bottle at least 3 times bigger than the volume of required SCFM-**

728 **PC.**

729 Under a biosafety cabinet:

730 1 Rinse Paster pipette several times with chloroform,

731 2 Add DOPC slowly 20µL per 20µL, making it run along the inside of the Erlenmeyer flask —in

732 order to evaporate the chloroform,

733 **!\ Lipid traces and lipid droplets will be visible on the inside of the flask, and in the bottom**

734 **of the flask, respectively.**

735 3 Gently manually shake to "rinse" the lipids traces,

736 4 Shake until lipids droplets can't be seen anymore: the medium will appear cloudy.

737 If lipids drops are hard to dissolve, alternate shaking and resting (5 min) under the biosafety

738 cabinet: This allows to evaporate last traces of chloroform which are trapped in lipid vesicles.

739 Note: If the lipid droplets are fully dissolved, the medium can be stored a week, at 4°C, in the

740 dark!

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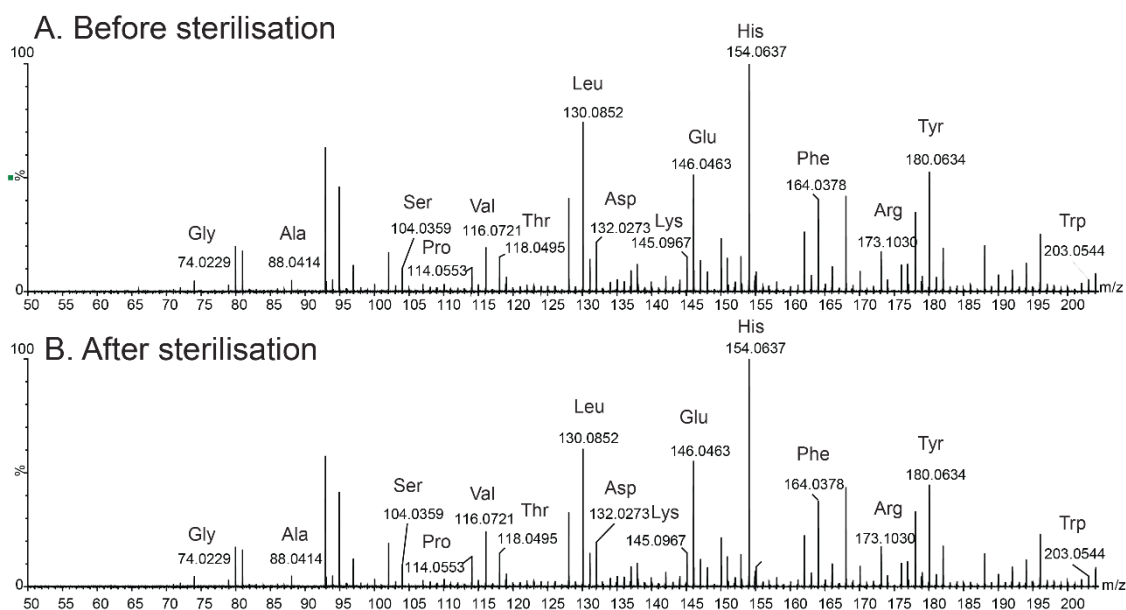
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Supplementary Information S2

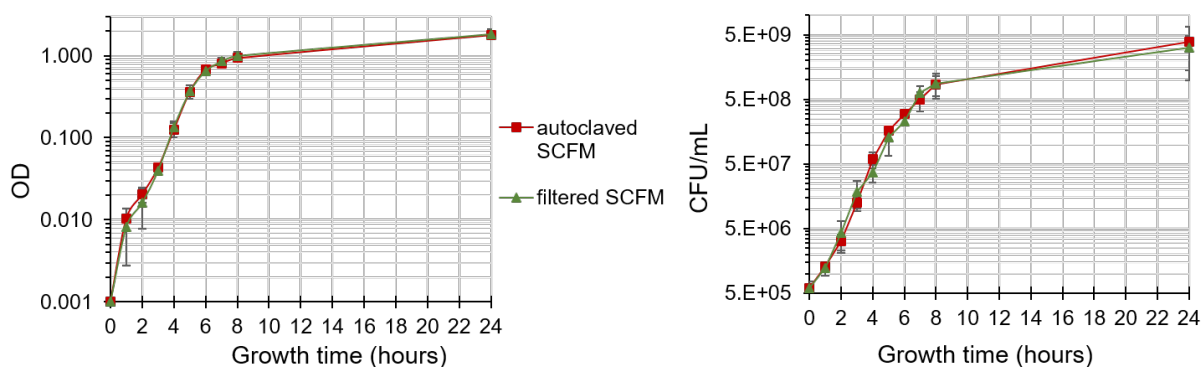
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744 **Fig. S2.1.** Mass spectra, in ion negative mode, of SCFM media collected during preparation,
745 before (A) or after (B) autoclave sterilisation. The ions detected whose m/z are specified
746 correspond to the deprotonated molecules $[M-H]^-$ of the amino acids.

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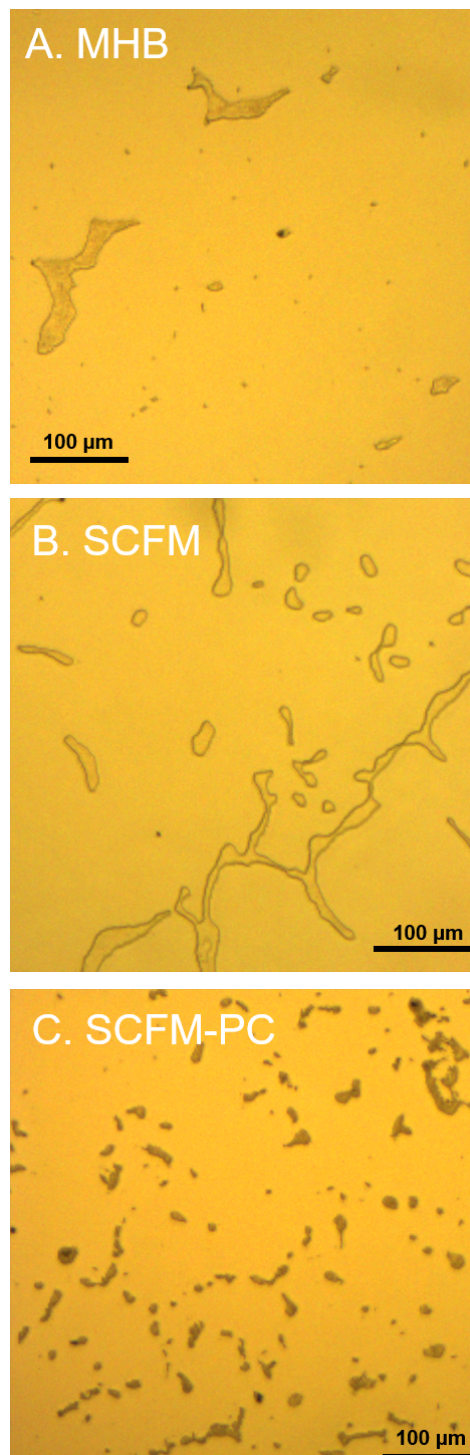
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749 **Fig. S2.2.** Optical Density (A) or Colony Forming Unit (B) plotted against growth time for PAK
750 grown into SCFM autoclave sterilised (red squares) or filtered sterilised (green triangles).
751 Experiments were made in biological triplicates. Standard deviations are represented by
752 error bars.

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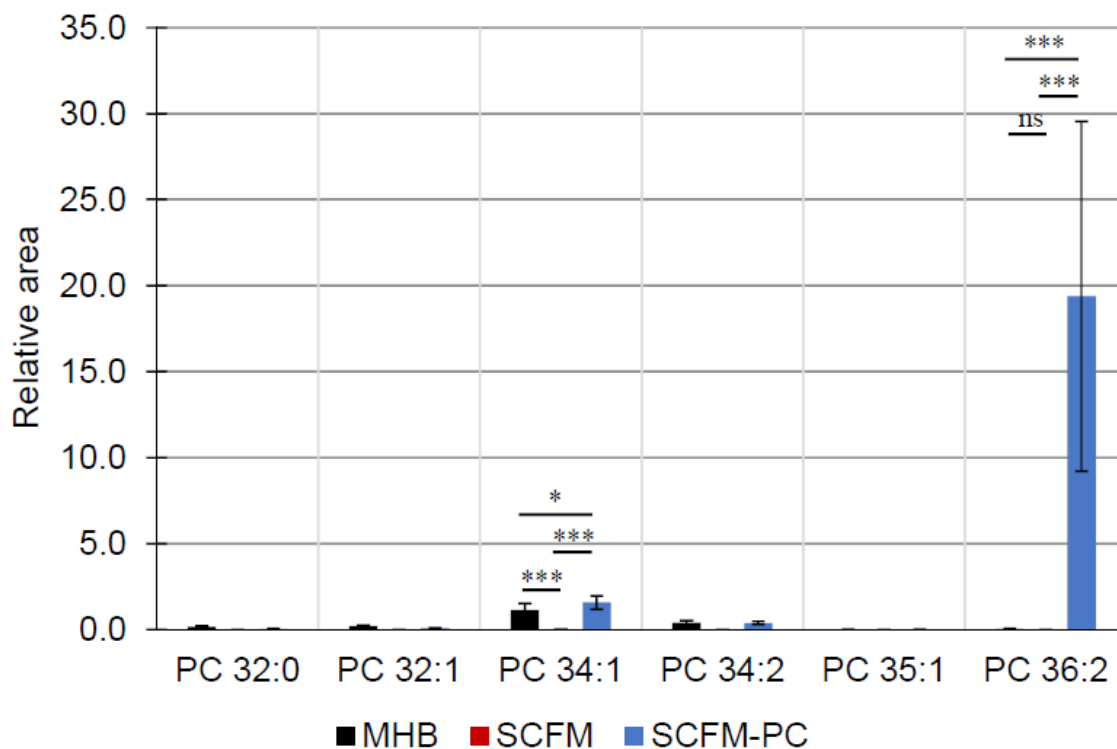
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754 **Fig S2.3.** Optical images of PAK grown into: A. MHB, B. SCFM, C. SCFM-PC. Influence on the
755 biofilm production depending on the growth medium was observed. Less biofilm were
756 observed for PAK grown into MHB rather than in SCFM or in SCFM-PC. In SCFM-PC, bacterial
757 colonies were smaller and more dispersed than the ones observed in the other two media.

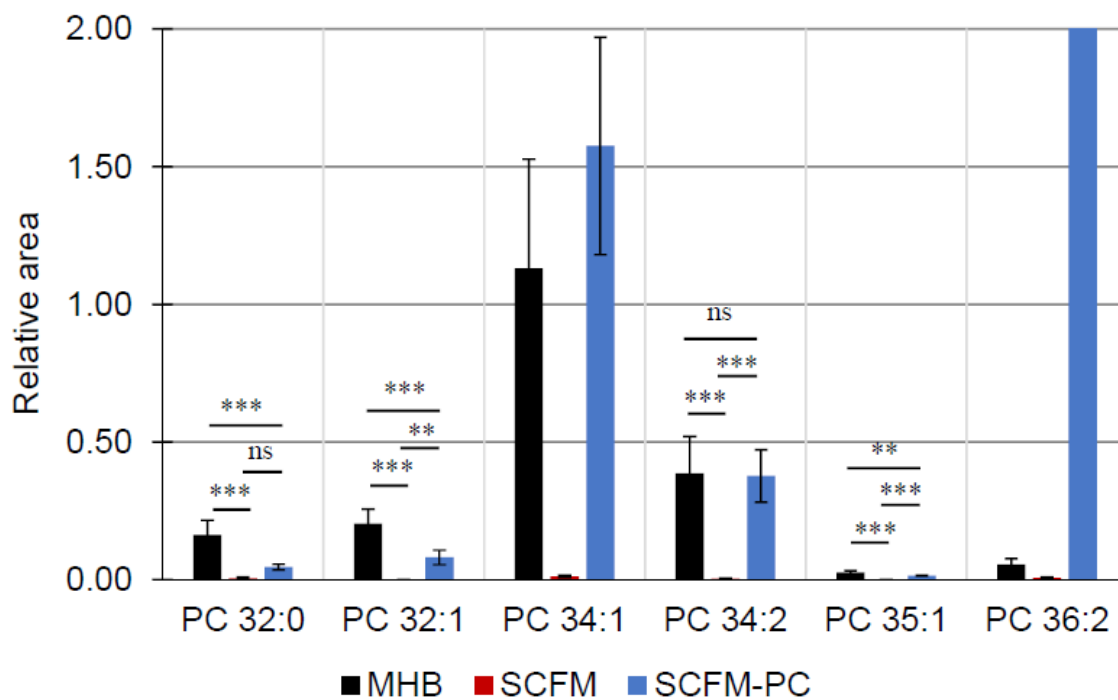
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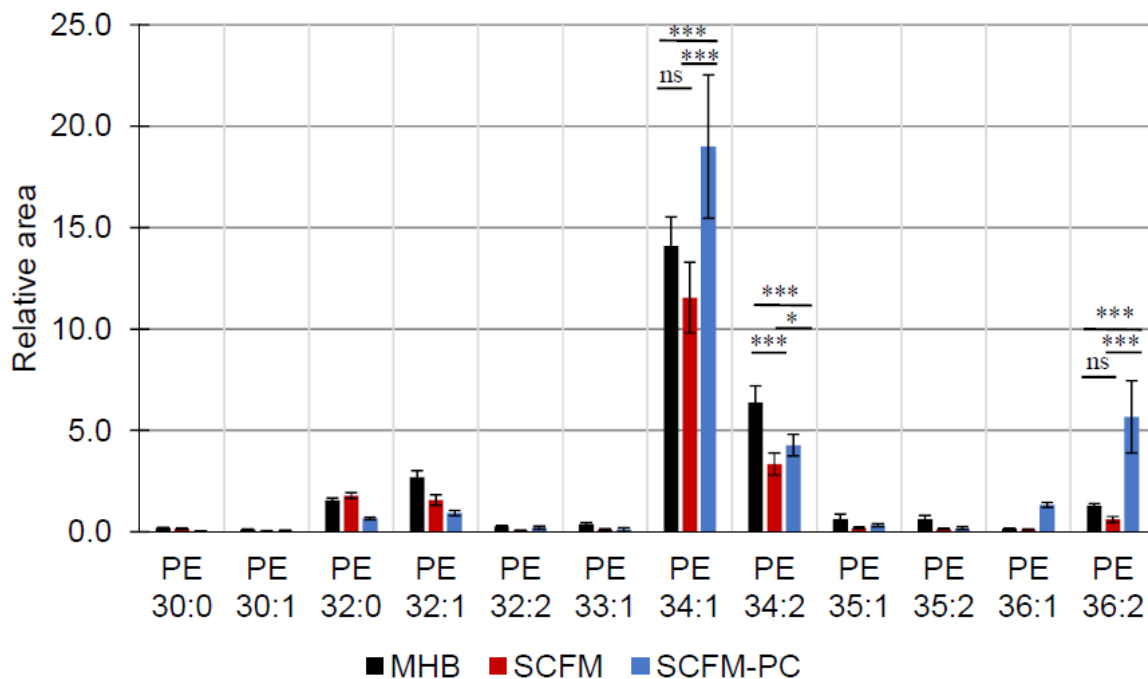
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760 **Fig S2.4.** Relative areas of phosphatidylcholines extracted from PAK grown into MHB (black),
 761 SCFM (red) or SCFM-PC (blue). Error bars: standard deviation ($7 \leq n \leq 9$). P-values are reported
 762 —between MHB and SCFM, SCFM and SCFM-PC and MHB and SCFM-PC— as: * for $p < 0.05$,
 763 ** for $p < 0.01$, *** for $p < 0.001$, ns for non-significant.

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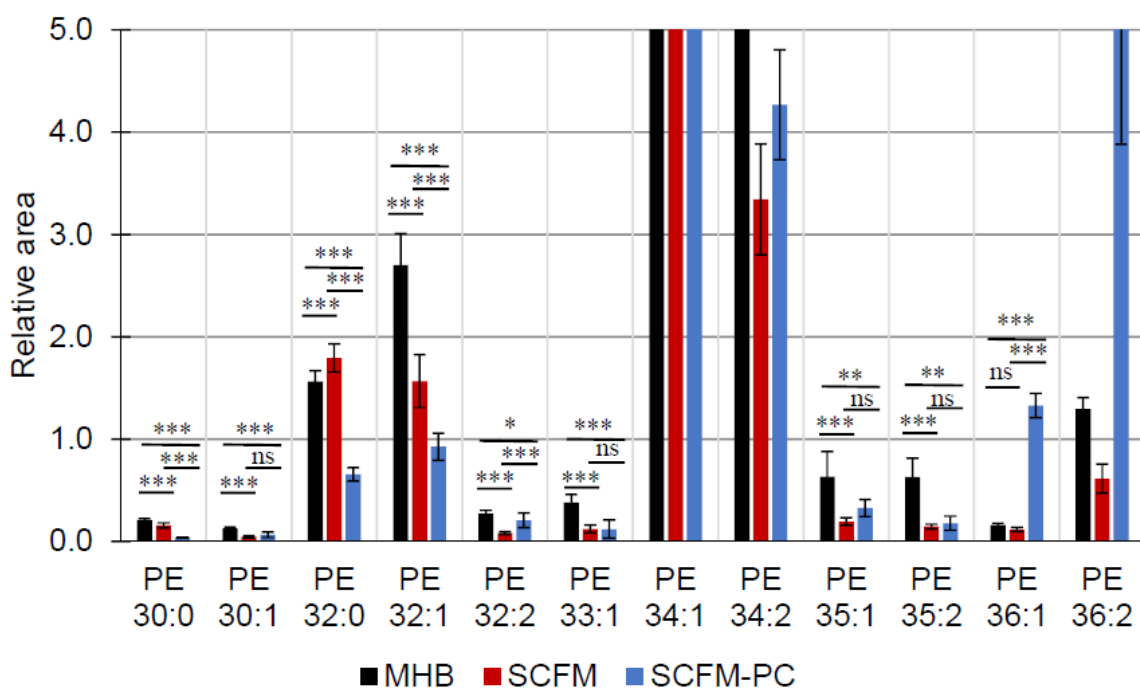
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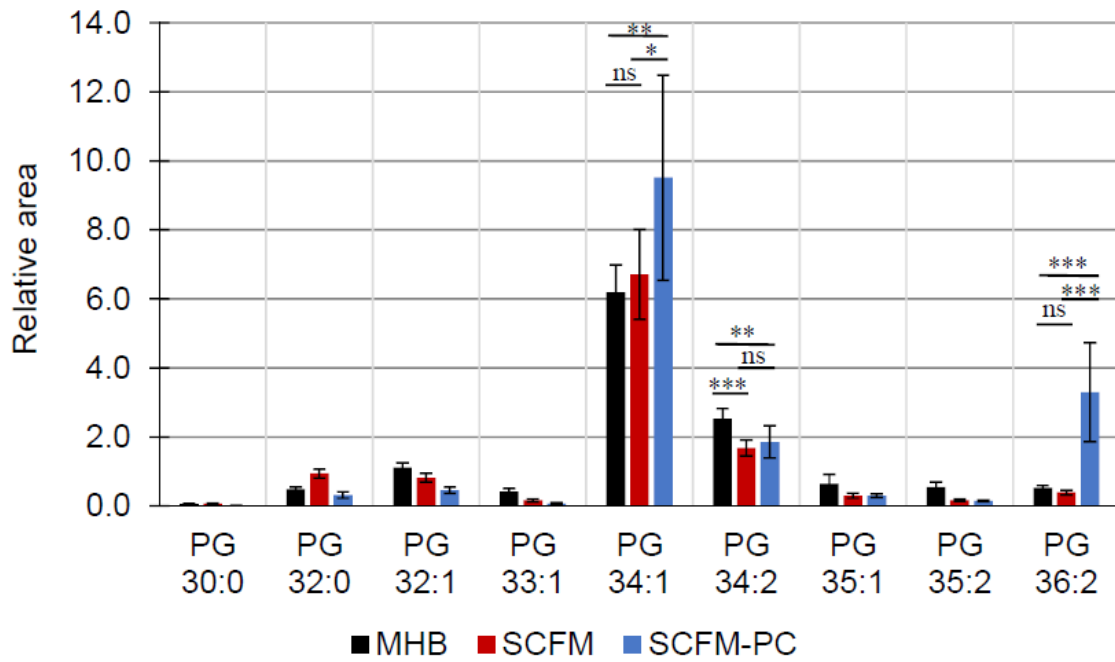
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767 **Fig S2.5.** Relative areas of phosphatidylethanolamines extracted from PAK grown into MHB
 768 (black), SCFM (red) or SCFM-PC (blue). Error bars: standard deviation (7≤n≤9). P-values are
 769 reported —between MHB and SCFM, SCFM and SCFM-PC and MHB and SCFM-PC— as: * for
 770 p<0.05, ** for p<0.01, *** for p<0.001, ns for non-significant.

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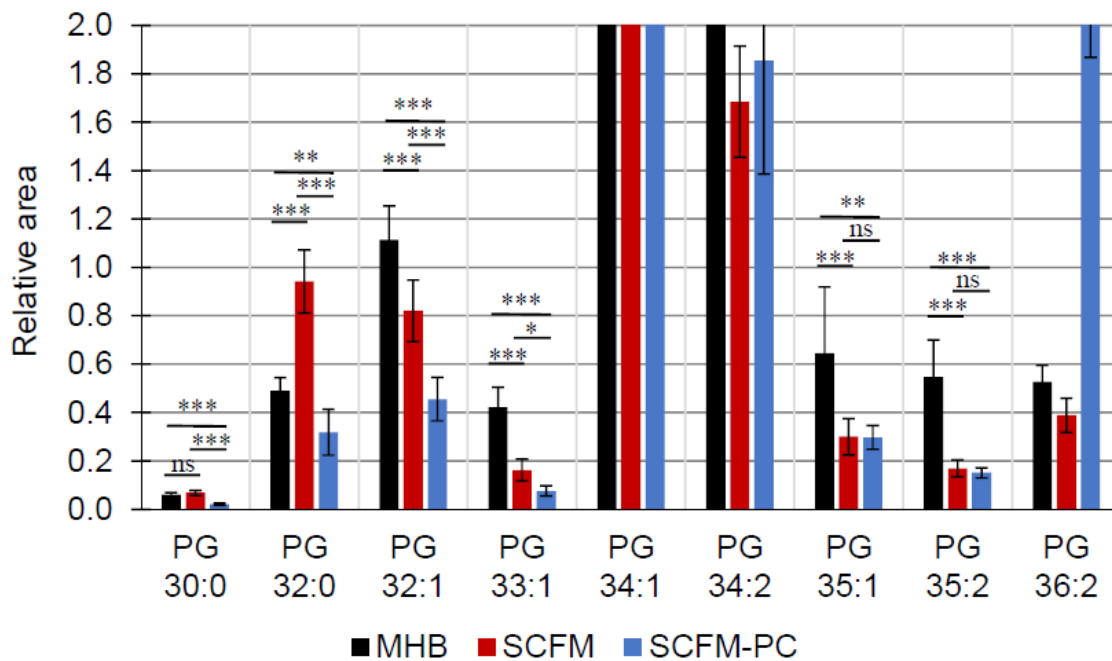
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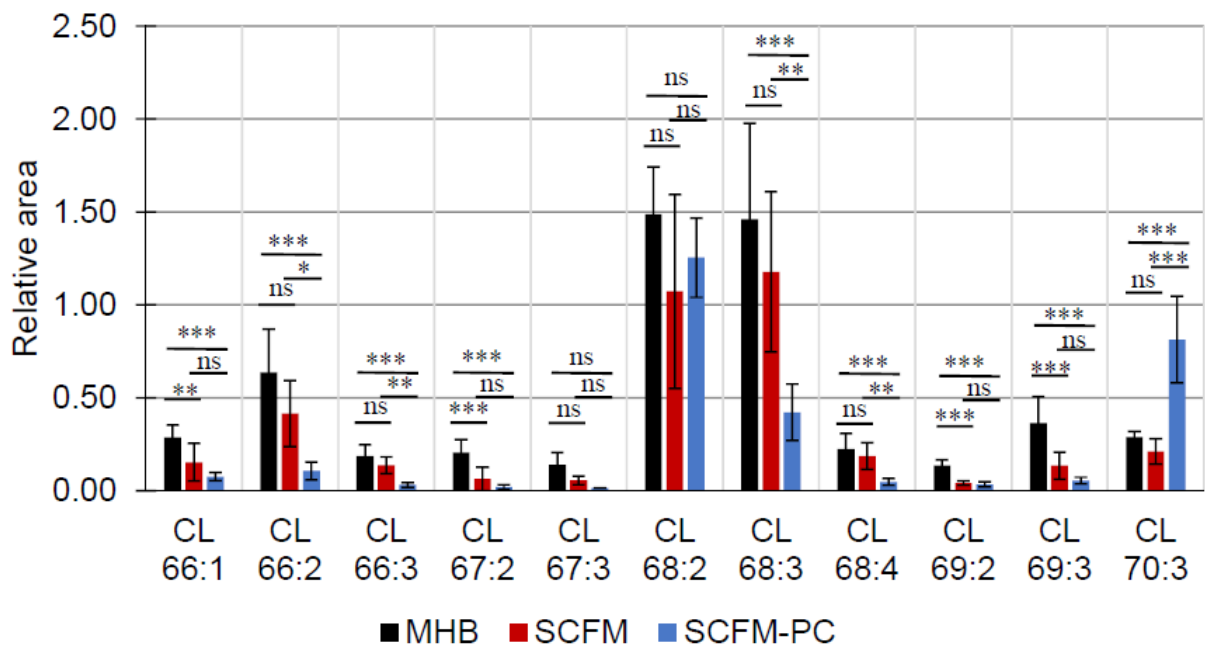
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774 **Fig S2.6.** Relative areas of phosphatidylglycerols extracted from PAK grown into MHB (black),
 775 SCFM (red) or SCFM-PC (blue). Error bars: standard deviation ($7 \leq n \leq 9$). P-values are reported
 776 —between MHB and SCFM, SCFM and SCFM-PC and MHB and SCFM-PC— as: * for $p < 0.05$,
 777 ** for $p < 0.01$, *** for $p < 0.001$, ns for non-significant.

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780 **Fig S2.7.** Relative areas of cardiolipins extracted from PAK grown into MHB (black), SCFM
 781 (red) or SCFM-PC (blue). Error bars: standard deviation ($3 \leq n \leq 6$). P-values are reported —
 782 between MHB and SCFM, SCFM and SCFM-PC and MHB and SCFM-PC— as: * for $p < 0.05$, **
 783 for $p < 0.01$, *** for $p < 0.001$, ns for non-significant.

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Supplementary Information S3: MS/MS

Full Excel file downloadable at <https://doi.org/10.1016/j.bbamem.2020.183482>

FA: Fatty Acid iso1: most intense isomer iso2: second most intense isomer nd: not detected

PC 32:1				PC 16:0/16:1								PC 14:0/18:1									
Growth medium - replicates	Phospholipid fragment			FA(iso1) sn2			FA(iso1) sn1			sn-1/sn-2 ratio (isomer 1)		FA(iso2) sn2			FA(iso2) sn1			isomer 2/ isomer 1 ratio		sn-1/sn-2 ratio (isomer 2)	
	m/z	intensity	identification	m/z	intensity	identification	m/z	intensity	identification			m/z	intensity	identification	m/z	intensity	identification				
PAK - MHB1 PAK - MHB2 PAK - MHB3	716.5381	50 203 257	PC 32:1-CH3	253.2192	341 4384 3414	16:1	nd nd nd	nd nd nd	nd nd nd			281.2545	157 1465 1740	18:1	227.2069	131 440 487	14:0	18:1/16:1	0.46 0.33 0.51 0.43 0.09 20.82	14:0/18:1	0.83 0.30 0.28 0.47 0.31 66.68
PAK - SCFM-PC1 PAK - SCFM-PC2 PAK - SCFM-PC3	716.5381	25 75 303	PC 32:1-CH3	253.2192	573 953 1392	16:1	255.2407	159 616 1175	16:0	16:0/16:1	0.28 0.65 0.84 0.59 0.29 48.80	281.2545	1529 3719 6302	18:1	227.2069	47 373 1286	14:0	18:1/16:1	2.67 3.90 4.53 3.70 0.95 25.57	14:0/18:1	0.03 0.10 0.20 0.11 0.09 78.09

PC 34:1				PC 18:1/16:0								PC 18:0/16:1							
Growth medium - replicates	Phospholipid fragment			FA(iso1) sn2			FA(iso1) sn1			sn-1/sn-2 ratio (isomer 1)		FA(iso2) sn2			FA(iso2) sn1			isomer 2/ isomer 1 ratio	
	m/z	intensity	identification	m/z	intensity	identification	m/z	intensity	identification			m/z	intensity	identification	m/z	intensity	identification		
PAK - MHB1 PAK - MHB2 PAK - MHB3	744.5784	700 2556 2168	PC 34:1-CH3	255.2475	2492 12843 13518	16:0	281.2545	2104 7448 7568	18:1	18:1/16:0	0.84 0.58 0.56 0.66 0.16 24.00	253.2261	26 216 222	16:1	nd nd nd	nd nd nd	nd nd nd	16:1/16:0	0.01 0.02 0.02 0.01 0.00 24.57
PAK - SCFM-PC1 PAK - SCFM-PC2 PAK - SCFM-PC3	744.5784	1041 3835 10562	PC 34:1-CH3	255.2475	4457 20425 46507	16:0 16:0 16:0	281.2545	4662 17718 40921	18:1	18:1/16:0	1.05 0.87 0.88 0.93 0.10 10.71	nd 253.2261	nd 73 43	nd 16:1	nd nd nd	nd nd nd	nd nd nd	nd 16:1/16:0	nd 0.00 0.00 0.00 0.00 83.29
										<i>Note:</i> in SCFM-PC, close abundance of PC 18:1/16:0 & PC 16:0/18:1 (sn-1/sn-2 ratio close to 1)									

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FA: Fatty Acid

iso1: most intense isomer

iso2: second most intense isomer

iso3: third most intense isomer

nd: not detected

Growth medium - replicates	PE 18:1/18:1			PE 19:1/17:1									PE 20:1/16:1														
	FA(iso2) sn2 & sn1			FA(iso2) sn2			FA(iso2) sn1			isomer 2/ isomer 1 ratio		sn-1/sn-2 ratio (iso2)		FA(iso3) sn2			FA(iso3) sn1			Isomer 3/ isomer 1 ratio							
	m/z	intensity	identification	m/z	intensity	identification	m/z	intensity	identification					m/z	intensity	identification	m/z	intensity	identification								
PAK - MHB1 PAK - MHB2 PAK - MHB3	281.2545	579 8579 8188	18:1	nd 267.2331	nd 733 490	nd 17:1	nd 295.2615	nd 214 299	nd 19:1	nd 17:1/18:1	nd 0.09 0.06	nd 0.29 0.61	nd 19:1/17:1	nd 253.2139	nd 75 86	nd 16:1	nd nd nd	nd nd nd	nd nd nd	nd 16:1/18:1	nd 0.01 0.01						
PAK - SCFM 1 PAK - SCFM 2 PAK - SCFM 3	281.2545	5143 4380 1887	18:1	267.2331	251 324 59	17:1	295.2615	59 56 nd	19:1 nd	17:1/18:1	0.05 0.07 0.03	19:1/17:1	0.24 0.17 nd	253.2139	26 94 43	16:1	nd nd nd	nd nd nd	nd nd nd	16:1/18:1	0.01 0.02 0.02						
PAK - SCFM-PC1 PAK - SCFM-PC2 PAK - SCFM-PC3	281.2545	98226 25714 53411	18:1	267.2331 nd 267.2331	953 nd 120	17:1 nd 17:1	295.2615 nd 295.2615	370 nd 51	19:1 nd 19:1	17:1/18:1 nd 17:1/18:1	0.01 nd 0.00	19:1/17:1 nd 0.43	0.39 nd 0.41	253.2139 nd 253.2139	66 nd 34	16:1 nd 16:1	nd nd nd	nd nd nd	nd nd nd	16:1/18:1 nd 16:1/18:1	0.00 nd 0.00						
											RSD %	19.43												RSD %	15.02	RSD %	1.94

Growth medium - replicates	PE 18:1/16:0							PE 18:0/16:1									
	FA(iso1) sn2			FA(iso1) sn1			sn-1/sn-2 ratio	FA(iso2) sn2			FA(iso2) sn1			isomer 2/ isomer 1 ratio			
	m/z	intensity	identification	m/z	intensity	identification		m/z	intensity	identification	m/z	intensity	identification				
PAK - MHB1 PAK - MHB2 PAK - MHB3	255.2475	4735 70116 69139	16:0	281.2545	2796 41641 42671	18:1	18:1/16:0	0.59 0.59 0.62	253.2261	202 2199 2314	16:1	nd nd nd	nd nd nd	16:1/16:0	0.04 0.03 0.03		
PAK - SCFM 1 PAK - SCFM 2 PAK - SCFM 3	255.2475	77871 80899 38588	16:0	281.2545	38154 30080 17722	18:1	18:1/16:0	0.49 0.37 0.46	253.2261	2273 2128 907	16:1	nd nd nd	nd nd nd	16:1/16:0	0.03 0.03 0.02		
PAK - SCFM-PC1 PAK - SCFM-PC2 PAK - SCFM-PC3	255.2475	732023 421282 368937	16:0	281.2545	535977 401387 398739	18:1	18:1/16:0	0.73 0.95 1.08	253.2261	7356 1917 1205	16:1	nd nd nd	nd nd nd	16:1/16:0	0.01 0.00 0.00		
							Note: In SCFM-PC, close abundance of PE 18:1/16:0 & PE 16:0/16:1 (sn-1/sn-2 ratio close to 1)	average std dev RSD %	0.92 0.17 18.90							average std dev RSD %	0.01 0.00 60.50

FA: Fatty Acid

iso1: most intense isomer

iso2: second most intense isomer

iso3: third most intense isomer

nd: not detected

PG 32:1	PG 16:0/16:1								PG 14:0/18:1									
	FA(iso1) sn2			FA(iso1) sn1			sn-1/sn-2 ratio (iso1)		FA(iso2) sn2			FA(iso2) sn1			isomer 2/ isomer 1 ratio		sn-1/sn-2 ratio (iso2)	
	m/z	intensité	identification	m/z	intensité	identification			m/z	intensité	identification	m/z	intensité	identification				
PAK - MHB1		595			215		0.36		124				56			0.21		0.45
PAK - MHB2	253.2192	6459	16:1	255.229	2608	16:0	0.40	227.2089	1820	14:0	281.2412	944	18:1	14:0/16:1	0.28	18:1/14:0	0.52	
PAK - MHB3		5617			2592		0.46		1500			832			0.27		0.55	
							average							average	0.25	average	0.51	
							std dev							std dev	0.04	std dev	0.05	
							RSD %							RSD %	15.38	RSD %	10.29	
PAK - SCFM 1		7023			3336		0.48		2313				887			0.33		0.38
PAK - SCFM 2	253.2192	8701	16:1	255.229	3123	16:0	0.36	227.2089	1924	14:0	281.2412	872	18:1	14:0/16:1	0.22	18:1/14:0	0.45	
PAK - SCFM 3		2263			1125		0.50		764			276			0.34		0.36	
							average							average	0.30	average	0.40	
							std dev							std dev	0.06	std dev	0.05	
							RSD %							RSD %	21.96	RSD %	12.02	
PAK - SCFM-PC1		12412			5984		0.48		4368				2852			0.35		0.65
PAK - SCFM-PC2	253.2192	708	16:1	255.229	503	16:0	0.71	227.2089	601	14:0	281.2412	603	18:1	14:0/16:1	0.85	18:1/14:0	1.00	
PAK - SCFM-PC3		1706			1172		0.69		1198			1068			0.70		0.89	
							average							average	0.63	average	0.85	
							std dev							std dev	0.26	std dev	0.18	
							RSD %							RSD %	40.25	RSD %	21.07	

PG 34:1	PG 18:1/16:0								PG 18:0/16:1								
	FA(iso1) sn2			FA(iso1) sn1			sn-1/sn-2 ratio (iso1)		FA(iso2) sn2			FA(iso2) sn1			isomer 2/ isomer 1 ratio		
	m/z	intensité	identification	m/z	intensité	identification			m/z	intensité	identification	m/z	intensité	identification			
PAK - MHB1		1175			660		0.56		115				nd	nd	nd	16:1/16:0	0.10
PAK - MHB2	255.2475	14066	16:0	281.255	9955	18:1	0.71	253.2261	1202	16:1			nd	nd	nd		0.09
PAK - MHB3		14509			10167		0.70		828				nd	nd	nd		0.06
							average									average	0.08
							std dev									std dev	0.02
							RSD %									RSD %	26.10
PAK - SCFM 1		23192			13486		0.58		1283				nd	nd	nd	16:1/16:0	0.06
PAK - SCFM 2	255.2475	23202	16:0	281.255	11623	18:1	0.50	253.2261	1012	16:1			nd	nd	nd		0.04
PAK - SCFM 3		8496			4613		0.54		601				nd	nd	nd		0.07
							average									average	0.06
							std dev									std dev	0.01
							RSD %									RSD %	24.05
PAK - SCFM-PC1		31396			32177		1.02		413				nd	nd	nd	16:1/16:0	0.01
PAK - SCFM-PC2	255.2475	129379	16:0	281.255	138995	18:1	1.07	253.2261	1312	16:1			nd	nd	nd		0.01
PAK - SCFM-PC3		171763			184963		1.08		1427				nd	nd	nd		0.01
				Note: In SCFM-PC, close abundance of PG 18:1/16:0 & PG 16:0/18:1 (sn-1/sn-2 ratio close to 1)			average									average	0.01
							std dev									std dev	0.00
							RSD %									RSD %	23.23