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► **To cite this version:**

Eve Hellequin, Sylvie Collin, Marina Seder-Colomina, Pierre Véquaud, Christelle Anquetil, et al.. Membrane lipid adaptation of soil Gram-negative bacteria isolates to temperature and pH. 2022. hal-03836117

**HAL Id: hal-03836117**

**<https://hal.science/hal-03836117v1>**

Preprint submitted on 1 Nov 2022

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# 1 Membrane lipid adaptation of soil Gram-negative bacteria isolates to 2 temperature and pH

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15 **Number of words: the main text manuscript is about 7900 words in length (excluding  
16 references) and contains 83 references**

17 **Number of figures: 3**

18 **Number of tables: 6**

19 **Keywords: 3-Hydroxy fatty acids<sup>1</sup>, Gram-negative bacteria<sup>2</sup>, soil<sup>3</sup>, temperature<sup>4</sup>, pH<sup>5</sup>,  
20 adaptation<sup>6</sup>, proxy<sup>7</sup>, *Bacteroidetes*<sup>8</sup>**

## 21 Abstract

22 3-hydroxy fatty acids (3-OH FAs) are characteristic components of the Gram-negative bacterial  
23 membrane, recently proposed as promising temperature and pH (paleo) proxies in soil. Nevertheless,  
24 to date, the relationships between the 3-OH FA distribution and temperature/pH are only based on  
25 empirical studies, with no work at the microbial level. This work investigated the influence of growth  
26 temperature and pH on the lipid profile in three strains of soil Gram-negative bacteria belonging to the  
27 *Bacteroidetes* phylum. Even though the non-hydroxy FAs were more abundant than the 3-OH FAs in  
28 the investigated strains, we showed the important role of the 3-OH FAs in the membrane adaptation of  
29 Gram-negative bacteria to temperature. The strains shared a common adaptation mechanism to  
30 temperature, with a significant increase in the ratio of *anteiso* vs. *iso* or *normal* 3-OH FAs at lower  
31 temperature. In contrast with temperature, no common adaptation mechanism to pH was noticed, the  
32 variations in the FA lipid profiles differing from one strain to another. The models envisioning the

33 reconstruction of environmental changes in soils should include the whole suite of 3-OH FAs present  
34 in the membrane of Gram-negative bacteria, as all of them can be influenced by temperature or pH at  
35 the microbial level.

## 36 **1 Introduction**

37 Microorganisms (bacteria, archaea and some eukaryotes) are able to adjust their membrane  
38 composition in response to the prevailing environmental conditions in order to maintain an appropriate  
39 fluidity and ensure the optimal state of the cellular membrane (Hazel and Williams, 1990; Denich et  
40 al., 2003; Mykytczuk et al., 2007). Such a strategy is termed ‘homeoviscous adaptation’ (Sinensky,  
41 1974). Bacteria can thus change the number of unsaturations, ramifications or chain length of their  
42 membrane lipids – mainly fatty acids – in response to varying environmental conditions (Reizer et al.,  
43 1985; Prado et al., 1988; Suutari and Laakso, 1992).

44 In the same way, the structure of glycerol dialkyl glycerol tetraethers (GDGTs), which are  
45 membrane lipids biosynthesized by archaea and some bacteria, is known to be related to environmental  
46 parameters (Schouten et al., 2013). In aquatic environments, the relative abundance of the different  
47 GDGTs produced by members of the archaeal phylum *Thaumarchaeota* was correlated with the water  
48 surface temperature, leading to the development of a temperature proxy (Schouten et al., 2002), the  
49 TEX<sub>86</sub> index, largely applied to marine and lacustrine paleorecords (Castañeda et al., 2010; Berke et  
50 al., 2012). Another type of GDGTs with branched alkyl chains – so-called branched GDGTs  
51 (brGDGTs) – were suggested to be produced by bacteria and are ubiquitous in aquatic and terrestrial  
52 environments (Schouten et al., 2013). Their analysis in soils/peats (Véquaud et al., 2022) and lake  
53 sediments (Martínez Sosa et al., 2021) distributed worldwide showed that their structure varies mainly  
54 with air temperature and soil pH, making them increasingly used as temperature and pH paleoproxies.  
55 BrGDGTs are the only microbial organic proxies which can be used for temperature reconstructions  
56 in both aquatic and terrestrial settings. Nevertheless, paleoenvironmental data derived from these  
57 molecules have to be interpreted with care, as (i) their source microorganisms remain unknown,  
58 although some might belong to the phylum *Acidobacteria* (Sinninghe Damsté et al., 2011, 2018) and  
59 (ii) high uncertainty is associated with global brGDGT-mean annual air temperature (MAAT)  
60 calibrations (> 4 °C; Véquaud et al., 2022). The development of new environmental proxies,  
61 independent and complementary to brGDGTs, is crucial to improve the reliability and accuracy of  
62 continental reconstructions.

## Temperature/pH effect on Gram- negative bacteria lipids

63           Recently, Wang et al. (2016) suggested that other bacterial lipids, so called 3-hydroxy fatty  
64 acids (3-OH FAs), could be used as such a proxy. These compounds, containing 10 to 18 C and a  
65 hydroxyl group in third position, are characteristic components of the Gram-negative bacterial  
66 membrane (e.g. Szponar et al., 2002, 2003; Keinänen et al., 2003). The membrane component of the  
67 cell wall of Gram-negative bacteria is more complex than that of Gram-positive cells, since it is  
68 composed of two distinct membranes, the outer membrane and the inner membrane, enclosing a thin  
69 space, the periplasm. The inner leaflet of the outer membrane is composed of phospholipids, while its  
70 outer leaflet is mainly composed of lipopolysaccharide (LPS) (Kamio and Nikaido, 1976). The LPS  
71 has a tripartite structure with the hydrophobic lipid A, the core oligosaccharide and the O-antigen  
72 polysaccharide, the two latter being hydrophilic. The lipid A, mainly constituted of 3-OH FAs (Raetz  
73 et al., 2007), anchors the LPS to the outer membrane and acts as a signaling molecule for the innate  
74 immune system. Three types of 3-OH FAs can be distinguished according to the presence and position  
75 of a methyl group on the carbon chain: *normal* (i.e. straight and unbranched), *iso* (methyl in penultimate  
76 position) or *anteiso* (methyl group in the antepenultimate position).

77           Gram-negative bacteria are ubiquitous in aquatic and terrestrial environments. 3-OH FAs were  
78 previously used to detect and quantify Gram-negative communities in various types of samples  
79 originating from terrestrial, aquatic and atmospheric environments (Lee et al., 2004; Wakeham et al.,  
80 2003; Tyagi et al., 2015, 2016). Nevertheless, until recently, these lipids have been largely overlooked  
81 for paleoclimate applications. Indeed, only limited information is available regarding the response of  
82 Gram-negative bacteria and associated 3-OH FA membrane lipids to the variations of environmental  
83 parameters.

84           Significant local correlations were recently obtained between the relative abundance of 3-OH FAs  
85 and temperature/pH in 26 soils collected along Mt. Shennongjia (China; Wang et al., 2016). Thus, the  
86 ratio of the summed *iso* and *anteiso* to the total amount of *normal* 3-OH FAs (the so-called RIAN  
87 index) was shown to vary with soil pH (Wang et al., 2016). In addition, the *anteiso* to *normal* 3-OH  
88 FA ratios of the C<sub>15</sub> and C<sub>17</sub> compounds (RAN<sub>15</sub> and RAN<sub>17</sub> indices, respectively) were negatively and  
89 linearly correlated with MAAT. The application of 3-OH FA based-proxies to a Holocene Chinese  
90 stalagmite reinforced the potential of these compounds as terrestrial paleoenvironmental proxies  
91 (Wang et al., 2018). Very recently, the applicability of 3-OH FAs as temperature and pH proxies was  
92 further investigated at the global level using extended soil datasets with samples from all over the world  
93 (Véquaud et al., 2021a; Wang et al., 2021). Significant relationships between the relative abundance

94 of 3-OH FAs and temperature/pH at the global scale could only be obtained using non-linear models  
95 based on machine-learning algorithms (Véquaud et al., 2021a; Wang et al., 2021), highlighting the  
96 complexity of the response of 3-OH membrane lipids to changes in environmental parameters. Such a  
97 complexity may be related to the fact that (i) the diversity of Gram-negative bacteria varies from one  
98 soil to another (Margesin et al., 2009; Siles and Margesin, 2016) and that (ii) the 3-OH FA distribution  
99 differs between Gram-negative genera and species (Oyaizu and Komagata, 1983; Goossens et al., 1986;  
100 Hedrick et al., 2009; Wang et al., 2018). Therefore, the applicability of 3-OH FAs as environmental  
101 proxies in soils could be strongly influenced by the Gram-negative bacterial diversity, as the response  
102 to temperature and pH changes may vary between species. Nevertheless, to the best of our knowledge,  
103 no work to date has been performed at the microbial level to assess the impact of environmental  
104 parameters on the 3-OH FA profiles of various Gram-negative bacterial strains from soils.

105 In this study, we examined for the first time the influence of temperature and pH on three different  
106 strains of soil Gram-negative bacteria belonging to the same phylum (*Bacteroidetes*) in order to better  
107 understand the effect of variable environmental parameters on the membrane lipid profile, with a focus  
108 on 3-OH-FAs and their applicability as temperature and pH proxies. We hypothesized that (i) different  
109 temperature and pH culture conditions could lead to changes in 3-OH FA distribution and that (ii) the  
110 variations in 3-OH FA lipid profile in response to temperature and pH variations could differ from one  
111 strain to another.

## 112 **2 Material and methods**

### 113 **2.1 Strain isolation**

#### 114 **2.1.1 Isolation of Gram-negative bacteria from surface soils**

115 Gram-negative bacterial strains were isolated from surface soils (0–10 cm depth) collected in  
116 October 2017 along a well-documented composite altitudinal transect (234–2748 m) representative of  
117 the variations in temperature, soil characteristics and plant communities in the French Alps (Véquaud  
118 et al., 2021b). Isolation of Gram-negative bacterial strains was carried out from four contrasting soils  
119 collected in lowland, subalpine, mountainous and alpine land sites (samples 4, 19, 39 and 44,  
120 respectively, in Véquaud et al. (2021b). The physicochemical characteristics of these soil samples are  
121 detailed in Véquaud et al. (2021b).

122 Bacterial strains were isolated by resuspending two grams of each soil in 200 mL of sterile 0.9%  
123 (w/v) NaCl, incubated at 225 rpm on a rotary shaker for 20 min then left to sediment at room  
124 temperature for 40 min. The supernatant was diluted to  $10^{-3}$  and  $10^{-4}$  in sterile NaCl 0.9% before plating

## Temperature/pH effect on Gram- negative bacteria lipids

125 on R2A agar. Bacterial isolation was carried out at pH 7.2 or pH 5.0 (representative of the pH measured  
126 in the French Alps; Véquaud et al., 2021b) on R2A agar plates supplemented with vancomycin 10  
127 mg/L, teicoplanin 10 mg/L, daptomycine 10 mg/L and cycloheximide 400 mg/L. For each soil, growth  
128 was monitored on eight plates at pH 7.2 and eight plates at pH 5.0, incubated at 5 °C or at 25 °C until  
129 no new colonies appeared. Twenty different clones covering the visible morphological differences of  
130 the colonies (colony size, shape, color, smoothness, general aspect), observed in each condition were  
131 re-isolated twice on the same medium. A single colony of each clone was then used to inoculate 10 mL  
132 of LB medium 0.1X, grown overnight at 25 °C or for 48h at 5 °C before storage of the cell pellet at -  
133 80 °C after resuspension in 60% glycerol, 7.5mM MgSO<sub>4</sub>. Three hundred and twenty cultivable species  
134 of Gram-negative bacteria were isolated from the four aforementioned soils (Véquaud et al., 2021b)  
135 and constituted the library for our study.

### 136 2.1.2 Taxonomic identification

137 For each bacterial clone, a single colony was used for PCR amplification of rDNA sequences.  
138 Amplification was carried out with primers 27F and 1391R (Lane, 1991) using GoTaq® DNA  
139 polymerase (Promega) according to the manufacturer's instructions, i.e. 95 °C for 2min, followed by  
140 94 °C for 30s, 60 °C for 30 s, 72 °C for 1min40s (35 cycles), and 72 °C for 5 min. The PCR fragments  
141 were directly sequenced (Eurofins Genomics) using primer T7. A preliminary assignation was made  
142 using Standard Nucleotide Blast (Altschul et al., 1990). *Pseudomonas* generated a total of 193 first  
143 hits. The next most abundant genera were *Flavobacterium* (26 hits), *Colimonas* (17 hits),  
144 *Mucilaginibacter* (12 hits) and *Pedobacter* (9 hits). Three strains were selected from the library for  
145 further study, identified as belonging to the *Flavobacterium*, *Mucilaginibacter* and *Pedobacter* genera  
146 in the *Bacteroidetes* phylum. Further taxonomic affiliation was carried out on these clones after PCR  
147 amplification of the rDNA genes using the same primers as above and *Pfu* DNA polymerase  
148 (Promega), following the manufacturer's instructions. The PCR fragments were cleaned, A-tailed and  
149 cloned into the pGEM-T vector. The identification of each strain was based on the sequencing of two  
150 positive plasmids using T7 and SP6 primers (Eurofins Genomics). The entire sequence was used for  
151 affiliation using EZBioCloud, database version 2121.07.07. The selected clones belonged to the  
152 *Bacteroidetes* phylum, as the C<sub>15</sub> and C<sub>17</sub> 3-OH FA homologues used in the RAN<sub>15</sub> and RAN<sub>17</sub>  
153 temperature proxies defined by Wang et al. (2016) were shown to be abundant in these bacteria (cf.  
154 section 4.1 for discussion).

155 The first strain, isolated from a calcosol collected at 232 m altitude (MAAT=12.5 °C; pH=  
156 7.3; soil 44 in Véquaud et al., 2021b), was most closely related to *Flavobacterium pectinovorum*  
157 (98.78% similarity; GenBank accession no. OP204417). The second strain, isolated from a brunisol  
158 collected at 932 m altitude (MAAT=8.6 °C; pH=7.2; soil 39 in Véquaud et al., 2021b), was most  
159 closely related to *Pedobacter lusitanus* NL19 (98.54% similarity; GenBank accession no.  
160 OP204418). The third strain, isolated from a rendisol collected at 2695 m altitude (MAAT=0.4 °C;  
161 pH=6.5; soil 4 in Véquaud et al., 2021b), was most closely related to phylotype LGEL\_s strain 048,  
162 *Pedobacter panaciterrae* (99.27% similarity; GenBank accession no. OP204416). In the rest of this  
163 paper, the three aforementioned strains will be referred to as strains A, B and C, respectively.

## 164 **2.2 Cultivation of the strains**

### 165 **2.2.1 Identification of the optimal growth conditions**

166 For each strain, the optimal growth temperature at the intrinsic pH of the R2A medium (pH 7.2)  
167 was first identified. Triplicate cultures were performed for each temperature tested (5 °C, 10 °C, 15  
168 °C, 20 °C, 25 °C and 30 °C). For each culture, a colony isolated on R2A agar medium was incubated  
169 in 7 mL of sterile R2A medium at 180 rpm (Infors HT Celltron) at the temperature to be tested to set  
170 up a subculture. After a few hours or a few days (depending on the strain and the tested temperature),  
171 the optical density (OD) of the subculture was measured at 600 nm (Spectrostar nano, BMG LabTech).  
172 One hundred ml of sterile R2A medium were inoculated with a volume of the subculture, chosen to  
173 reach an OD value between 0.05 and 0.10. The growth kinetics of each strain was then established.  
174 Cultures were shaken at 180 rpm under the desired conditions in a thermostatically controlled cabinet  
175 (Pol-Eko Aparatura®). OD was measured every hour until the stationary phase of bacterial growth was  
176 reached. The optimum pH (5, 6, 7 or 8) was defined under the same conditions, but with the temperature  
177 set at the defined optimal growth temperature (25 °C, see below). The pH of the R2A medium was  
178 adjusted with a pH meter using 1 M NaOH (pH above 7.2) or 1 M HCl (pH below 7.2). For each  
179 culture, bacterial growth curves were generated and the generation time was calculated in order to  
180 identify the optimal growth temperature and pH. 25 °C was identified as the optimal growth  
181 temperature for all the strains. At 25 °C, the optimal growth pH was 8 for strain A and 5 for strains B  
182 and C.

### 183 **2.2.2 Cultivation of the strains under different temperature and pH conditions**

184 Once the optimum temperature and pH were identified, the strains were cultivated in triplicate in  
185 order to obtain a reference bacterial growth curve for each condition tested. The strains were cultivated

## Temperature/pH effect on Gram- negative bacteria lipids

186 at 180 rpm, either at fixed temperature (optimal growth temperature) and different pH (5, 6, 7 or 8) or  
187 at fixed pH (optimal growth pH) and different temperatures (5, 10, 15, 20 and 25 °C). These reference  
188 curves were used for regular measurements of OD, in order to determine the time until stationary phase  
189 is reached. Growth phase has been shown to have an influence on the membrane lipid composition of  
190 microorganisms (Männistö and Puhakka, 2001; Elling et al., 2014). Thus, in order to reliably compare  
191 the influence of temperature and pH on the distribution of 3-OH FAs between the different strains, the  
192 bacterial biomass was collected at the same stage of growth. For all the strains, the biomass was  
193 collected during the exponential phase by dividing by two the OD obtained at the stationary phase  
194 (reference OD). After subculturing as described above, the final cultures were performed in triplicate  
195 in a final volume of 150 mL of sterile R2A medium incubated at 180 rpm until reaching the reference  
196 OD. The cells were collected in 50 mL Falcon® tubes and centrifuged at 7000 rpm (JA12 rotor,  
197 Beckman) for 10 min at 20 °C. Each bacterial pellet was resuspended in 10 mL of 0.9 g/L NaCl to  
198 remove traces of R2A medium and then centrifuged again at the same speed as before. The supernatant  
199 was removed and the cell pellets were frozen at -20 °C before being freeze-dried for 48 h at -80 °C at  
200 a pressure of 0.93 Pa. The freeze-dried cell pellets were kept frozen at -20 °C before lipid extraction  
201 and analysis.

### 202 **2.3 Lipid extraction**

203 The freeze-dried cells were first subjected to acid methanolysis in 1M HCl /MeOH at 100 °C for 3  
204 h. The suspension was then centrifuged at 15 °C and 3000 rpm for 5 min. The supernatant was collected  
205 in an Erlenmeyer flask. The pellet was extracted 3 times with a mixture of dichloromethane  
206 (DCM):MeOH (1:1, v/v; 25 mL). Each extraction was followed by centrifugation and pooling of all  
207 extracts. The extracts were then transferred to a separation funnel and washed with ultrapure water in  
208 order to neutralize the organic phase. The organic phase was recovered in a new Erlenmeyer flask. The  
209 aqueous phase was extracted twice with DCM (20 mL). The organic phase was recovered and pooled  
210 with the organic phase previously obtained. The organic phase containing the lipids was dried with  
211 sulfate sodium, then rotary-evaporated, diluted with a mixture of DCM:MeOH (5:1), transferred in a 4  
212 mL vial and dried under nitrogen flow. The lipid extract was resuspended in 1 mL DCM and stored at  
213 -20 °C until 3-OH FA analysis.

### 214 **2.4 Lipid analysis**

215 The lipid extracts were first derivatised with a solution of *N,O*- bis(trimethylsilyl)trifluoroacetamide  
216 (BSTFA) at 70 °C for 45 min. Fatty acids were analysed by gas chromatography coupled to mass



217 spectrometry using an Agilent Network 6980 GC System coupled with a 5973 Mass Selective Detector,  
218 with electron impact at 70 eV. A Restek RXI-5 Sil MS silica column (60 m x 0.25 mm, i.d. 0.50  $\mu$ m  
219 film thickness) was used with He as the carrier gas at 1 mL/min, as previously described (Huguet et  
220 al., 2019). The GC oven program was: 70 °C to 200 °C at 10 °C/min, then to 310 °C (held 20min) at 2  
221 °C/min. Samples were injected in splitless mode and the injector was set at 280 °C. The individual  
222 fatty acids (either hydroxy or non-hydroxy homologues) were first identified from the total ion  
223 chromatogram. The corresponding peaks were integrated, allowing the determination of the relative  
224 abundances of the different fatty acids.

225 A focus was then made on the different 3-OH FAs after extraction of the characteristic  $m/z$  175  
226 fragment (cf. Huguet et al., 2019), allowing the identification of the low-abundant homologues based  
227 on their relative retention times. 3-OH FAs were quantified by integrating the appropriate peak on the  
228 ion chromatogram and comparing the area with an analogous deuterated internal standard (3-  
229 hydroxytetradecanoic acid, 2,2,3,4,4-d<sub>5</sub>; Sigma-Aldrich, France). The internal standard (0.5 mg/mL)  
230 was added just before injection, with a proportion of 3  $\mu$ L of standard to 100  $\mu$ L of sample, as detailed  
231 by Huguet et al. (2019). The  $m/z$  178 fragment was used for identification and integration of the  
232 deuterated internal standard.

233 The RIAN index was calculated as follows in the range C<sub>10</sub>-C<sub>18</sub> (Wang et al., 2016):

234 
$$\text{RIAN} = -\log [(I + A)/N] \quad (1)$$

235 where I, A, N represent the sum of all *iso*, *anteiso* and *normal* 3-OH FAs, respectively.

236 RAN<sub>15</sub> and RAN<sub>17</sub> indices were calculated as follows (Wang et al., 2016):

237 
$$\text{RAN}_{15} = [\textit{anteiso} - \text{C15}] / [\textit{normal} - \text{C15}] \quad (2)$$

238 
$$\text{RAN}_{17} = [\textit{anteiso} - \text{C17}] / [\textit{normal} - \text{C17}] \quad (3)$$

## 239 **2.5 Statistical analyses**

240 All the statistical analyses were performed using R (v3.6.3, Core Team, 2020). To investigate the  
241 statistical differences in the relative abundances of 3-OH FAs depending on the pH or temperature of  
242 the culture experiments, analysis of variance (ANOVA) and post-hoc Tukey tests were used. In order  
243 to investigate the correlations between the temperature or pH and the relative abundances of 3-OH  
244 FAs, pairwise Spearman correlation matrices and single linear regressions were performed for each  
245 strain. The *p*-value was set at 0.05 for all the statistical analyses.

## Temperature/pH effect on Gram- negative bacteria lipids

### 246 3 Results

#### 247 3.1 Global distribution of fatty acids in bacterial strains isolated from soils

##### 248 3.1.1 Non-hydroxy fatty acids

249 The membrane lipid profile of the three strains was investigated under different conditions of  
250 temperature and pH (Tables 1-3). The non-hydroxy FAs were generally more abundant than 3-OH  
251 FAs, especially for strain A, where non-hydroxy FAs represented more than 70 % of the total relative  
252 abundance in FAs (FAs; Table 1). For strains B and C, non-hydroxy FAs were generally slightly more  
253 abundant (> 55 % of total FAs) than 3-OH FAs. Straight-chain (i.e. *normal*) monounsaturated FAs  
254 were the major group of non-hydroxy FAs in strains B and C (Tables 2 and 3). In contrast, in strain A,  
255 the branched saturated FAs were as abundant as the normal monounsaturated FAs (Table 1). Strain A  
256 also differed from strains B and C by the presence of branched unsaturated FAs (*iso*-C<sub>15:1</sub> and to a  
257 lesser extent *iso*-C<sub>16:1</sub>; Table 1). At the individual level, *normal*-C<sub>16:1</sub> was observed to be the dominant  
258 non-hydroxy FA in strains B and C, followed, to a lesser extent, by *normal*-C<sub>16:0</sub>, *normal*-C<sub>18:0</sub> and *iso*-  
259 C<sub>15:0</sub> (Tables 2 and 3). Regarding strain A, the most abundant non-hydroxy FAs were the *iso*-C<sub>15:0</sub> and  
260 *normal*-C<sub>16:1</sub> homologues, followed by the *normal*-C<sub>15:0</sub> and -C<sub>16:0</sub> isomers.

##### 261 3.1.2 3-OH FAs

262 The distribution of the 3-OH FAs in the three investigated strains was also examined in detail  
263 (Figure 1 and Tables 4-6). The 3-OH FAs detected had a carbon number of between 13C and 18C.  
264 Whatever the cultivation conditions, the *iso* homologues were the most abundant for strain A (Table  
265 4). This was also the case for strain B, except at a growth temperature of 5 °C (Table 5), where the  
266 *anteiso* homologues were slightly more abundant than the *iso* homologues. In addition, the relative  
267 abundance of the *normal* homologues was higher than the one of the *anteiso* isomers for strain A (Table  
268 4), whereas an opposite trend was observed for strain C (Table 6). Regarding strain B, the relative  
269 abundance of *normal* vs. *anteiso* isomers was highly dependent on the cultivation conditions (Table  
270 4).

271 The distribution of the individual 3-OH FAs was also observed to differ between the strains. Thus,  
272 for strain A, the *iso*-C<sub>15</sub> homologue was by far the most abundant 3-OH FA, followed by the *iso*-C<sub>17</sub>  
273 and *normal*-C<sub>16</sub> compounds, whatever the cultivation conditions (Figure 1 and Table 4). Regarding  
274 strain B, the 3-OH FA distribution was dominated by either *iso*-C<sub>15</sub>, *iso*-C<sub>17</sub> or *anteiso*-C<sub>17</sub> homologues  
275 (depending on the cultivation conditions), followed by the *n*-C<sub>16</sub> isomer (Table 4). As for strain C, the  
276 most abundant 3-OH FAs were *anteiso*-C<sub>17</sub>, *iso*-C<sub>17</sub>, *anteiso*-C<sub>15</sub> and *iso*-C<sub>15</sub>, with a strong effect of

277 the cultivation conditions on the relative proportion of these isomers. It should be noted that part of the  
278 3-OH FAs were present at very low abundance (< 1% of total abundance in 3-OH FAs), and were even  
279 not detected in some of the samples. For example, the *iso*-C<sub>13</sub> and *normal*-C<sub>15</sub> homologs were mainly  
280 detected in strain A isolates (Table 4). In contrast, the *normal*-C<sub>18</sub> isomer was only detected in strains  
281 B and C (Tables 5 and 6).

## 282 **3.2 Influence of temperature on the FA distribution of the different strains**

### 283 **3.2.1 Non-hydroxy fatty acids**

284 The influence of temperature on the relative abundance of the individual FAs was compound- and  
285 strain-dependent. Regarding the non-hydroxy fatty acids, the relative abundance of *anteiso*-C<sub>15:0</sub> and  
286 *normal*-C<sub>17:1</sub> was observed to significantly decrease with an increase in temperature for both strains A  
287 and B (Tables 1 and 2). In addition to the two aforementioned homologues, temperature variations had  
288 a strong influence on the relative abundance of other isomers in strain A, as the relative abundances in  
289 *normal*-C<sub>15:0</sub> and *iso*-C<sub>15:0</sub> were shown to significantly increase with temperature ( $R^2 = 0.94$  and  $0.54$ ,  
290 respectively), whereas an opposite trend was observed for *iso*-C<sub>15:1</sub> ( $R^2 = 0.95$ ; Table 1). In contrast,  
291 temperature had only a limited impact on the non-hydroxy FA lipid profile in strain C (Table 3).

### 292 **3.2.2 3-OH FAs**

293 In all strains, the relative abundance of the total *anteiso* 3-OH FAs was observed to significantly  
294 decrease with increasing temperature (Tables 4-6). In addition, in strains A and C, the relative  
295 abundance of the total *normal* 3-OH FAs was significantly and positively correlated with temperature  
296 (Tables 4 and 6), whereas an opposite trend was observed for strain B (Table 5). In strains B and C,  
297 the relative abundance of the total *iso* 3-OH FAs was also positively and strongly correlated with  
298 temperature (Table 5 and 6).

299 The relative abundance of some of the individual 3-OH FAs was also observed to be similarly  
300 affected by temperature changes, whatever the strain. Thus, in strain C, the relative abundance of the  
301 *anteiso*-C<sub>17</sub> and *anteiso*-C<sub>15</sub> isomers significantly decreased with temperature ( $R^2 = 0.77$  and  $0.67$ ,  
302 respectively; Figure 1 and Table 6). Such a significant decrease in the relative abundances of the  
303 *anteiso*-C<sub>17</sub> and *anteiso*-C<sub>15</sub> 3-OH FAs with temperature was similarly observed in strains B ( $R^2 = 0.95$   
304 and  $0.82$ , respectively; Table 5) and strain A ( $R^2 = 0.79$  and  $0.85$ , respectively; Table 4) despite the  
305 low abundance of the two *anteiso* isomers in the latter strain (< 5% of total 3-OH FAs). It should be  
306 noted that the relative abundance of the *iso*-C<sub>14</sub> 3-OH FA was also observed to significantly decrease

## Temperature/pH effect on Gram- negative bacteria lipids

307 with temperature in strains A and C ( $R^2 = 0.55$  and  $0.69$ , respectively; Tables 4 and 6), despite its low  
308 proportion (this compound was even not detected in most of the strain B samples).

309 In contrast, the effect of temperature differed between the strains for some of the individual 3-OH  
310 FAs. The relative abundance of the *iso*-C<sub>15</sub> isomer, predominant in strain A, did not change  
311 significantly with temperature ( $R^2 = 0.02$ ; Table 4), whereas it significantly increased for the B and C  
312 strains ( $R^2 = 0.72$  and  $0.68$ , respectively; Tables 5 and 6). Similarly, the relative abundance in the *iso*-  
313 C<sub>17</sub> 3-OH FA was observed to strongly ( $R^2 = 0.86$  and  $0.83$ ) and significantly increase with increasing  
314 temperature for strains B and C (Tables 5 and 6), whereas no significant correlation was observed for  
315 strain A (Table 4). As for the relative abundance of the *iso*-C<sub>16</sub> homolog, it was observed to strongly  
316 and significantly decrease with temperature for strains A and B (Tables 4 and 5), whereas no significant  
317 change was noticed for strain C.

318 Regarding the *normal* isomers, the relative abundance of the *normal*-C<sub>16</sub> homologue significantly  
319 increased with temperature in strains A and C (Tables 4 and 6), while it significantly decreased in strain  
320 B (Table 5). As for the relative abundance of the *normal*-C<sub>15</sub> homologue, it significantly increased with  
321 temperature in strain A ( $p < 0.001$ ), the only strain where it was detected.

### 322 3.3 Influence of pH on the FA distribution of the different strains

323 Only weak to moderate significant correlations were observed between some of the individual non-  
324 hydroxy fatty acids and pH, with different trends from one strain to another (Tables 4-6). Similarly,  
325 the influence of pH on the relative abundance of 3-OH FAs was strain-dependent (Figure 2). In strain  
326 A, the relative abundances of the *iso*-C<sub>14</sub>, *anteiso*-C<sub>15</sub>, *iso*-C<sub>16</sub>, *iso*-C<sub>17</sub> and *anteiso*-C<sub>17</sub> homologues  
327 were significantly and positively correlated with pH, whereas an opposite trend was observed for the  
328 *iso*-C<sub>13</sub> and *normal*-C<sub>15</sub> homologues (Table 4). No clear variations of the 3-OH FA relative abundance  
329 with pH were not observed for strain B (Table 5). Last, in strain C, a significant increase in the relative  
330 abundance of *iso*-C<sub>14</sub> and also *normal*-C<sub>18</sub> 3-OH FAs was observed with pH (Table 6).

### 331 3.4 Relationship between 3-OH FA-derived proxies and temperature/pH

332 Wang et al. (2016) proposed three indices based on the relative abundance of 3-OH FAs and related  
333 to pH (RIAN, Eq. 1) or temperature (RAN<sub>15</sub> and RAN<sub>17</sub>, Eqs. 2 and 3). The *normal*-C<sub>15</sub> homologue  
334 was not detected in strain B and in most of the cultures of strain C, preventing the calculation of the  
335 RAN<sub>15</sub> for the corresponding samples of these two strains. In strain A, the RAN<sub>15</sub> was observed to be  
336 significantly negatively correlated with temperature ( $R^2 = 0.92$ ; Table 4 and Figure 3A). Regarding the

337 RAN<sub>17</sub>, a significant negative relationship was observed between this index and the temperature for  
338 strains A ( $R^2 = 0.93$ ) and B ( $R^2 = 0.51$ ; Figure 3B and Tables 4-5). The RAN<sub>17</sub> also tended to decrease  
339 with temperature for strain C ( $R^2 = 0.25$ ; Figure 3B and Table 6), even though this correlation was non-  
340 significant ( $p = 0.057$ ). The RIAN (Eq. 1) did not show any correlation with pH for the three strains  
341 (Figure 3C and Tables 4-6).

## 342 **4 Discussion**

### 343 **4.1 Choice of the investigated strains**

344 The carbon number of the 3-OH FAs present in the lipopolysaccharides (LPS) of Gram-negative  
345 bacteria is exclusively comprised between 10 and 18 (Wollenweber and Rietschel, 1990; Szponar et  
346 al., 2003). In most of the Gram-negative bacteria, the major 3-OH FAs are normal, even-carbon  
347 compounds, with variations of the dominant homolog between different species (e.g. Oyaizu and  
348 Komagata, 1982; Wilkinson, 1988). The natural variability in the distribution of 3-OH FAs in the LPS  
349 of the Gram-negative bacterial species logically leads to variations in the 3-OH FA profile of  
350 environmental samples, as recently observed in soils from all over the world (Véquaud et al., 2021a;  
351 Wang et al., 2021). Nevertheless, despite their lower relative abundance vs. normal homologues, the  
352 straight chain, odd-carbon numbered 3-OH FAs are of special interest. They are at the basis of the  
353 different indices (RIAN, RAN<sub>15</sub> and RAN<sub>17</sub>; Eqs. 1, 2 and 3) based on the relative abundances of the  
354 3-OH FAs and shown to be linearly correlated with soil pH and MAAT (Wang et al., 2016). Therefore,  
355 in this study, we purposefully selected, among 320 Gram-negative bacterial clones isolated from  
356 French Alps soils, three strains from the same phylum, *Bacteroidetes*, whose LPS was reported to be  
357 enriched in C<sub>15</sub> and C<sub>17</sub> 3-OH FAs (Wilkinson, 1988). Such a choice was made to ascertain the presence  
358 of the C<sub>15</sub> and/or C<sub>17</sub> 3-OH FAs in the investigated strains and to allow a proper investigation of the  
359 relationships between the aforementioned indices and temperature/soil pH.

360 The lipid profiles of the three strains were first determined at their optimal growth conditions  
361 (temperature of 25 °C; pH = 5 for strain A and 8 for strains B and C). Strain A, most closely related to  
362 *Flavobacterium pectinovorum*, contained mainly *iso*-C<sub>15:0</sub> and *normal*-C<sub>16:1</sub> FAs and to a lower extent  
363 *iso*-C<sub>15</sub> and *iso*-C<sub>17</sub> 3-OH FAs (Tables 1 and 4), consistent with the lipid profile reported by Cousin et  
364 al. (2007) for related strains *F. aquidurense* and *F. hercynium*. As for the strains B and C, most closely  
365 related to *Pedobacter lusitanus* and *Pedobacter panaciterrae*, respectively, their FA profiles were  
366 dominated by the *normal*-C<sub>16:1</sub> FA (Tables 2 and 3) and the *iso*-C<sub>17</sub> and *iso*-C<sub>15</sub> 3-OH FAs (Tables 5  
367 and 6), in agreement with the distribution reported by Steyn et al. (1998), Yoon et al. (2007) and Covas

## Temperature/pH effect on Gram- negative bacteria lipids

368 et al. (2017) for related *Pedobacter* strains. Even though the predominant 3-OH FAs were, as expected,  
369 the same for the three investigated strains, the whole 3-OH FA distribution differed from one strain to  
370 another (Figures 1 and 2), which is related to the fact that a large amount of phylogenetic and  
371 physiological diversity exists at the phylum or even at the genus level (Torsvik and Øvreås, 2002;  
372 Fierer et al. 2007).

### 373 4.2 Effect of growth temperature and pH on the FA lipid profile

374 The fatty acid composition of the cell membrane plays a leading role in the bacterial adaptation (i.e.  
375 homeoviscous adaptation; Sinensky, 1974) to environmental changes. Previous studies mainly  
376 investigated the membrane lipid adaptation of bacteria to changes in temperature and to a lesser extent  
377 pH, showing that bacteria were able to modify the degree of fatty acid unsaturation and cyclization,  
378 chain length or branching to cope with such changes (e.g. Suutari and Lakso, 1974; Diomande et al.,  
379 2015). The present study aimed to study for the first time the influence of temperature and pH on both  
380 non-hydroxy and 3-OH FA lipid profile of soil Gram-negative bacteria, as 3-OH FAs represent major  
381 components of the Gram-negative bacteria lipid outer membrane (Tables 1-6; Wilkinson, 1988).

#### 382 4.2.1 Effect of temperature

383 Changes in the growth temperature led to some similar shifts in the FA lipid profile of the three  
384 investigated strains. Thus, whatever the strain, an increase in the proportion of (i) the *anteiso*-C<sub>15:0</sub> FA  
385 (Tables 1-3) and (ii) the total *anteiso* 3-OH FAs (Tables 4-6) with decreasing growth temperature was  
386 observed. Such a trend is consistent with the homeoviscous adaptative mechanism classically adopted  
387 by bacteria. Indeed, at low temperature the fluidity of bacterial membranes decreases. To control the  
388 phase transition temperature of the membrane lipids and maintain adequate membrane properties in  
389 response to temperature changes, bacteria are able to modulate the membrane composition and  
390 incorporate lower-melting point fatty acids (unsaturated, short chain and branched chain fatty acids),  
391 which have a fluidizing effect on the membrane (Suutari and Laakso, 1994; Denich et al., 2003; Koga,  
392 2012). As *anteiso* fatty acids have a lower melting point than *iso* and *normal* fatty acids (Russel and  
393 Fukunga, 1990; Kaneda, 1991), an increase in the relative abundance of *anteiso* isomers is commonly  
394 observed in the bacterial cell membrane as growth temperature decreases (e.g. Oshima and Miyagawa,  
395 1974; Mazzota and Motville, 1997; Koga et al., 2012). The increase in the relative abundance of the  
396 all *anteiso* FAs – non-hydroxy but also 3-hydroxy – detected in the three Gram-negative bacterial  
397 strains of the present study appears as a common mechanism to maintain the cell membrane fluidity at  
398 low temperatures.

## Temperature/pH effect on Gram-negative bacteria lipids

399 A decrease in the relative proportion of the *iso* FAs at lower temperatures is often concomitant with  
400 higher relative abundances in *anteiso* FAs (e.g. Kaneda, 1991; Prakash et al., 2015). For example, a  
401 higher relative abundance of lower-melting point *anteiso* FAs was observed at lower temperature for  
402 Gram-positive bacteria species *Bacillus subtilis* and *Bacillus megaterium*, together with a lower  
403 relative abundance of *iso* FAs (Suutari and Lakso, 1992). Similarly, the proportion of the *iso* C<sub>15</sub> and  
404 C<sub>17</sub> FAs of ten Gram-negative bacterial *Thermus* strains decreased and the one of the *anteiso* C<sub>15</sub> and  
405 C<sub>17</sub> FA increased with decreasing temperature (Nordström and Laakso, 1992). Here, the ratio of the  
406 total *iso* 3-OH FAs versus total *anteiso* 3-OH FAs was observed to increase with the temperature  
407 whatever the strain (Tables 4-6), confirming the importance of the branching pattern in response to  
408 changing temperature.

409 This conclusion is strengthened by a concomitant decrease in the total *anteiso* 3-OH FAs vs. total  
410 *normal* 3-OH FAs observed for the three investigated strains (Tables 4-6). A similar trend was recently  
411 observed in Gram-negative and positive psychrophilic bacteria isolated from Pakistanese glaciers, for  
412 which a higher temperature resulted in a significant increase in the relative abundance of saturated  
413 *normal* FAs and a substantial decrease in the relative abundance of branched FAs (Hassan et al., 2020).  
414 The change of the 3-OH FA branching ratio with temperature, reflected by the relative increase and  
415 decrease of the ratios in *anteiso/normal* and *iso/anteiso* 3-OH FAs at lower temperature, respectively,  
416 should enable the membrane to maintain a normal liquid-crystalline state and the cell to function  
417 normally even at low temperature, as previously suggested for non-hydroxy fatty acids (Suutari and  
418 Laakso, 1994; Sun et al., 2012). This appears as a common adaptation mechanism to temperature for  
419 all the Gram-negative bacterial strains investigated in this study.

420 Despite shared variations in the ratios of the main 3-OH FAs isomers with temperature, strain-  
421 specific trends were observed when examining the individual relative abundances of the 3-OH FAs.  
422 Thus, the variations of the relative abundance of the *iso* 3-OH FAs with temperature generally differed  
423 between strain A and the two other strains. For strains B and C (Tables 5 and 6), the total relative  
424 abundance in *iso* 3-OH FAs was weakly correlated with temperature and only for strain A (Table 4).  
425 This might be related to the fact that strain A is related to a different genus (*Flavobacterium*) and class  
426 (*Flavobacteria*) than strains B and C (genus *Pedobacter*, class *Sphingobacteria*) (Hahnke et al, 2016).  
427 Such strain-specific trends were also observed for the *normal* 3-OH FAs, despite their lower abundance  
428 than the *iso* and *anteiso* isomers. However, the relative abundance of the odd and even *normal* 3-OH  
429 FAs (C<sub>14</sub>-C<sub>18</sub>) significantly increased with temperature, especially at 20 and 25 °C, for strains A and C  
430 (Tables 4 and 6), whereas no correlation was observed for strain B (Table 5). This implies that the

## Temperature/pH effect on Gram- negative bacteria lipids

431 relatedness to a different genus cannot explain alone the observed trends, suggesting that even within  
432 a genus two strains could respond differently to environmental changes (e.g. strains B and C).

433 In addition to the branched FAs, the role of unsaturated FAs in cold adaptation was previously  
434 reported, with higher relative abundances of monounsaturated FAs observed at lower temperatures in  
435 several Gram-negative bacteria species (e.g. *Rhizobium leguminosarum*, Theberge et al., 1996;  
436 *Chryseobacterium frigidisoli*, Bajerski et al., 2017). Such a trend was explained by the lower melting  
437 point of monounsaturated FAs vs. saturated counterparts (Russell, 1984, 1989) and is consistent with  
438 the increase in the relative abundance of (i) the *normal*-C<sub>17:1</sub> FA observed in strains A and B (Tables 1  
439 and 2) and (ii) *normal*-C<sub>16:1</sub> and *iso*-C<sub>15:1</sub> FA observed in strain A (Table 1) at lower temperatures.

440 Overall, even though the 3-OH FAs are generally less abundant than the non-hydroxy FAs in the  
441 cell membrane, we showed for the first time the essential role of the former compounds in the  
442 membrane adaptation to temperature related to the maintenance of the membrane fluidity. Thus, a  
443 notable increase in the ratio of *anteiso* vs. *iso* or *normal* 3-OH FAs at lower temperature was observed  
444 for all the investigated strains isolated from soils. Such a shared temperature adaptation mechanism  
445 should be further confirmed by investigating additional Gram-negative bacterial strains from different  
446 settings. The relative proportions of the individual 3-OH FAs were also affected by temperature  
447 variations, but the corresponding trends were strain-dependent.

### 448 4.2.2 Effect of pH

449 In contrast with temperature, less data is available on the lipid membrane adaptation to pH  
450 variations, with contrasting observations, based on limited groups of organisms. Thus, Prakash et al.  
451 (2015) observed an increase of the relative proportion of branched-chain FAs (*iso* C<sub>15</sub>, *iso* C<sub>16</sub>, *anteiso*  
452 C<sub>15</sub>, *anteiso* C<sub>17</sub>) at higher pH for the Gram-positive species *Micrococcus yunnanensis* and  
453 *Micrococcus aloeverae*. Giotis et al. (2017) similarly observed the important role of the *iso* and *anteiso*  
454 FAs for pH adaptation of the Gram-negative species *Listeria Monocytogenes*, with an increase in the  
455 relative abundance of branched-chain FAs in alkaline conditions and an opposite trend in acid  
456 conditions. An increase in the relative proportion of short-chain *iso*- and *anteiso*-C<sub>15:0</sub> FAs at higher  
457 pH was observed for *C. frigidisoli*, isolated from Antarctic glacier soils (Bajerski et al., 2017). In  
458 contrast, Russell (1984) reported an increase of *iso* and *anteiso* FAs at low pH, and Bååth and Anderson  
459 (2003) a decrease of *iso*-C<sub>15:0</sub> and *iso*-C<sub>16:0</sub> at higher pH, with no obvious change in the relative  
460 abundance of *anteiso* FAs.

461 As reported in previous work, the interpretation of the fatty acid variations in response to changing  
462 pH is complex, with different trends between the three Gram-negative bacterial strains investigated. In



463 strain B, the pH did not have a major effect on the FA composition. In strain C, the relative abundance  
464 in *iso*-C<sub>17</sub> 3-OH FA was observed to increase at higher pH (Table 6), as also observed for strain A  
465 (Table 4). Nevertheless, in these two strains, the relative abundance in *iso*-C<sub>15</sub> 3-OH FA, the most  
466 abundant of the 3-OH FAs, decreased with increasing pH (Tables 4 and 6), thus showing an opposite  
467 trend to *iso*-C<sub>17</sub> 3-OH FA. In parallel, the relative abundances in *anteiso*-C<sub>15</sub> and C<sub>17</sub> 3-OH FAs were  
468 observed to increase at higher pH for strain A (Table 4). Regardless of the increasing or decreasing  
469 variations in the relative abundances of the *iso* and *anteiso* isomers with pH, the ratio of the *iso/anteiso*  
470 3-OH FAs was shown to be significantly and negatively correlated with pH in strain A, concomitantly  
471 with a significant increase of the *anteiso/normal* 3-OH FA ratio (Table 4). The relative increase in  
472 branched *anteiso* 3-OH FAs could help in maintaining the membrane flexibility at higher pH (Russell,  
473 1989), as was also assumed for *C. frigididoli* (Bajerski et al., 2017). Nevertheless, it is unclear why  
474 such a change would only occur at alkaline pH and only for strain A. Further studies are needed to  
475 better understand the structural membrane adaptation of Gram-negative bacteria in response to pH  
476 variations.

#### 477 **4.3 Implications for the use of 3-OH FAs as temperature and pH proxies**

478 3-OH FAs were recognized as potential temperature and pH proxies in soils six years ago after  
479 their analysis in 26 Chinese soils (Wang et al., 2016). Significant correlations were obtained between  
480 the relative abundance of these compounds and MAAT/pH, through the use of the RIAN, RAN<sub>15</sub> and  
481 RAN<sub>17</sub> indices (Eqs. 1, 2 and 3). We recently analysed 3-OH FAs in surficial soils from seven other  
482 altitudinal transects distributed worldwide (Tanzania, Italy, French Alps, Chile, Peru, Tibet), leading  
483 to an extended dataset of ca. 170 soils, and observed that strong linear relationships between the 3-OH  
484 FA-derived indices defined by Wang et al. (2016) and MAAT/pH could only be obtained locally for  
485 some of the individual transects (Huguet et al., 2019; Véquaud et al., 2021a). This could be due to the  
486 fact that local parameters (e.g. soil moisture, grain size, vegetation and soil types) may exert a strong  
487 influence on 3-OH FA distribution in soils, as shown in a detailed study we performed in the French  
488 Alps (Véquaud et al., 2021). Nevertheless, to date, the relationships between environmental variables  
489 and 3-OH FA distribution were mainly obtained from bulk soil samples and no study was performed  
490 the microorganism level. This work is the first one to report the effect of temperature and pH on the 3-  
491 OH FA composition of Gram-negative bacterial strains from soils.

492 When considering the different indices proposed by Wang et al. (2016), the RAN<sub>17</sub> appears to be  
493 weakly to strongly correlated with the growth temperature of the three Gram-negative bacterial strains  
494 isolated from French Alps soils (Fig. 3b; Tables 4-6). Moreover, the RAN<sub>17</sub>/temperature relationships

## Temperature/pH effect on Gram- negative bacteria lipids

495 for these three strains present different slopes and y-intercept. This can explain, at least partly, the  
496 difficulty in establishing global RAN<sub>17</sub>/MAAT calibrations from soils distributed worldwide, since the  
497 bacterial diversity is expected to widely vary between soils, especially for 3-OH FA-producing Gram-  
498 negative microorganisms (e.g. Margesin et al., 2009; Siles and Margesin, 2016). Instead, our study  
499 confirms the potential of the RAN<sub>17</sub> as a temperature proxy in terrestrial settings at a local scale  
500 (Véquaud et al., 2021a; Wang et al., 2021).

501 The global application of the RAN<sub>15</sub> as a temperature proxy appears even more challenging as the  
502 one of the RAN<sub>17</sub>, since the 3-OH FA isomers required for the calculation of this index can only be  
503 detected in some Gram-negative bacterial strains. In the only strain (A) for which the RAN<sub>15</sub> could be  
504 calculated, a strong correlation with MAAT was obtained (Fig. 3a; Table 4). This implies that the  
505 application of the RAN<sub>15</sub> as a temperature proxy is soil-dependent, with the prerequisite that  
506 corresponding soil Gram-negative bacteria produce *anteiso*- and *normal*-C<sub>15</sub> 3OH FAs in sufficient  
507 amounts.

508 Regarding the RIAN index, no significant correlations with pH were observed for the three  
509 investigated strains (Fig. 3c; Tables 4-6). In soils, the weakness of the correlations between the RIAN  
510 and pH were notably explained by the fact that (i) MAAT rather than soil pH could rather influence  
511 the bacterial diversity and composition and by (ii) the intrinsic heterogeneity of soils, with a large  
512 diversity of bacterial communities encountered at a given site (Véquaud et al., 2021a). The first  
513 hypothesis is consistent with the fact that the RIAN index was significantly and moderately correlated  
514 with MAAT for the three strains ( $R^2 = 0.51 - 0.67$ ; Tables 4-6), reflecting the stronger influence of the  
515 temperature than pH on this ratio. In addition, the response of Gram-negative bacterial communities  
516 to pH was shown to be complex, making difficult the use of the RIAN as a pH proxy in soils, where  
517 the microbial diversity naturally varies from one sample to another.

518 As linear models did not appear as the most suitable to establish global relationships between the  
519 3-OH FA-based indices (RIAN, RAN<sub>15</sub> and RAN<sub>17</sub>) and MAAT/pH in soils, non-parametric machine  
520 learning models were used instead (Véquaud et al., 2021a; Wang et al., 2021), allowing to take into  
521 account non-linear environmental influences, as well as the intrinsic complexity of the environmental  
522 settings. Véquaud et al. (2021) especially used a random forest algorithm, which was shown to be the  
523 most robust and allowed the development of strong global calibrations with MAAT/pH. The MAAT  
524 random forest model included all the 3-OH FAs involved in the calculation of the RAN<sub>15</sub> and RAN<sub>17</sub>  
525 indices (*anteiso*-C<sub>15</sub> and -C<sub>17</sub> and *normal*-C<sub>15</sub> and -C<sub>17</sub>), with other individual 3-OH FAs also having a  
526 major weight (e.g. *iso*-C<sub>13</sub>, *iso*-C<sub>14</sub>, *normal*-C<sub>16</sub>; Véquaud et al., 2021). This is consistent with the

527 results we obtained at the microbial level, where (i) the adaptation mechanism of the Gram-negative  
528 bacterial strains to temperature variations involved a concomitant change in the ratios of the *iso*, *anteiso*  
529 and *normal* isomers and (ii) the growth temperature was observed to have a significant effect on the  
530 relative abundance of most of the individual 3-OH FAs (Tables 4-6). As the whole suite of 3-OH FAs  
531 can be influenced by the temperature, our results highlight the fact that the global models based on  
532 these compounds should preferentially include all of them rather than a limited set as in the RAN<sub>15</sub> and  
533 RAN<sub>17</sub> indices to reflect the natural response of Gram-negative bacteria to temperature changes.

534 A similar conclusion can be drawn out for the global calibrations between pH and 3-OH FA  
535 distribution. Indeed, even though the *iso*-C<sub>13</sub>, *iso*-C<sub>16</sub> and *normal*-C<sub>15</sub> 3-OH FAs were shown to have  
536 a major weight in the random forest pH model proposed by Véquaud et al. (2021a), all the C<sub>10</sub> to C<sub>18</sub>  
537 3-OH FAs had a non-negligible influence in the latter. This is in agreement with the results derived  
538 from the three Gram-negative bacterial species investigated in the present study, as pH was shown to  
539 have a potential influence on the relative distribution of all the individual 3-OH FAs (Tables 4-6),  
540 despite different variations from one strain to another.

541 Additional strains of Gram-negative bacteria should be investigated to better understand the lipid  
542 adaptation mechanism of these microorganisms to temperature and pH changes and better constrain  
543 the applicability of 3-OH FAs as environmental proxies in terrestrial settings.

## 544 **5 Conclusion**

545 3-OH FAs were recently proposed as promising temperature and pH (paleo)proxies in soil.  
546 Nevertheless, to date, the relationships between the 3-OH FA distribution and temperature/pH were  
547 only based on empirical studies in soil, with no work performed at the microbial level. Here, we  
548 examined the influence of growth temperature and pH on the lipid profile of three different strains of  
549 soil Gram-negative bacteria belonging to the same phylum (*Bacteroidetes*). Even though the non-  
550 hydroxy FAs were more abundant than the 3-OH FAs in the three investigated strains, we showed the  
551 important role of the 3-OH FAs in the membrane adaptation of Gram-negative bacteria to temperature.  
552 The Gram-negative bacterial strains shared a common temperature adaptation mechanism, with a  
553 significant increase in the ratio of *anteiso* versus *iso* or *normal* 3-OH FAs at lower temperature, which  
554 would help in maintaining the membrane fluidity in colder conditions. Nevertheless, the variations of  
555 the relative abundances of the individual 3-OH FAs with temperature were shown to be strain-  
556 dependent. In contrast with the growth temperature, no common adaptation mechanism to pH was  
557 noticed for the investigated Gram-negative bacterial strains, the variations of the fatty acid lipid profiles

## Temperature/pH effect on Gram- negative bacteria lipids

558 differing from one species to another. As the entire suite of 3-OH FAs present in the lipid membrane  
559 of Gram-negative bacteria can be influenced by either temperature or pH, our results suggest that  
560 models based on these compounds and envisioning the reconstruction of environmental changes in  
561 terrestrial settings at the global scale should include all compounds rather than only indices based on a  
562 sub-selection as initially proposed. In a context of global change, additional studies on a larger number  
563 of Gram-negative bacterial strains from contrasting settings (soils, marine and lake environments) are  
564 now required to better understand the adaptation mechanism of these microorganisms to environmental  
565 variations.

### 566 **6 Conflict of Interest**

567 The authors declare that the research was conducted in the absence of any commercial or financial  
568 relationships that could be construed as a potential conflict of interest.

### 569 **7 Author Contributions**

570 EH, SC and AH designed the research work. PV collected the soil samples from the French Alps. MS-  
571 C and SC isolated and identified bacterial strains. EH carried out the cultivation of the strains, lipid  
572 extraction and analysis. CA carried out the GC/MS analysis. EH and AH carried out the statistical  
573 analyses. EH and AH generated the figures and tables. AH and SC supervised the whole research work.  
574 EH, AH, SC wrote the original draft. AK provided critical reading and assisted in the writing and  
575 revision of the manuscript. All authors contributed and approved the final manuscript.

### 576 **8 Funding**

577 Funds for the research work were provided by Sorbonne Université through the TEMPO project  
578 (Emergence 2019-2020). In addition, the EC2CO program (CNRS/INSU –  
579 BIOHEFFECT/MICROBIEN) also supported this study through the SHAPE project.

### 580 **9 Acknowledgments**

581 We are thankful to Pr. Jérôme Poulénard for assisting during the soils collection in the French Alps.  
582 Lucile Daubercies is gratefully acknowledged for assistance in taxonomic affiliation following Blast  
583 analysis.

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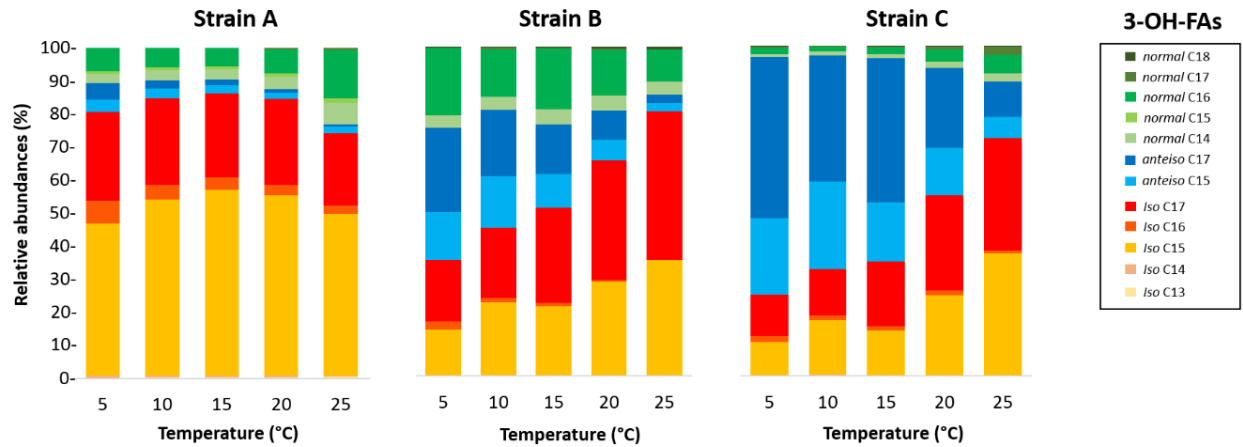
### 798 **11 Data Availability Statement**

799 All datasets generated for this study are included in the article.

800

### 801 **12 Figures**

## Temperature/pH effect on Gram-negative bacteria lipids

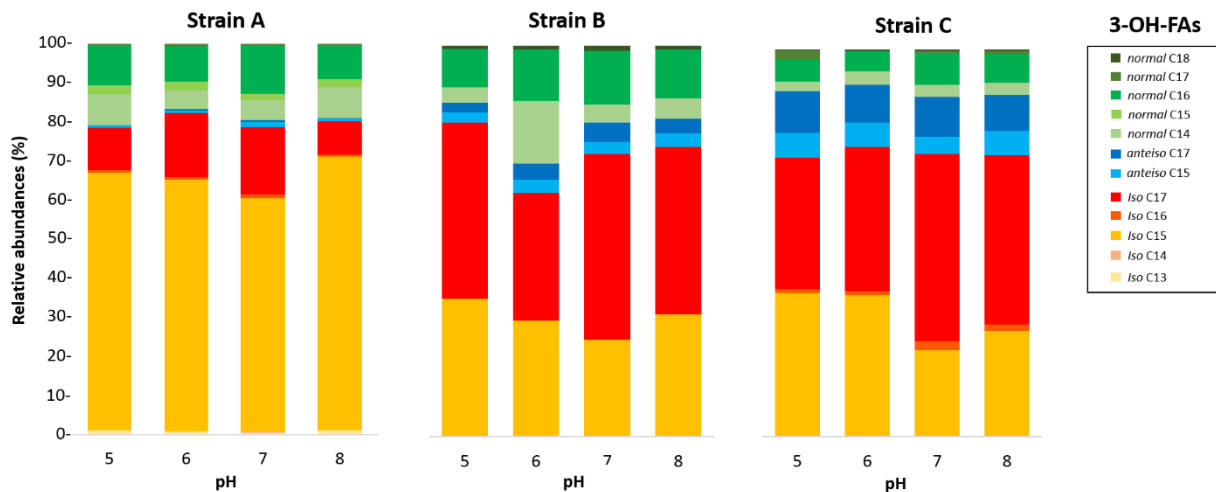


802

803 **Figure 1: Variation of the relative abundances of 3-OH-FAs with temperature for the three**  
804 **Gram-negative bacterial strains isolated from soils of the French Alps. Strain A: *Flavobacterium***  
805 ***pectinovorum* (98.78% similarity). Strain B: *Pedobacter lusitanus* NL19 (98.54% similarity). Strain C:**  
806 ***Pedobacter panaciterrae* (99.27% similarity)**

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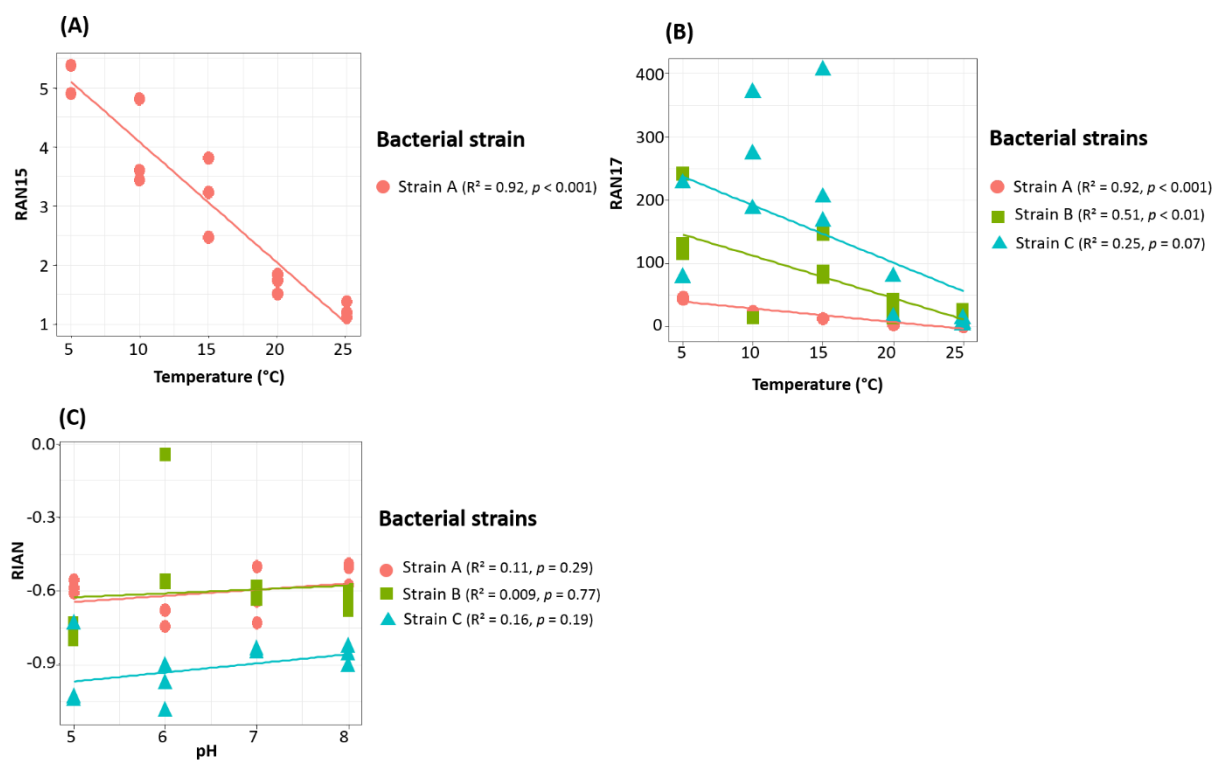


809

810 **Figure 2: Variation of the relative abundances of 3-OH FAs with pH for the three Gram-negative**  
811 **bacterial strains isolated from soils of the French Alps. Strain A: *Flavobacterium pectinovorum***  
812 **(98.78% similarity). Strain B: *Pedobacter lusitanus* NL19 (98.54% similarity). Strain C: *Pedobacter***  
813 ***panaciterrae* (99.27% similarity)**

814

## Temperature/pH effect on Gram- negative bacteria lipids



815

816 **Figure 3: Relationships between temperature and RAN<sub>15</sub> (A) and RAN<sub>17</sub> (B) and between pH**  
817 **and RIAN (C) for the three Gram-negative bacterial strains isolated from soils of the French**  
818 **Alps. Strain A: *Flavobacterium pectinovorum* (98.78% similarity). Strain B: *Pedobacter lusitanus***  
819 **NL19 (98.54% similarity). Strain C: *Pedobacter panaciterrae* (99.27% similarity). Each point**  
820 **corresponds to one replicate.**

821

822 **13 Tables**

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825

**Table 1.** Relative abundances of non-hydroxy fatty acids (%) vs. total fatty acids for strain A at different temperature conditions (pH 8) and at different pH conditions (temperature 25 °C). Data are means of three biological replicates. Values after ( $\pm$ ) are standard deviations. Correlations of the relative abundances of each fatty acid (FA) with either temperature or pH are provided with corresponding *p*-values. Significant correlations (*p* < 0.005) are indicated in bold. n.d.: not detected.

Strain A	Temperature conditions (°C)							pH conditions					
	5 °C	10 °C	15 °C	20 °C	25 °C	R <sup>2</sup>	<i>p</i>	5	6	7	8	R <sup>2</sup>	<i>p</i>
<b>Normal saturated</b>													
15:0	3.08 ± 0.16	4.60 ± 0.18	5.59 ± 0.42	7.45 ± 0.57	9.15 ± 1.10	<b>0.94</b>	2.11E-09	8.41 ± 3.27	12.39 ± 0.53	10.34 ± 2.34	9.15 ± 1.10	8.14E-05	0.98
16:0	8.75 ± 5.91	6.25 ± 1.29	6.13 ± 0.92	8.43 ± 0.74	12.81 ± 2.84	0.17	0.12	7.93 ± 2.36	7.50 ± 0.41	7.76 ± 0.70	12.81 ± 2.84	<b>0.38</b>	0.032
18:0	2.39 ± 0.83	3.71 ± 0.46	1.57 ± 0.76	1.27 ± 0.49	2.84 ± 0.42	0.05	0.45	0.86 ± 0.16	0.75 ± 0.28	0.62 ± 0.07	2.84 ± 0.42	<b>0.48</b>	0.013
Total	14.21 ± 5.83	14.57 ± 1.78	13.30 ± 1.50	17.17 ± 0.77	24.80 ± 4.11	<b>0.45</b>	6.29E-03	17.21 ± 5.44	20.62 ± 0.33	18.73 ± 2.95	24.80 ± 4.11	0.31	0.06
<b>Branched saturated</b>													
iso 14:0	0.63 ± 0.33	0.47 ± 0.03	0.19 ± 0.32	n.d.	0.68 ± 0.02	0.05	0.42	0.77 ± 0.26	0.42 ± 0.03	0.47 ± 0.05	0.68 ± 0.02	0.02	0.65
iso 15:0	18.34 ± 0.37	19.27 ± 0.81	22.86 ± 3.19	24.18 ± 0.55	22.77 ± 1.65	<b>0.54</b>	1.75E-03	26.47 ± 0.93	25.37 ± 1.24	30.48 ± 1.16	22.77 ± 1.65	0.05	0.48
anteiso 15:0	5.89 ± 0.07	4.59 ± 0.25	4.01 ± 0.43	2.67 ± 0.14	2.20 ± 0.20	<b>0.96</b>	3.88E-10	1.07 ± 0.11	2.06 ± 1.72	1.54 ± 0.15	2.20 ± 0.20	0.15	0.22
iso 16:0	1.07 ± 0.31	1.48 ± 0.19	1.19 ± 0.21	0.86 ± 0.75	0.98 ± 0.03	0.06	0.37	0.09 ± 0.16	n.d.	n.d.	0.98 ± 0.03	n.d.	n.d.
Total	25.94 ± 0.73	25.82 ± 1.22	28.24 ± 3.11	27.71 ± 1.08	26.63 ± 1.85	0.07	0.34	28.40 ± 1.19	27.85 ± 1.10	32.50 ± 1.27	26.63 ± 1.85	8.33E-04	0.93
<b>Normal unsaturated</b>													
15:1	1.41 ± 0.19	1.93 ± 0.18	1.92 ± 0.11	1.90 ± 0.09	1.86 ± 0.25	0.25	0.057	3.77 ± 0.70	2.70 ± 2.09	2.31 ± 0.41	1.86 ± 0.25	<b>0.35</b>	0.045
16:1	15.61 ± 0.35	15.58 ± 0.70	17.85 ± 1.34	17.88 ± 1.13	18.08 ± 0.63	<b>0.58</b>	1.05E-03	16.02 ± 2.56	15.49 ± 0.80	18.85 ± 0.87	18.08 ± 0.63	<b>0.34</b>	0.047
17:1	7.47 ± 2.93	10.86 ± 0.91	8.93 ± 0.81	4.95 ± 2.85	3.87 ± 1.81	<b>0.37</b>	0.017	4.00 ± 2.20	4.27 ± 0.87	3.15 ± 0.64	3.87 ± 1.81	0.017	0.69
18:1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	24.49 ± 3.29	28.37 ± 0.75	28.45 ± 2.23	24.74 ± 2.45	23.81 ± 1.10	0.07	0.34	23.80 ± 1.59	22.46 ± 2.10	24.32 ± 1.89	23.81 ± 1.10	0.019	0.67
<b>Branched unsaturated</b>													
iso 15:1	9.73 ± 0.43	8.16 ± 0.44	6.96 ± 0.67	4.54 ± 0.19	4.04 ± 0.14	<b>0.95</b>	8.90E-10	3.41 ± 0.38	3.23 ± 0.14	3.39 ± 0.23	4.04 ± 0.14	<b>0.38</b>	0.032
iso 16:1	0.22 ± 0.38	0.48 ± 0.42	0.24 ± 0.41	n.d.	n.d.	<b>0.56</b>	1.27E-03	n.d.	n.d.	n.d.	n.d.	0.09	0.14
Total	9.95 ± 0.54	8.64 ± 0.36	7.20 ± 0.33	4.54 ± 0.19	4.04 ± 0.14	<b>0.95</b>	4.29E-10	3.41 ± 0.38	3.23 ± 0.14	3.39 ± 0.23	4.04 ± 0.14	<b>0.38</b>	0.03
<b>Total non-hydroxy FAs</b>	<b>74.60 ± 2.86</b>	<b>77.40 ± 2.90</b>	<b>77.18 ± 5.06</b>	<b>74.15 ± 3.22</b>	<b>79.28 ± 5.00</b>	0.05	0.41	<b>72.81 ± 5.95</b>	<b>74.16 ± 1.55</b>	<b>78.93 ± 5.83</b>	<b>79.28 ± 5.00</b>	0.30	0.065

## Temperature/pH effect on Gram- negative bacteria lipids

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**Table 2.** Relative abundances of non-hydroxy fatty acids (%) vs. total fatty acids for strain B at different temperature conditions (pH 5) and at different pH conditions (temperature 25 °C). Data are means of three biological replicates. Values after (±) are standard deviations. Correlations of the relative abundances of each fatty acid (FA) with either temperature or pH are provided with corresponding *p*-values. Significant correlations (*p* < 0.005) are indicated in bold. n.d.: not detected.

Strain B	Temperature conditions (°C)							pH conditions					
	5 °C	10 °C	15 °C	20 °C	25 °C	R <sup>2</sup>	<i>p</i>	5	6	7	8	R <sup>2</sup>	<i>p</i>
<b>Normal saturated</b>													
15:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
16:0	11.80 ± 0.99	13.18 ± 3.99	12.13 ± 0.98	11.31 ± 1.59	12.09 ± 1.44	0.010	0.73	12.09 ± 1.44	13.94 ± 4.77	12.69 ± 1.02	17.08 ± 1.84	<b>0.27</b>	0.081
18:0	4.94 ± 1.54	5.90 ± 3.17	3.42 ± 0.41	6.19 ± 3.21	2.39 ± 0.62	<b>0.23</b>	0.28	2.39 ± 0.62	1.87 ± 0.87	2.40 ± 0.64	3.62 ± 0.67	0.30	0.067
Total	16.74 ± 2.53	19.07 ± 5.50	15.56 ± 1.39	17.50 ± 4.80	14.48 ± 2.06	0.83	0.35	14.48 ± 2.06	15.82 ± 3.91	15.08 ± 1.35	20.70 ± 2.28	0.38	0.032
<b>Branched saturated</b>													
iso 14:0	n.d.	2.80 ± 2.52	n.d.	n.d.	0.13 ± 0.22	n.d.	n.d.	0.13 ± 0.22	0.31 ± 0.53	0.22 ± 0.38	n.d.	0.26	0.092
iso 15:0	5.61 ± 0.37	7.09 ± 1.08	6.03 ± 0.32	7.27 ± 0.61	10.10 ± 1.00	<b>0.59</b>	7.83E-04	10.10 ± 1.00	6.35 ± 0.99	6.50 ± 1.22	9.23 ± 2.24	0.02	0.68
anteiso 15:0	2.52 ± 0.22	1.72 ± 1.54	1.49 ± 0.16	0.25 ± 0.44	0.23 ± 0.40	<b>0.81</b>	4.84E-06	0.23 ± 0.40	n.d.	0.14 ± 0.24	n.d.	<b>n.d.</b>	1.30E-02
iso 16:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	8.13 ± 0.59	11.60 ± 1.37	7.52 ± 0.20	7.53 ± 0.73	10.45 ± 1.62	0.002	0.88	10.45 ± 1.62	6.65 ± 0.48	6.85 ± 0.91	9.23 ± 2.24	0.04	0.55
<b>Normal unsaturated</b>													
15:1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
16:1	33.69 ± 1.43	33.71 ± 1.87	32.65 ± 2.33	33.11 ± 2.59	30.86 ± 1.29	0.21	0.083	30.86 ± 1.29	33.05 ± 1.30	31.54 ± 2.03	7.70 ± 0.78	<b>0.57</b>	4.68E-03
17:1	7.89 ± 1.70	4.49 ± 1.41	2.33 ± 0.28	n.d.	0.57 ± 0.52	<b>0.77</b>	1.85E-05	0.57 ± 0.52	0.46 ± 0.09	0.71 ± 0.05	n.d.	<b>0.05</b>	0.49
18:1	0.36 ± 0.34	n.d.	n.d.	n.d.	0.32 ± 0.29	0.09	0.29	0.32 ± 0.29	3.18 ± 5.50	n.d.	n.d.	n.d.	n.d.
Total	41.94 ± 0.38	38.20 ± 0.78	34.99 ± 2.04	33.11 ± 2.59	31.76 ± 2.05	<b>0.83</b>	2.31E-06	31.76 ± 2.05	36.69 ± 6.62	32.25 ± 1.99	7.70 ± 0.78	0.53	7.10E-03
<b>Branched unsaturated</b>													
iso 15:1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Iso 16:1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
<b>Total non-hydroxy FAs</b>	<b>66.82 ± 2.89</b>	<b>68.89 ± 6.23</b>	<b>58.06 ± 3.62</b>	<b>58.13 ± 6.64</b>	<b>56.69 ± 5.08</b>	<b>0.45</b>	6.54E-03	<b>56.69 ± 5.08</b>	<b>59.16 ± 10.09</b>	<b>54.18 ± 3.91</b>	<b>37.62 ± 0.10</b>	<b>0.51</b>	9.20E-03

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**Table 3.** Relative abundances of non-hydroxy fatty acids (%) vs. total fatty acids for strain C at different temperature conditions (pH 5) and at different pH conditions (temperature 25 °C). Data are means of three biological replicates. Values after (±) are standard deviations. Correlations of the relative abundances of each fatty acid (FA) with either temperature or pH are provided with corresponding *p*-values. Significant correlations (*p* < 0.005) are indicated in bold. n.d.: not detected.

Strain C	Temperature conditions (°C)							pH conditions					
	5 °C	10 °C	15 °C	20 °C	25 °C	R <sup>2</sup>	<i>p</i>	5	6	7	8	R <sup>2</sup>	<i>p</i>
<b>Normal saturated</b>													
15:0	n.d.	0.17 ± 0.29	n.d.	n.d.	n.d.	0.07	0.23	n.d.	0.35 ± 0.30	0.37 ± 0.32	0.48 ± 0.42	<b>0.41</b>	1.94E-05
16:0	11.82 ± 0.78	9.02 ± 1.96	7.06 ± 1.29	8.17 ± 0.72	6.69 ± 1.12	<b>0.56</b>	1.32E-03	6.69 ± 1.12	8.03 ± 1.97	18.15 ± 5.54	9.39 ± 2.66	0.15	0.21
18:0	10.58 ± 1.06	4.08 ± 0.81	1.85 ± 0.39	5.76 ± 0.59	6.20 ± 0.68	0.12	0.21	6.20 ± 0.68	1.28 ± 0.07	2.74 ± 1.13	1.47 ± 1.29	<b>0.45</b>	0.016
Total	22.40 ± 1.58	13.27 ± 2.55	8.91 ± 1.67	13.93 ± 1.14	12.89 ± 1.68	<b>0.31</b>	0.031	12.89 ± 1.68	9.31 ± 1.94	21.26 ± 5.38	11.34 ± 3.69	0.02	0.64
<b>Branched saturated</b>													
iso 14:0	n.d.	0.57 ± 0.52	0.33 ± 0.29	n.d.	0.66 ± 0.11	0.07	0.33	0.66 ± 0.11	0.35 ± 0.30	n.d.	n.d.	n.d.	n.d.
iso 15:0	5.13 ± 0.24	4.28 ± 3.71	5.29 ± 0.93	7.06 ± 3.48	8.96 ± 3.64	0.26	0.053	8.96 ± 3.64	8.00 ± 2.11	8.99 ± 0.54	11.69 ± 1.07	0.21	0.14
anteiso 15:0	2.20 ± 0.23	3.85 ± 3.45	0.82 ± 0.73	n.d.	0.45 ± 0.02	0.23	0.070	0.45 ± 0.02	n.d.	0.53 ± 0.04	0.63 ± 0.04	<b>0.85</b>	2.18E-05
iso 16:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	7.33 ± 0.29	8.70 ± 0.76	6.44 ± 1.02	7.06 ± 3.48	10.06 ± 3.56	0.06	0.40	10.06 ± 3.56	8.35 ± 2.23	9.34 ± 0.85	12.33 ± 1.12	0.14	0.23
<b>Normal unsaturated</b>													
15:1	n.d.	n.d.	n.d.	n.d.	n.d.	0.03	0.41	n.d.	n.d.	n.d.	0.22 ± 0.38	n.d.	n.d.
16:1	26.58 ± 1.29	30.16 ± 4.05	26.96 ± 2.18	33.36 ± 5.15	29.27 ± 4.60	0.10	0.261	29.27 ± 4.60	32.75 ± 3.00	32.22 ± 1.98	38.65 ± 4.60	<b>0.46</b>	0.016
17:1	3.26 ± 1.52	5.64 ± 0.66	2.77 ± 2.42	0.95 ± 1.63	2.04 ± 1.12	0.24	0.062	2.04 ± 1.12	2.39 ± 0.13	2.95 ± 0.32	4.19 ± 0.47	<b>0.66</b>	1.32E-03
18:1	n.d.	n.d.	n.d.	1.02 ± 1.77	n.d.	0.07	0.21	n.d.	n.d.	0.33 ± 0.05	0.64 ± 0.16	n.d.	n.d.
Total	29.84 ± 0.29	35.80 ± 3.97	29.73 ± 0.68	35.33 ± 7.20	31.31 ± 3.52	0.007	0.77	31.31 ± 3.52	35.14 ± 2.90	35.40 ± 2.41	43.49 ± 5.12	<b>0.59</b>	3.33E-03
<b>Branched unsaturated</b>													
iso 15:1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
iso 16:1	n.d.	0.21 ± 0.37	n.d.	n.d.	n.d.	0.07	0.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	n.d.	0.21 ± 0.37	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
<b>Total non-hydroxy FAs</b>	<b>59.58 ± 1.46</b>	<b>57.99 ± 5.08</b>	<b>45.08 ± 0.48</b>	<b>56.32 ± 7.35</b>	<b>54.27 ± 8.56</b>	<b>0.06</b>	<b>0.36</b>	<b>54.27 ± 8.56</b>	<b>52.80 ± 5.43</b>	<b>66.01 ± 3.85</b>	<b>67.16 ± 3.07</b>	<b>0.52</b>	<b>7.74E-03</b>

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**Table 4.** Relative abundances of individual 3-hydroxy fatty acids (%) vs. total 3-hydroxy fatty acids and related indices (defined by Wang et al., 2016) for strain A at different temperature conditions (pH 8) and at different pH conditions (temperature 25 °C). Data are means of three biological replicates. Values after ( $\pm$ ) are standard deviations. Correlations of the relative abundances of each 3-hydroxy fatty acid with either temperature or pH are provided with corresponding  $p$ -values. Significant correlations ( $p < 0.005$ ) are indicated in bold. n.d.: not detected.

Strain A	Temperature conditions (°C)							pH conditions					
	5 °C	10 °C	15 °C	20 °C	25 °C	R <sup>2</sup>	$p$	5	6	7	8	R <sup>2</sup>	$p$
iso C <sub>13</sub>	0.15 ± 0.006	0.28 ± 0.02	0.33 ± 0.06	0.42 ± 0.03	0.48 ± 0.03	<b>0.91</b>	2.59E-08	1.15 ± 0.36	0.79 ± 0.08	0.54 ± 0.21	0.48 ± 0.03	<b>0.64</b>	1.73E-03
iso C <sub>14</sub>	0.52 ± 0.03	0.45 ± 0.04	0.44 ± 0.15	0.37 ± 0.04	0.31 ± 0.001	<b>0.55</b>	1.45E-03	n.d.	0.09 ± 0.08	0.21 ± 0.07	0.31 ± 0.001	<b>0.88</b>	7.93E-06
normal C <sub>14</sub>	2.88 ± 0.15	3.06 ± 0.45	3.22 ± 0.31	3.69 ± 0.08	6.35 ± 0.88	<b>0.64</b>	3.51E-04	7.81 ± 2.25	4.66 ± 0.52	5.03 ± 1.05	6.35 ± 0.88	0.07	0.39
iso C <sub>15</sub>	46.17 ± 1.43	53.41 ± 5.32	56.37 ± 10.02	54.64 ± 4.40	49.02 ± 6.17	0.02	0.58	65.92 ± 7.93	64.32 ± 4.78	59.69 ± 9.43	49.02 ± 6.17	<b>0.48</b>	0.012
anteiso C <sub>15</sub>	3.74 ± 0.16	3.08 ± 0.26	2.45 ± 0.29	1.88 ± 0.22	1.98 ± 0.19	<b>0.85</b>	1.10E-6	0.61 ± 0.11	0.71 ± 0.09	1.28 ± 0.34	1.98 ± 0.19	<b>0.84</b>	2.74E-05
normal C <sub>15</sub>	0.72 ± 0.008	0.79 ± 0.08	0.81 ± 0.28	1.10 ± 0.05	1.60 ± 0.08	<b>0.73</b>	4.78E-05	2.35 ± 0.32	2.41 ± 0.03	1.61 ± 0.28	1.60 ± 0.08	<b>0.64</b>	1.79E-03
iso C <sub>16</sub>	6.92 ± 0.62	4.27 ± 0.89	3.77 ± 0.28	3.14 ± 0.54	2.47 ± 0.68	<b>0.68</b>	1.42E-04	0.58 ± 0.35	0.71 ± 0.10	1.07 ± 0.32	2.47 ± 0.68	<b>0.67</b>	1.09E-03
normal C <sub>16</sub>	7.10 ± 0.21	5.88 ± 0.76	5.53 ± 2.43	7.44 ± 1.64	14.45 ± 2.69	<b>0.41</b>	9.94E-03	10.20 ± 1.90	8.94 ± 1.58	12.29 ± 5.48	14.45 ± 2.69	0.28	0.078
iso C <sub>17</sub>	26.93 ± 0.79	26.30 ± 3.79	25.40 ± 3.92	25.95 ± 2.23	21.89 ± 3.66	0.23	0.073	10.84 ± 7.68	16.33 ± 3.67	17.17 ± 5.92	21.89 ± 3.66	<b>0.41</b>	0.026
anteiso C <sub>17</sub>	4.81 ± 0.30	2.44 ± 0.53	1.62 ± 0.86	1.05 ± 0.19	0.75 ± 0.23	<b>0.79</b>	8.29E-06	0.13 ± 0.12	0.32 ± 0.05	0.56 ± 0.13	0.75 ± 0.23	<b>0.79</b>	1.07E-04
normal C <sub>17</sub>	0.07 ± 0.06	0.04 ± 0.07	0.07 ± 0.12	0.32 ± 0.04	0.70 ± 0.05	<b>0.71</b>	7.94E-05	0.42 ± 0.37	0.72 ± 0.13	0.54 ± 0.07	0.70 ± 0.05	0.14	0.23
normal C <sub>18</sub>	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total iso 3-OH FAs	80.69 ± 0.16	84.71 ± 0.74	86.31 ± 4.27	84.52 ± 1.75	74.17 ± 1.88	0.16	0.15	78.49 ± 1.03	82.24 ± 1.18	78.69 ± 3.85	74.17 ± 1.88	0.29	0.070
Total anteiso 3-OH FAs	8.55 ± 0.13	5.52 ± 0.29	4.07 ± 1.15	2.93 ± 0.35	2.72 ± 0.05	<b>0.85</b>	1.15E-06	0.73 ± 0.02	1.04 ± 0.05	1.84 ± 0.35	2.72 ± 0.05	<b>0.93</b>	5.20E-07
Total normal 3-OH FAs	10.77 ± 0.13	9.77 ± 0.45	9.62 ± 3.13	12.56 ± 1.61	23.10 ± 1.86	<b>0.54</b>	1.81E-03	20.78 ± 1.04	16.72 ± 1.22	19.47 ± 4.20	23.10 ± 1.86	0.12	0.25
Iso/anteiso 3-OH FAs	9.44 ± 0.15	15.39 ± 0.97	22.42 ± 6.39	29.16 ± 3.75	27.27 ± 1.06	<b>0.79</b>	1.05E-05	107.0 ± 2.81	79.48 ± 3.30	43.57 ± 7.11	27.27 ± 1.06	<b>0.97</b>	6.22E-09
Iso/normal 3-OH FAs	7.50 ± 0.10	8.68 ± 0.49	9.65 ± 3.17	6.82 ± 1.06	3.23 ± 0.36	<b>0.34</b>	0.023	3.79 ± 0.23	4.94 ± 0.45	4.19 ± 1.06	3.23 ± 0.36	0.11	0.28
Anteiso/normal 3-OH FAs	0.79 ± 0.02	0.56 ± 0.005	0.43 ± 0.03	0.24 ± 0.04	0.12 ± 0.009	<b>0.98</b>	6.79E-13	0.04 ± 0.02	0.06 ± 0.008	0.10 ± 0.04	0.12 ± 0.009	<b>0.79</b>	1.05E-04
RIAN	-0.92 ± 0.006	-0.97 ± 0.02	-0.99 ± 0.15	-0.85 ± 0.07	-0.52 ± 0.05	<b>0.51</b>	2.87E-03	-0.58 ± 0.03	-0.70 ± 0.04	-0.62 ± 0.12	-0.52 ± 0.05	0.11	0.29
RAN <sub>15</sub>	5.22 ± 0.27	3.96 ± 0.75	3.17 ± 0.67	1.70 ± 0.16	1.24 ± 0.14	<b>0.92</b>	2.31E-08	0.26 ± 0.07	0.30 ± 0.04	0.79 ± 0.08	1.24 ± 0.14	<b>0.88</b>	5.57E-06
RAN <sub>17</sub>	44.80 ± 2.33	24.44	12.51	3.30 ± 0.21	1.07 ± 0.34	<b>0.92</b>	1.26E-08	0.31 ± 0.03	0.46 ± 0.02	1.02 ± 0.10	1.07 ± 0.34	<b>0.73</b>	4.20E-04

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**Table 5.** Relative abundances of individual 3-hydroxy fatty acids (%) vs. total 3-hydroxy fatty acids and related indices (defined by Wang et al., 2016) for strain B at different temperature conditions (pH 5) and at different pH conditions (temperature 25 °C). Data are means of three biological replicates. Values after ( $\pm$ ) are standard deviations. Correlations of the relative abundances of each 3-hydroxy fatty acid with either temperature or pH are provided with corresponding  $p$ -values. Significant correlations ( $p < 0.005$ ) are indicated in bold. n.d.: not detected.

Strain B	Temperature conditions (°C)							pH conditions					
	5 °C	10 °C	15 °C	20 °C	25 °C	R <sup>2</sup>	$p$	5	6	7	8	R <sup>2</sup>	$p$
iso C <sub>13</sub>	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
iso C <sub>14</sub>	0.19 $\pm$ 0.06	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
normal C <sub>14</sub>	3.70 $\pm$ 0.62	3.89 $\pm$ 0.47	4.57 $\pm$ 1.04	4.66 $\pm$ 1.12	4.08 $\pm$ 1.18	0.07	0.36	4.08 $\pm$ 1.18	16.07 $\pm$ 17.17	4.53 $\pm$ 0.51	5.37 $\pm$ 0.46	0.010	0.76
iso C <sub>15</sub>	13.93 $\pm$ 3.19	22.37 $\pm$ 0.47	21.24 $\pm$ 3.39	28.68 $\pm$ 6.91	35.18 $\pm$ 5.23	<b>0.72</b>	6.29E-05	35.18 $\pm$ 5.23	29.61 $\pm$ 0.85	24.80 $\pm$ 3.99	31.27 $\pm$ 4.10	0.08	0.35
anteiso C <sub>15</sub>	14.61 $\pm$ 3.09	15.57 $\pm$ 1.77	10.24 $\pm$ 2.26	6.31 $\pm$ 1.73	2.55 $\pm$ 0.64	<b>0.82</b>	3.12E-06	2.55 $\pm$ 0.64	3.56 $\pm$ 0.85	3.15 $\pm$ 0.80	3.43 $\pm$ 0.25	0.14	0.24
normal C <sub>15</sub>	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
iso C <sub>16</sub>	2.38 $\pm$ 0.27	1.40 $\pm$ 0.30	0.88 $\pm$ 0.11	0.37 $\pm$ 0.14	0.03 $\pm$ 0.05	<b>0.93</b>	9.88E-09	0.03 $\pm$ 0.05	n.d.	n.d.	n.d.	n.d.	n.d.
normal C <sub>16</sub>	20.44 $\pm$ 1.59	14.45 $\pm$ 1.26	18.51 $\pm$ 1.48	13.87 $\pm$ 2.03	9.77 $\pm$ 0.30	<b>0.62</b>	4.59E-04	9.77 $\pm$ 0.30	13.11 $\pm$ 1.98	13.71 $\pm$ 0.91	12.34 $\pm$ 0.95	0.27	0.08
iso C <sub>17</sub>	18.76 $\pm$ 1.50	21.28 $\pm$ 2.06	29.04 $\pm$ 2.73	36.42 $\pm$ 6.20	45.15 $\pm$ 6.57	<b>0.86</b>	7.20E-07	45.15 $\pm$ 6.57	32.63 $\pm$ 4.49	47.44 $\pm$ 4.38	42.78 $\pm$ 2.62	0.016	0.70
anteiso C <sub>17</sub>	25.53 $\pm$ 3.49	20.25 $\pm$ 1.95	14.96 $\pm$ 2.35	8.71 $\pm$ 1.39	2.43 $\pm$ 0.44	<b>0.95</b>	5.19E-10	2.43 $\pm$ 0.44	4.02 $\pm$ 0.46	4.99 $\pm$ 0.49	3.79 $\pm$ 0.50	0.32	0.053
normal C <sub>17</sub>	0.18 $\pm$ 0.08	0.54 $\pm$ 0.94	0.16 $\pm$ 0.06	0.36 $\pm$ 0.14	0.07 $\pm$ 0.06	0.02	0.61	0.07 $\pm$ 0.06	n.d.	n.d.	n.d.	<b>0.36</b>	0.039
normal C <sub>18</sub>	0.29 $\pm$ 0.02	0.25 $\pm$ 0.21	0.40 $\pm$ 0.005	0.63 $\pm$ 0.09	0.74 $\pm$ 0.15	<b>0.70</b>	1.08E-03	0.74 $\pm$ 0.15	1.01 $\pm$ 0.29	1.37 $\pm$ 0.13	1.02 $\pm$ 0.12	0.25	0.097
Total iso 3-OH FAs	35.26 $\pm$ 1.50	45.05 $\pm$ 1.65	51.16 $\pm$ 0.60	65.47 $\pm$ 0.71	80.36 $\pm$ 1.31	<b>0.97</b>	2.19E-11	80.36 $\pm$ 1.31	62.23 $\pm$ 13.74	72.25 $\pm$ 1.61	74.05 $\pm$ 1.70	0.01	0.72
Total anteiso 3-OH FAs	40.13 $\pm$ 0.41	35.82 $\pm$ 1.04	25.20 $\pm$ 0.09	15.02 $\pm$ 0.40	4.97 $\pm$ 0.52	<b>0.98</b>	7.88E-13	4.97 $\pm$ 0.52	7.58 $\pm$ 1.24	8.14 $\pm$ 0.76	7.22 $\pm$ 0.27	<b>0.36</b>	0.04
Total normal 3-OH FAs	24.60 $\pm$ 1.11	19.13 $\pm$ 1.35	23.64 $\pm$ 0.52	19.51 $\pm$ 0.95	14.66 $\pm$ 0.95	<b>0.57</b>	1.12E-03	14.66 $\pm$ 0.95	30.18 $\pm$ 14.97	19.61 $\pm$ 1.07	18.73 $\pm$ 1.43	4.7E-04	0.95
Iso/Anteiso 3-OH FAs	0.88 $\pm$ 0.05	1.26 $\pm$ 0.07	2.03 $\pm$ 0.03	4.36 $\pm$ 0.11	16.29 $\pm$ 1.97	<b>0.68</b>	1.69E-04	16.29 $\pm$ 1.97	8.15 $\pm$ 0.55	8.94 $\pm$ 0.06	10.28 $\pm$ 0.62	<b>0.33</b>	0.05
Iso/normal 3-OH FAs	1.44 $\pm$ 0.13	2.37 $\pm$ 0.25	2.17 $\pm$ 0.07	3.36 $\pm$ 0.20	5.50 $\pm$ 0.44	<b>0.82</b>	3.23E-06	5.50 $\pm$ 0.44	2.50 $\pm$ 1.32	3.69 $\pm$ 0.27	3.97 $\pm$ 0.39	0.10	0.33
Anteiso/normal 3-OH FAs	1.63 $\pm$ 0.06	1.88 $\pm$ 0.15	1.07 $\pm$ 0.02	0.77 $\pm$ 0.06	0.34 $\pm$ 0.03	<b>0.86</b>	7.36E-07	0.34 $\pm$ 0.03	0.30 $\pm$ 0.15	0.42 $\pm$ 0.03	0.39 $\pm$ 0.01	0.14	0.24
RIAN	-0.49 $\pm$ 0.03	-0.63 $\pm$ 0.04	-0.51 $\pm$ 0.01	-0.62 $\pm$ 0.03	-0.77 $\pm$ 0.03	<b>0.57</b>	1.08E-03	-0.77 $\pm$ 0.03	-0.39 $\pm$ 0.30	-0.61 $\pm$ 0.03	-0.64 $\pm$ 0.04	0.009	0.77
RAN <sub>15</sub>	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
RAN <sub>17</sub>	162.0 $\pm$ 69.0	n.d.	103.7 $\pm$ 37.4	27.89 $\pm$ 13.98	24.38 $\pm$ 2.65	<b>0.51</b>	2.92E-03	24.38 $\pm$ 2.65	n.d.	n.d.	n.d.	n.d.	n.d.

## Temperature/pH effect on Gram- negative bacteria lipids

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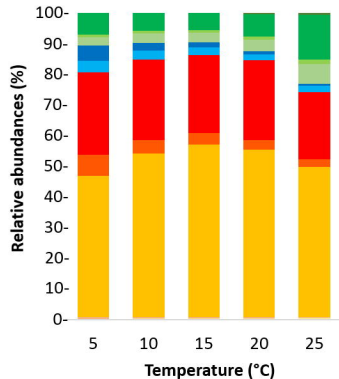
**Table 6.** Relative abundances of individual 3-hydroxy fatty acids (%) vs. total 3-hydroxy fatty acids and related indices (defined by Wang et al., 2016) for strain C at different temperature conditions (pH 8) and at different pH conditions (temperature 25 °C). Data are means of three biological replicates. Values after ( $\pm$ ) are standard deviations. Correlations of the relative abundances of each 3-hydroxy fatty acid with either temperature or pH are provided with corresponding  $p$ -values. Significant correlations ( $p < 0.005$ ) are indicated in bold. n.d.: not detected.

Strain C	Temperature conditions (°C)							pH conditions					
	5 °C	10 °C	15 °C	20 °C	25 °C	R <sup>2</sup>	$p$	5	6	7	8	R <sup>2</sup>	$p$
iso C <sub>13</sub>	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
iso C <sub>14</sub>	0.31 ± 0.04	0.34 ± 0.14	0.16 ± 0.01	0.10 ± 0.09	0.03 ± 0.06	<b>0.69</b>	1.39E-04	0.03 ± 0.06	0.12 ± 0.06	0.23 ± 0.06	0.27 ± 0.04	<b>0.79</b>	1.24E-04
normal C <sub>14</sub>	0.79 ± 0.17	1.25 ± 0.54	1.32 ± 0.33	2.01 ± 0.26	2.48 ± 1.40	<b>0.50</b>	3.37E-03	2.48 ± 1.40	3.55 ± 1.29	3.11 ± 0.64	3.17 ± 0.62	0.04	0.54
iso C <sub>15</sub>	9.98 ± 1.84	16.57 ± 4.20	13.60 ± 0.82	24.36 ± 5.12	37.08 ± 9.96	<b>0.68</b>	1.41E-04	37.08 ± 9.96	36.44 ± 5.78	22.08 ± 2.60	26.93 ± 4.94	<b>0.37</b>	0.036
anteiso C <sub>15</sub>	23.04 ± 5.28	26.57 ± 5.97	17.95 ± 1.64	14.45 ± 3.63	6.45 ± 3.19	<b>0.67</b>	1.73E-04	6.45 ± 3.19	6.33 ± 1.00	4.55 ± 0.49	6.16 ± 1.26	0.03	0.58
normal C <sub>15</sub>	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.14 ± 0.25	0.13 ± 0.02	0.17 ± 0.07	0.19	0.15
iso C <sub>16</sub>	1.78 ± 0.23	1.54 ± 0.32	1.28 ± 0.13	1.40 ± 0.39	0.91 ± 0.05	<b>0.55</b>	1.47E-03	0.91 ± 0.05	1.02 ± 0.40	2.29 ± 0.18	1.79 ± 0.34	<b>0.51</b>	8.88E-03
normal C <sub>16</sub>	1.91 ± 0.48	1.54 ± 0.49	2.09 ± 0.30	3.77 ± 1.64	5.58 ± 1.78	<b>0.58</b>	9.16E-04	5.58 ± 1.78	5.03 ± 1.13	8.26 ± 0.52	7.54 ± 1.41	<b>0.36</b>	0.040
iso C <sub>17</sub>	12.65 ± 2.68	13.85 ± 2.78	19.64 ± 0.83	28.86 ± 2.92	34.01 ± 7.05	<b>0.83</b>	2.03E-06	34.01 ± 7.05	37.15 ± 7.24	48.26 ± 1.93	43.61 ± 3.99	<b>0.39</b>	0.029
anteiso C <sub>17</sub>	48.96 ± 3.95	38.19 ± 8.68	43.64 ± 1.73	24.11 ± 4.32	10.81 ± 5.00	<b>0.77</b>	1.61E-05	10.81 ± 5.00	9.65 ± 1.40	10.21 ± 1.93	9.25 ± 1.13	0.04	0.55
normal C <sub>17</sub>	0.50 ± 0.26	0.14 ± 0.02	0.19 ± 0.08	0.76 ± 1.07	2.41 ± 1.25	<b>0.37</b>	0.016	2.41 ± 1.25	0.30 ± 0.27	0.48 ± 0.26	0.71 ± 0.07	0.31	0.062
normal C <sub>18</sub>	0.07 ± 0.06	n.d.	0.12 ± 0.03	0.18 ± 0.03	0.24 ± 0.10	<b>0.59</b>	8.08E-04	0.24 ± 0.10	0.27 ± 0.03	0.40 ± 0.05	0.41 ± 0.09	<b>0.53</b>	7.21E-03
Total iso 3-OH FAs	24.71 ± 1.84	32.30 ± 2.07	34.69 ± 0.80	54.72 ± 3.29	72.03 ± 3.52	<b>0.90</b>	5.75E-08	72.03 ± 3.52	74.73 ± 1.25	72.85 ± 2.42	72.60 ± 0.89	7.99E-5	0.98
Total anteiso 3-OH FAs	72.00 ± 2.27	64.76 ± 3.05	61.59 ± 1.00	38.56 ± 0.71	17.26 ± 7.54	<b>0.88</b>	1.97E-07	17.26 ± 7.54	15.97 ± 0.50	14.76 ± 2.25	15.41 ± 0.18	0.05	0.48
Total normal 3-OH FAs	3.28 ± 0.57	2.94 ± 0.98	3.72 ± 0.22	6.72 ± 2.75	10.71 ± 4.18	<b>0.57</b>	1.12E-03	10.71 ± 4.18	9.30 ± 1.75	12.38 ± 0.18	11.99 ± 0.93	0.12	0.27
Iso/anteiso 3-OH FAs	0.34 ± 0.04	0.50 ± 0.06	0.56 ± 0.02	1.42 ± 0.11	5.09 ± 3.17	<b>0.48</b>	4.41E-03	5.09 ± 3.17	4.68 ± 0.07	5.03 ± 0.91	4.71 ± 0.08	4.23E-3	0.84
Iso/normal 3-OH FAs	7.63 ± 0.97	11.61 ± 2.82	9.33 ± 0.37	9.07 ± 3.40	7.26 ± 2.08	0.04	0.49	7.26 ± 2.08	8.26 ± 1.76	5.89 ± 0.28	6.08 ± 0.55	0.20	0.15
Anteiso/normal 3-OH FAs	22.46 ± 4.64	23.80 ± 7.98	16.59 ± 1.20	6.29 ± 2.04	1.92 ± 1.18	<b>0.78</b>	1.33E-05	1.92 ± 1.18	1.77 ± 0.40	1.19 ± 0.16	1.29 ± 0.11	0.21	0.14
RIAN	-1.47 ± 0.08	-1.53 ± 0.14	-1.41 ± 0.03	-1.16 ± 0.18	-0.94 ± 0.18	<b>0.67</b>	1.74E-04	-0.94 ± 0.18	-0.99 ± 0.09	-0.85 ± 0.007	-0.87 ± 0.04	0.16	0.19
RAN <sub>15</sub>	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	17.39	34.49 ± 7.10	40.13 ± 11.40	<b>0.45</b>	0.017
RAN <sub>17</sub>	126.5 ± 86.2	275.4 ± 91.9	258.3 ± 127.3	46.6 ± 45.6	6.27 ± 5.46	0.25	0.057	6.27 ± 5.46	22.98 ± 2.18	23.58 ± 7.50	13.10 ± 0.33	0.11	0.29

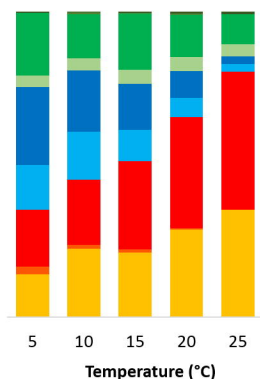
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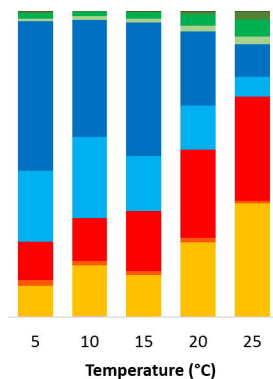
Strain A



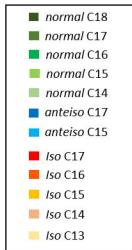
Strain B

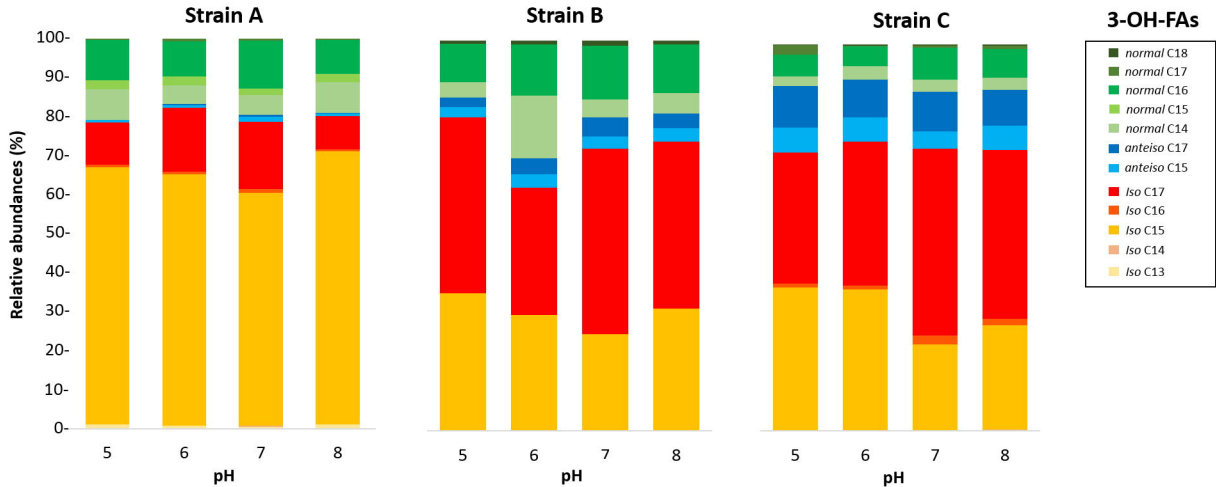


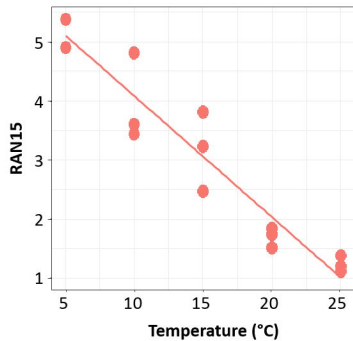
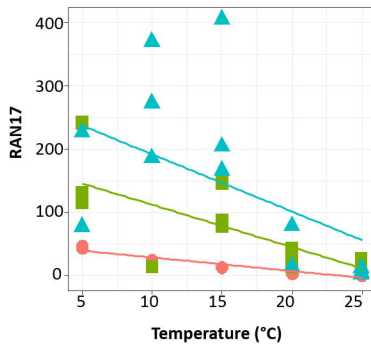
Strain C



3-OH-FAs





**(A)****(B)****(C)**