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1           **Identification and quantification of natural compounds produced by**  
2                           **antifungal bioprotective cultures in dairy products**

3

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17

18 ABSTRACT

19

20 In the context of growing consumer demand for clean label foods, antifungal cultures  
21 offer alternatives to chemical preservatives for the reduction of food fungal spoilage. Selected  
22 binary combinations of lactobacilli strains were recently successfully used to inhibit  
23 *Penicillium commune* and *Mucor racemosus* in four dairy products. Our aim was to identify  
24 the compounds most likely involved in their antifungal activity. Four chromatographic  
25 methods, targeting 56 antifungal compounds as well as volatiles, were combined. Overall, 53  
26 antifungal compounds were detected, of which 33 were in significantly higher amounts in at  
27 least one product inoculated with an antifungal culture compared to the controls. They were  
28 present at concentrations below their MIC and thus could act in synergy. Among them, the  
29 most commonly identified were acetic, hydroxyphenyllactic, phenyllactic, 3-phenylpropanoic,  
30 3-(4-hydroxyphenyl)propanoic and 5-oxopyrrolidine-2-carboxylic acids, diacetyl, acetoin,  
31 and an unidentified volatile. This extensive study contributes to improve the knowledge about  
32 the action mode of antifungal lactobacilli.

33

34 **Keywords:** fatty acids; organic acids; volatile compounds; antifungal bioprotective cultures;  
35 sour cream; yogurt, cheese.

## 36        **1. Introduction**

37

38        Dairy products constitute a favorable environment for the growth of fungal contaminants  
39 that leads to food waste, economic losses and brand image deterioration (Pitt & Hocking,  
40 2009). Molds and yeasts frequently found in contaminated dairy products belong to the  
41 *Penicillium*, *Mucor* and *Cladosporium*, and *Candida*, *Kluyveromyces* and *Yarrowia* genera,  
42 respectively (Garnier et al., 2016; Pitt & Hocking, 2009).

43        To control fungal contaminants in the dairy industry, both preventive and control methods  
44 are used. The hurdle technologies are implemented to prevent contamination or  
45 recontamination during product manufacturing and/or to inhibit or slow down the growth of  
46 fungal contaminants. During processing and storage, good hygiene practices, a hazard  
47 analysis critical control point (HACCP) system, air and surfaces decontamination, cleanroom  
48 technologies, heat treatment, refrigeration and brining can prevent and control contamination  
49 (Fellows, 2000). In addition, additives such as benzoate, propionate, sorbate and natamycin  
50 can be added to dairy products to extend their shelf-life, according to the relevant legislation  
51 (Silva & Lidon, 2016). However, consumer preference for preservative-free, more natural and  
52 less processed food has led to the study of preservation alternatives such as biopreservation.  
53 Biopreservation is the use of microorganisms (bioprotective cultures) or their metabolites  
54 exhibiting antimicrobial activities to extend food shelf-life and/or increase food safety (Stiles,  
55 1996).

56        Lactic acid bacteria (LAB) are good candidates for biopreservation, as they have a long  
57 history of safe use in various dairy products, such as fermented milks, kefir, or cheese (Stiles,  
58 1996). In this context, several antifungal bioprotective LAB cultures are available on the  
59 market to be used in dairy products (e.g., Holdbac (DuPont Danisco), FreshQ (CHR Hansen)).  
60 The production of antifungal molecules and pH decrease are the main factors contributing to

61 LAB antifungal activity in foods (Leyva Salas et al., 2017). LAB produce lactic and acetic  
62 acid as the main fermentation end-products. Lactic acid *per se*, despite being produced at high  
63 quantities, is not very effective against spoilage fungi in contrast to acetic acid (Dagnas,  
64 Gauvry, Onno, & Membré, 2015). Nevertheless, acetic acid minimal inhibitory concentrations  
65 (MIC) are increased in the presence of lactic acid, indicating that they can act in synergy  
66 (Dagnas et al., 2015). Despite this, the latter mentioned acids are not sufficient to explain the  
67 antifungal activity of LAB. LAB can also produce a variety of compounds at low  
68 concentrations and below their individual MIC, which are also likely to act in synergy with  
69 lactic and acetic acids (Dagnas et al., 2015; Honoré et al., 2016). The nature of the antifungal  
70 compounds corresponds to other organic acids, fatty acids, cyclopeptides, reuterin, hydrogen  
71 peroxide, and volatile compounds such as diacetyl (Crowley, Mahony, & van Sinderen, 2013;  
72 Leyva Salas et al., 2017; Sjogren, Magnusson, Broberg, Schnurer, & Kenne, 2003).

73 In a recent study, 3 binary combinations of lactobacilli with antifungal activity were  
74 selected after an *in vitro* screening in yogurt and cheese models and the applicability of two of  
75 them as bioprotective cultures in dairy products was validated at a pilot-scale after  
76 incorporation in sour cream and semi-hard cheese production (Leyva Salas et al., 2018). The  
77 aim of the present study was to identify the compounds potentially responsible for their  
78 observed antifungal activity in these four dairy products. We hypothesized that a few  
79 compounds would be specifically identified in the dairy products inoculated with the  
80 bioprotective cultures. Different analytical methods, including liquid and gas chromatography  
81 coupled to mass spectrometry, were applied to detect and quantify antifungal compounds,  
82 followed by statistical analyses.

83

## 84 **2. Materials and methods**

85

86           2.1. *Microorganisms and culture conditions*

87

88           The strains constituting the antifungal cultures, namely, *Lactobacillus plantarum* L244,  
89 *Lactobacillus harbinensis* L172, *Lactobacillus brevis* L128 and *L. rhamnosus* CIRM-BIA113  
90 were obtained from the Université de Bretagne Occidentale (UBOCC, France) and CIRM-  
91 BIA (INRA, France) culture collections. Previously selected A1, A2 and A3 antifungal  
92 cultures corresponded to binary combination of *L. plantarum* L244 and either *L. harbinensis*  
93 L172, *L. rhamnosus* CIRM-BIA1113, or *L. brevis* L128, respectively. Moreover, three strains  
94 isolated on de Man-Rogosa-Sharpe (MRS) agar from commercial bioprotective cultures  
95 (*Lactobacillus paracasei* CIRM-BIA1759 and *Lactobacillus rhamnosus* CIRM-BIA1761  
96 from commercial culture CC1, and *L. plantarum* CIRM-BIA1758 from commercial culture  
97 CC2) were used as reference antifungal strains. Culture X1 corresponded to a 1:1 combination  
98 of *L. paracasei* CIRM-BIA1759 and *L. rhamnosus* CIRM-BIA1761 strains, while culture X2  
99 corresponded to *L. plantarum* CIRM-BIA1758 alone. Another commercial culture (CC3) was  
100 also used as detailed in Leyva Salas et al. (2018). All strains were stored at –80 °C in MRS  
101 broth (BD Difco laboratories, Sparks, MD) supplemented with glycerol (20%) (Thermo  
102 Fisher Scientific, Waltham, MA). Before inoculation as adjunct cultures, strains were  
103 cultivated twice in MRS broth at 30 °C for 24 h. Culture concentration was then adjusted to  
104 the target concentrations using sodium chloride peptone broth (Merck, Darmstadt, Germany).  
105 After centrifugation, the cells were suspended in sterile skimmed milk.

106

107           2.2. *Dairy products preparation*

108

109           The cheese model and yogurt were prepared as previously reported (Leyva Salas et al.,  
110 2018) with the antifungal cultures A1, A2, A3, X1 and X2 added at  $2 \times 10^7$  colony forming

111 units (CFU)/mL together with the starter cultures. The cheese model was prepared as detailed  
112 by Garnier et al. (2018). Briefly, 10 mL of heat-treated, salted (0.7%, w/w), ultrafiltered milk  
113 retentate was poured into 60-mL sterile polypropylene containers. Chymosin rennet (1.5  
114 mL/L of 5× diluted and 0.22 µm filtered Maxiren 180; DSM Food Specialties, The  
115 Netherlands), starter culture MA016 (*Lactococcus lactis* subsp. *cremoris* and *L. lactis* subsp.  
116 *lactis*, Elimeca, Thoissey, France) and cell suspensions in milk of the antifungal strains at a  
117 final concentration of 10<sup>7</sup> CFU/mL of each strain forming the binary combinations was added  
118 to the cheese. The containers were then incubated for 1 h at 30 °C then for 3 days at 20 °C,  
119 leading to a final pH of 4.8 ± 0.1. The yogurt was prepared from semi-skimmed milk (Candia,  
120 France) supplemented with skimmed milk powder at 4% (Carrefour, France). After heat  
121 treatment for 30 min at 85 °C, milk was rapidly cooled to 45 °C before adding 0.1 g/L of the  
122 lyophilized starter culture MY800 (*Streptococcus thermophilus*, *Lactobacillus delbrueckii*  
123 subsp. *lactis* and *L. delbrueckii* subsp. *bulgaricus*, Choozit, Danisco, Sassenage, France).  
124 After homogenization, the preparation was distributed into sterile polypropylene containers  
125 (10 mL/container) and the antifungal strain suspensions were added under the same  
126 conditions as for the cheese model. Containers were then incubated at 42 °C for 4 h, until pH  
127 4.97 ± 0.03 was reached. At the end of the yogurt and cheese model fermentation, the exudate  
128 formed was removed and samples were stored at -20°C.

129 Samples from sour cream and semi-hard cheese produced at pilot scale with the A1, A3,  
130 X1 or CC3 antifungal cultures in the previous study (Leyva Salas et al., 2018) were stored at  
131 -20 °C before analysis. Sour cream (final fat 35%) was produced from pasteurized (75 °C for  
132 20 s) cow milk, as previously described by Leyva Salas et al. (2018). It was inoculated with  
133 the Choozit MM100 starter culture (Danisco, Sassenage, France) at 3 U/100 L and the  
134 antifungal combinations A1 or A3 (at either 10<sup>6</sup>, 5.10<sup>6</sup> or 2.10<sup>7</sup> CFU/mL) or the X1  
135 combination (10<sup>6</sup> CFU/mL) before packaging in 100-g plastic tubs with removable lids and

136 incubated for 22 h at 23 °C. Sour cream samples were stored at –20 °C before antifungal  
137 molecules analyses. Semi-hard cheese was manufactured from pasteurized (72 °C for 20 s),  
138 standardized cow milk as previously described (Leyva Salas et al., 2018). Antifungal cultures  
139 were added at  $5 \times 10^6$  CFU/mL (each strain at  $2.5 \times 10^6$  CFU/mL for the A1 and A3  
140 combinations and the commercial culture CC3) with the commercial starter. Samples of semi-  
141 hard cheese were stored at –20 °C before ripening ( $Ch_i$ ) and after 4 weeks of ripening at 12  
142 °C ( $Ch_f$ ) before antifungal compound analyses.

143

144 Control samples corresponded to the yogurt, cheese model, sour cream and semi-hard  
145 cheese containing only the corresponding commercial starters. Yogurt and cheese models  
146 were tested in triplicate, sour cream and semi-hard cheese manufactured in duplicate per assay  
147 performed on separate weeks (2 assays per product).

148

### 149 *2.3. Antifungal compounds analysis*

150

151 Each biological replicate ( $n = 2$  to 4) was analyzed once by high-performance liquid  
152 chromatography (HPLC), liquid chromatography–quadrupole–time-of-flight mass  
153 spectrometry (LC–Q–ToF) and gas chromatography–mass spectrometry (GC–MS). For the  
154 quantification of free fatty acids by gas chromatography–flame ionization detector (GC–FID),  
155 each biological replicate was extracted once and injected twice.

156

#### 157 *2.3.1. Quantification by HPLC*

158 Cheese model and semi-hard cheese were first blended with MilliQ water (Millipore,  
159 Saint-Quentin-en-Yvelines, France) using a stomacher (Merck Eurolab, Strasbourg, France)  
160 (1:2 and 2:3  $w/v$ , respectively). The obtained suspensions were then centrifuged at 10 000  $g$   
161 for 30 min and supernatants were diluted (2:1 and 1:5  $v/v$  ratios) with  $H_2SO_4$  to reach 5 mM



162 final concentration and stored overnight at  $-20^{\circ}\text{C}$ . Sour cream and yogurt samples were  
163 directly dissolved at proportions of 2:1 and 1:5 *w/v* with  $\text{H}_2\text{SO}_4$  to reach 5 mM. After thawing,  
164 all samples were centrifuged at 10 000 *g* for 30 min, the upper phase recovered, filtered  
165 through a PTFE membrane (0.45  $\mu\text{m}$  pore size; Fisher Scientific) and centrifuged again in  
166 centrifugal concentrators (Vivaspin 6 Polyethersulfone, 10 kDa; Sartorius, Göttingen,  
167 Germany) at 4 000 *g* for 5 min. Samples were then poured into 2-mL vials for analysis. The  
168 compounds were separated using a Dionex HPLC system (Sunnydale, CA) equipped with an  
169 Aminex HPX-87H column (Bio-Rad, Hercules, CA), using 5 mM  $\text{H}_2\text{SO}_4$  as the mobile phase,  
170 and detected using both UV (210 nm) and refractometer detectors. In order to quantify  
171 organic acids at high concentrations and low concentrations, the two prepared dilutions were  
172 injected for each sample. The compounds were identified and quantified using standard  
173 solutions (concentrations ranging from 0.06 to 1 mg/mL) of acetic, benzoic, butanoic, citric,  
174 hydroxyphenyllactic, lactic, phenylpropanoic, phenyllactic, propanoic, pyruvic, succinic and  
175 2-pyrrolidone-5-carboxylic acids.

176

### 177 2.3.2. Quantification of organic acids by LC-Q-ToF

178 A total of 31 compounds with known antifungal activity were searched for as previously  
179 described (Brosnan, Coffey, Arendt, & Furey, 2012, 2014; Le Lay et al., 2016) (Table 1). For  
180 sample preparation, 10 g of each dairy product were first blended in sterile bags with 9 mL of  
181 water for mass spectrometry (Sigma-Aldrich, St Louis, MO) using a stomacher (Merck  
182 Eurolab, Strasbourg, France). Then, 10 mL of the resulting suspensions were mixed with 4 g  
183 of  $\text{MgSO}_4$ , 1 g of NaCl and 10 mL ethyl acetate supplemented with 1% formic acid. The  
184 obtained mixtures were then vigorously shaken and centrifuged for 10 min at 8500 *g* at  $4^{\circ}\text{C}$ .  
185 The organic phase was transferred into a dSPE tube (dispersive solid-phase extraction,  
186 Agilent Technologies, Santa Clara, CA), vortexed and centrifuged for 10 min at 2600 *g*. The

187 liquid phase was then recovered in a 15-mL tube, mixed with 100  $\mu$ L of dimethyl sulfoxide  
188 (DMSO) (Sigma-Aldrich, St Louis, MO) and evaporated under nitrogen gas until a volume of  
189 100  $\mu$ L, corresponding to the added DMSO. The 100- $\mu$ L volume (non-volatile under nitrogen  
190 gas) was reconstituted with 900  $\mu$ L of H<sub>2</sub>O/acetonitrile (90/10, *v/v*), filtered at 0.22  $\mu$ m and  
191 stored in amber vials at  $-20$  °C until analysis.

192 Samples were analyzed by liquid chromatography (1260 Infinity binary HPLC) coupled  
193 with quadrupole–time-of-flight mass spectrometry (6540 Q–ToF with a dual ESI source  
194 Agilent Technologies, Santa Clara, CA), under the conditions described by Le Lay et al.  
195 (2016), except that injected volumes were 10 and 50  $\mu$ L LC-Q-ToF). Compounds were  
196 separated at 30 °C on a Zorbax Extend C18 column (201 x 150 mm, 5  $\mu$ m) equipped with a  
197 pre-column (2.1  $\times$  12.5 mm, 5  $\mu$ m, Agilent Technologies), using a binary solvent gradient (**A**:  
198 water with 10 mM ammonium formate and 0.1% formic acid; **B**: acetonitrile with 0.1%  
199 formic acid). The mass spectrometer was operated in negative electrospray ionization mode.  
200 Compounds were identified and quantified using a matrix-matched calibration curve, built by  
201 spiking a sample (blank extract of semi-skimmed milk acidified to pH 5 with lactic acid) with  
202 a standard mix of the 31 compounds at 0, 1, 5, 10, 30, and 50 ppm.

203

### 204 2.3.3. Quantification of free fatty acids by GC–FID

205 Free fatty acids were extracted and quantified with the method described by De Jong and  
206 Badings (1990) with some modifications by Thierry et al. (2006). Briefly, 1 g for yogurt or  
207 sour cream samples, or 10 g for cheese model or grated semi-hard cheese were first mixed  
208 with 3 g of Na<sub>2</sub>SO<sub>4</sub>. Then, 0.3 mL of H<sub>2</sub>SO<sub>4</sub> (2.5 M), 1 mL of internal standards and 15 mL of  
209 ether/heptane (1:1) were added before centrifugation (3 min at 1000 g). The organic layer was  
210 recovered with 100 mL of Na<sub>2</sub>SO<sub>4</sub> to capture residual water. The obtained pellet was

211 suspended in 15 mL of ether/heptane (1:1) and centrifuged (3 min at 1000 g). This step was  
212 repeated 3 times by pooling the organic layers.

213 Free fatty acid purification was performed with a manifold, using a 500-mg SPE column  
214 (Phenomenex, Macclesfield, UK) conditioned with 10 mL heptane. The organic layer was  
215 transferred onto the column and 10 mL heptane/2-propanol (3:2) were added for neutral lipids  
216 removal. Free fatty acids were eluted with 5 mL diethyl ether with 2% formic acid and  
217 transferred to an amber vial. Free fatty acids were quantified by gas chromatography (Varian  
218 CP-3800) equipped with a flame ionization detector (FID) and a capillary column (BP21 25m  
219  $\times$  0.53 mm, 0.5  $\mu$ m film thickness; J&W Scientific, Folsom, CA). Carrier gas was hydrogen at  
220 9.7 mL/min. A temperature gradient was applied from 65 to 240 °C with a ramp rate of 10  
221 °C/min then 10 min at 240 °C using direct injection. Quantification was performed using  
222 standard calibration curves with the Star Varian software (version 5.3).

223 The internal standard solution contained 3 standards at 0.5 g/L: heptanoic acid (C7:0),  
224 undecanoic acid (C11:0) and heptadecanoic acid (C17:0). Calibration curves were established  
225 based on the correction factors against each internal standard. The short-chain acids (acetic to  
226 hexanoic acids) were calculated against C7:0, the mid-chain acids (octanoic to tetradecanoic  
227 acids) against C11:0, and the long-chain acids (hexadecanoic to eicosanoic acids) against  
228 C17:0.

229

#### 230 2.3.4. Volatile compounds analysis of by GC–MS

231 Dairy product samples (2.5 g) were transferred into 22-mL Perkin Elmer vials and stored  
232 at –80 °C until analysis. Headspace (HS) trap extraction coupled to gas chromatography–  
233 mass spectrometry (GC–MS) analyses were performed using a Perkin Elmer Turbomatrix  
234 HS-40 trap automatic headspace sampler with trap enrichment and a Clarus 680 gas  
235 chromatograph coupled to a Clarus 600T quadrupole mass spectrometer (PerkinElmer,

236 Courtaboeuf, France), as described by Pogačić et al. (2015), with some modifications.  
237 Volatile compounds were separated on a Stabilwax MS capillary column (30 m × 0.25 mm ×  
238 0.25 μm; Restek, Bellefonte, PA), with helium as the mobile phase, under the following  
239 conditions: initial temperature 35 °C maintained for 10 min, then increased at 5 °C/min up to  
240 230 °C. The MS was operated within a mass range of  $m/z$  29–206 using electron ionization at  
241 70 eV.

242 Volatile compounds were identified by comparison to standards, retention indices and/or  
243 the mass spectral data from the NIST 2008 Mass Spectral Library (Scientific Instrument  
244 Services, Ringoes, NJ). GC–MS data were processed as described by Pogačić et al. (2015).  
245 Briefly, raw data files were converted to time- and mass-aligned chromatographic peaks  
246 areas, using the XCMS open source package implemented with the R statistical language  
247 (Smith, Want, O’Maille, Abagyan, & Siuzdak, 2006). Volatiles were semi-quantified using  
248 the abundance of one selected mass fragment ( $m/z$ ), in arbitrary units. In addition, diacetyl  
249 was quantified in 8 samples (cheese model, yogurt, sour cream and semi-hard cheese  
250 containing the antifungal culture A1 and in the corresponding control samples) by spiking two  
251 samples (a non-fermented cheese model and cream containing sodium azide at 0.02%  $w/w$  to  
252 prevent microbial development) at 6 to 1 500 ppb. Samples and spiked samples were analyzed  
253 by HS-trap/GC–MS as described above. The calibration curves of standards spiked in the  
254 cheese model and in sour cream were used to quantify the compounds in cheese model and  
255 semi-hard cheese, and in yogurt and sour cream, respectively.

256

#### 257 *2.4. Statistical analysis*

258 Statistical analyses were performed using the R software (Free Software Foundation’s  
259 GNU General Public License).

260 Analysis of variance (ANOVA) was applied to determine whether the presence of an  
261 antifungal culture significantly influenced the amount of antifungal compound for the data  
262 from LC–Q–ToF, HPLC, GC–FID and GC–MS analyses. When significant results ( $p < 0.05$ )  
263 were observed, means were compared using a post hoc Fisher's least significant difference  
264 (LSD) test.

265 A multiple factor analysis (MFA) was performed using the MFA function of the  
266 *FactoMineR* R package (Lê, Josse, & Husson 2008). The data set was constituted of three  
267 groups of active variables: concentrations of 21 organic acids, of 12 free fatty acids, and  
268 abundance of 12 volatile compounds for the four dairy products, including semi-hard cheeses  
269 before and after ripening. In addition, three groups of supplementary (*i.e.* illustrative)  
270 variables were used: the characteristics of the samples, *i.e.* the type of dairy product and the  
271 nature of the antifungal culture added, the pH, and two variables that were previously used to  
272 quantify the antifungal activity against *P. commune* and *M. racemosus* (Leyva Salas et al.  
273 2018). These variables were a score of inhibition which ranged from 0 (no inhibition) to 3  
274 (total inhibition) and a duration of total inhibition until fungal growth was visible, expressed  
275 as a number of days compared to the control (for more details about the antifungal evaluation  
276 see Leyva Salas et al., 2018).

277 A partial least squares discriminant analysis (PLS–DA) was performed using the  
278 *mixOmics* R package (Le Cao, Rohart, Gonzalez, & Dejean, 2016) to identify the compounds  
279 that best discriminated the samples according to the intensity of inhibition of the two targets.  
280 To do so, the number of days of total inhibition was used to generate a qualitative variable  
281 that allowed the classifying of the samples into three groups of inhibition: no inhibition; slight  
282 inhibition (1–2 days); strong inhibition (> 2 days).

283

### 284 3. Results

285 *3.1. Antifungal compounds detected*

286

287 3.1.1. Antifungal organic acids quantified

288 Out of the 56 antifungal compounds targeted using HPLC, LC–Q–ToF and GC–FID  
289 analyses, 34 compounds were detected in at least one dairy product, while 22 were below  
290 their limit of detection (LOD) (Table 1). Most of them (28 compounds out of 34) were  
291 detected in the four tested dairy products. For the latter compounds, the results from  
292 quantification by LC–Q–ToF–MS were chosen because MS provided lower limits of  
293 quantification (LOQ) and a higher identification accuracy compared to HPLC–UV–  
294 refractometry. Concerning GC, GC–FID was chosen rather than GC–MS because the GC–  
295 FID results were obtained as absolute concentrations.

296 The antifungal compounds detected were present either in abundant, moderate, or low  
297 concentrations, as shown in Fig. 1A, 1B and 1C, respectively. The five most abundant  
298 compounds, lactic, citric, acetic, succinic, and hexadecanoic acids (Fig. 1A), accounted for  
299 ~95% of the total amount of all the detected antifungal compounds. Their concentrations  
300 varied depending on the considered dairy product and ranged from 5.6 to 16.9 g/kg for lactic  
301 acid, 0.2 to 3.4 g/kg for citric acid, 0.04 to 0.9 g/kg for acetic acid, 0.02 to 0.4 g/kg for  
302 succinic acid and 0.03 to 0.2 g/kg for hexadecanoic acid (Fig. 1A). Nine other organic and  
303 fatty acids were present at intermediate concentrations (5–150 mg/kg) and accounted for 1 to  
304 5% of the total amount of antifungal compounds (Fig. 1B). Finally, 11 organic acids and 8  
305 fatty acids were present at lower concentrations (Fig. 1C).

306 In general, as shown in Fig. 1, organic acids were at higher concentrations in semi-hard  
307 cheese followed by cheese model, then yogurt and sour cream. The concentrations of fatty  
308 acids also differed according to the considered product and were the highest in ripened semi-  
309 hard cheese and in sour cream and the lowest in yogurt (Fig. 1).

310

### 311 3.1.2. Profile of volatile compounds

312 The untargeted GC–MS approach led to the identification of 27 to 35 volatile compounds,  
313 (excluding contaminants), depending on the dairy product. Some of these volatile compounds,  
314 such as diacetyl, acetoin, dimethyl sulfone, 2-butanone, and volatile fatty acids have been  
315 reported as having antifungal properties (Arrebola, Sivakumar, & Korsten, 2010; Aunbjerg et  
316 al., 2015; Caulier et al., 2019; Hashem, Alamri, Alrumman, & Moustafa, 2016). Some other  
317 volatile compounds detected in significantly higher abundance in products containing  
318 antifungal cultures compared to controls were also considered (listed in Table 2) because they  
319 are close to other compounds reported as antifungal (Caulier et al., 2019).

320

### 321 3.1.3. All detected compounds

322 A global picture of the composition of the four dairy products in antifungal compounds is  
323 shown on the MFA presented on Fig. 2. The variables characterizing the antifungal activities  
324 were projected on the MFA as supplementary variables, in order to visualize the potential  
325 correlations between the presence of some antifungal compounds and the intensity of the  
326 inhibition observed for the two fungi targeted, *P. commune* and *M. racemosus*.

327 The first two dimensions of the MFA accounted for more than 62% of the total variance  
328 and clearly distinguished the four dairy products, regardless of the presence or not of  
329 antifungal cultures (Fig. 2). The three groups of compounds strongly contributed to the first  
330 dimension of MFA (coordinates of 0.80, 0.90 and 0.88 for organic acids, fatty acids and  
331 volatiles, respectively), while organic acids were also well represented on the second  
332 dimension of MFA (coordinates of 0.79). In contrast, the four variables describing the  
333 inhibition of *P. commune* and *M. racemosus* were poorly represented on this plot (Fig. 2) and  
334 on the following dimensions (coordinates < 0.12 in the five first dimensions of MFA, data not

335 shown). The first dimension accounted for 40.0% of the total variance and separated yogurt  
336 and cheese model from semi-hard cheese and sour cream. Yogurt and, to a lesser extent,  
337 cheese model, were associated with the highest concentrations of most of the volatile  
338 compounds and some organic acids (e.g., PPA, HC10, 2HC12, 3HC12, and HC14). In  
339 contrast, semi-hard cheeses and sour creams were characterized by higher concentrations of  
340 most free fatty acids. The second dimension accounted for 22.7% of the total variance and  
341 separated sour cream from the three other products, which contained higher concentrations of  
342 many organic acids, such as AZA, HPLA, HPPA, LA and PLA, in particular semi-hard  
343 cheeses after ripening.

344

### 345 *3.2. Antifungal compounds produced by the antifungal cultures*

346 ANOVA was performed on 53 volatile and non-volatile compounds detected in the dairy  
347 products to identify the compounds that were present at significantly ( $p < 0.05$ ) higher  
348 amounts in the products containing antifungal cultures A1, A2, A3, X1, X2 and/or CC3  
349 compared to the controls, *i.e.* the compounds produced by the antifungal cultures. Out of the  
350 53 compounds, 33 were produced in at least one of the dairy products by at least one of the  
351 antifungal cultures (Tables 1 & 2). Six of these compounds were detected at significantly  
352 higher amounts in the four dairy products: acetic acid, HPLA, HPPA, PPA, PCA and diacetyl.  
353 A group of compounds produced by an antifungal culture is referred to as a ‘cocktail’  
354 hereafter.

355 Each antifungal culture produced a distinct cocktail of antifungal compounds, and this  
356 cocktail varied according to the dairy product, as shown in Fig. 3. The cocktails of  
357 compounds produced by cultures A1, A2, A3, X1, and/or CC3 in each of the 4 dairy products  
358 are illustrated by four Venn diagrams shown in Fig. 3. In cheese model, 17 compounds, in  
359 cocktails of 7 to 16, were produced by A1, A2, A3 and X1, A1 being the highest-producing



360 antifungal culture (Fig. 3). In yogurt, 16 out of 25 compounds were produced by A1, followed  
361 by A2, A3 and X1 cultures, with cocktails of 8 to 16 compounds (Fig. 3). In sour cream, 8  
362 compounds were produced by A1, and only 2 of them by A3 (Fig. 3). In semi-hard cheese, 13,  
363 12, and 6 compounds were produced by A1, A3 and CC3 cultures, respectively (Fig. 3).  
364 Regardless of the dairy product, the concentrations of antifungal compounds increased by  
365 factors of 1.2 to 26 (for pyruvic acid in unripened semi-hard cheese) in samples with  
366 antifungal cultures compared to controls, and only small differences in concentrations were  
367 observed between the antifungal cultures, as illustrated in Fig. 3. Globally, HPLA and acetic  
368 acid (C2) were the organic acids that exhibited the greatest increase in concentrations, with  
369 1.5 to 5 times more HPLA and 1.5 to 6 times more acetic acid with culture A1, compared to  
370 the controls (Fig. 3). Regarding volatile compounds, diacetyl, an unidentified volatile  
371 compound (named VX) and, to a lesser extent, acetoin and methyl-ketones (HTN, NN and  
372 UDN), exhibited the greatest differences in abundance, with, for example, ~40 times more  
373 diacetyl and ~8 times more acetoin in unripened cheeses containing antifungal cultures up to  
374 15 times more diacetyl in sour cream containing A1, compared to the corresponding controls.  
375 The absolute concentrations of diacetyl were determined in all the samples with A1 and in  
376 controls. Diacetyl concentrations were 3.3, 3.7, 6.2 and 13.4 mg/kg in sour cream, yogurt,  
377 semi-hard cheese and cheese model, respectively, *vs* 0.6, 1.9, < 0.2 and 3.7 mg/kg in the  
378 corresponding controls.

379 During cheese ripening, 15 antifungal compounds increased in concentration by a factor  
380 ranging from 1.3 to 15, as illustrated in Supplementary Fig. 1. One third (6/20 out of the 20  
381 compounds produced in cheeses by antifungal cultures) were only detected at significantly  
382 higher concentrations after the ripening, namely 2-pyrrolidone-5-carboxylic (PCA), succinic,  
383 butyric, hydroxydecanoic, benzoic and lactic acids (Table 1). Some compounds, such as  
384 acetoin (AC) and PCA, increased in concentrations during ripening in all cheeses including

385 the controls, while some others, such as PLA and HPLA, increased only in the presence of  
386 antifungal cultures. Differences were also observed between the antifungal cultures. For  
387 example, A1 produced the highest amounts of AC, diacetyl (D), C2, HPLA, PLA, SA, and  
388 PCA in unripened cheeses, whereas A3 produced the highest amounts of AC, C2, PLA and  
389 SA during the ripening (Supplementary Fig. 1).

390 The impact of the inoculation level of the antifungal cultures A1 and A3 was investigated  
391 in sour cream. The inoculation level impacted the concentration of most compounds produced  
392 by A1 and A3 in sour cream: the higher the inoculation level, the higher the concentration  
393 was, as illustrated in Supplementary Fig. 2.

394

### 395 *3.3. Identification of the most probable antifungal compounds by discriminant analysis*

396 Discriminant analyses were performed to identify the antifungal compounds which best  
397 discriminated the samples according to the intensity of inhibition (no/slight/strong inhibition)  
398 of the two fungal targets considered, *M. racemosus* and *P. commune*. When the whole dataset  
399 including the 4 dairy products was used, the samples could not be discriminated according to  
400 the intensity of inhibition, regardless of the fungal target (results not shown).

401 An individual discriminant analysis was thus performed for each dairy product. The  
402 results are illustrated in Supplementary Fig. 3 for the inhibition of *P. commune* in sour cream.  
403 The samples that exhibited a strong inhibition of *P. commune* were located in the upper-left  
404 quadrant of the score plot derived from PLS-DA (Supplementary Fig. 3). *P. commune*  
405 inhibition in sour cream was associated with higher amounts of 8 main compounds, *i.e.* five  
406 organic acids, HPLA, PAA, PPA, PyA and acetic acid, and three volatiles, diacetyl, acetoin  
407 and VX (Table 3).

408 The results of PLS-DA for the four dairy products and the two fungal targets are  
409 summarized in Table 3, which also gives the correlation coefficients calculated between the

410 compound concentrations and the number of days of inhibition. More than thirty compounds  
411 were associated with antifungal activity, including 12 organic acids, mevalonolactone, 14 free  
412 fatty acids including 3 hydroxyacids, and 6 volatiles (Table 3). One quarter of compounds  
413 were common to 3 or 4 dairy products, namely acetic acid (C2), HPLA, HPPA, PLA, PPA,  
414 diacetyl (D), acetoin (AC) and the unidentified VX volatile compound. Most compounds were  
415 common to the two fungal targets, even if acetic acid appeared as more strongly correlated to  
416 *M. racemosus* inhibition compared to that of *P. commune*.

417

#### 418 **4. Discussion**

419 This extensive study aimed to identify the antifungal compounds most likely involved in  
420 the antifungal activity previously observed in dairy products inoculated by antifungal  
421 bioprotective cultures (Leyva Salas et al., 2018). It is the first study that combines several  
422 antifungal cultures, assessed in four dairy products, including a cheese model, yogurt, sour  
423 cream, and semi-hard cheese. Moreover, four extraction methods coupled to different  
424 chromatographic methods were combined, because microbial antifungal activity can result  
425 from a large panel of active molecules that belong to various chemical families (Crowley et  
426 al., 2013) and exhibit different chemical properties (hydrophobicity, volatility). Both targeted  
427 and non-targeted approaches were used: major and minor metabolites such as organic acids  
428 were analyzed by two HPLC methods, free fatty acids by GC and profile of volatile  
429 compounds by headspace GC–MS. These four complementary methods led to the  
430 identification of 36 of the targeted antifungal compounds, whereas 22 of the targeted  
431 compounds were not detected in any of the samples (Table 1), plus a range of neutral volatile  
432 compounds (Table 2). Most of these compounds were present in the four dairy products  
433 studied (Tables 1 and 2).

434 Overall, 33 antifungal compounds were produced by the antifungal cultures used (Tables  
435 1 and 2), as cocktails of 2 to 16 compounds depending on the antifungal culture and on the  
436 dairy product. Surprisingly, antifungal compounds were detected in all samples (Fig. 3),  
437 including in the control samples that were inoculated only with the starters used for  
438 acidification purpose in the manufacture of these fermented dairy products. This result  
439 suggests that antifungal compounds were also produced by the starters used. It is important to  
440 note however that the fungal targets grew in the control products within 5 to 6 days after  
441 inoculation, as expected (Leyva Salas et al., 2018). This suggests that the amounts of  
442 antifungal compounds present in the controls were too low to inhibit the fungal targets used  
443 and demonstrates that the inhibition observed was associated with quantitative and not  
444 qualitative differences in the profile of antifungal compounds. The increase in concentration  
445 of most antifungal compounds did not exceed a factor of 2 in the presence of antifungal  
446 cultures, but some compounds in particular volatiles showed a far higher increase.  
447 Lactobacilli have been reported to produce many antifungal compounds (Brosnan et al., 2012;  
448 Crowley et al., 2013; Leyva Salas et al., 2018), especially in *L. plantarum* species (Broberg,  
449 Jacobsson, Strom, & Schnurer, 2007; Sjogren et al., 2003), from which one strain was  
450 included in the three antifungal cultures used here.

451 The pathways of production of the antifungal compounds identified include three main  
452 origins (Table 1). The formation of antifungal compounds that are involved in milk  
453 acidification and/or flavour formation in dairy products is well documented in LAB. Lactic  
454 and acetic acids, diacetyl, and acetoin result from the catabolism of the two main carbon  
455 substrates present in dairy products, lactose and citrate (Urbach, 1995). A range of volatile  
456 and nonvolatile compounds are derived from amino acid catabolism. They include some of  
457 the antifungal compounds identified in this study, for example, 2-hydroxy acids, HiC6, PLA  
458 and HPLA, derived from Leu, Phe and Tyr, respectively (Honoré et al. 2016; Yoon 2016),

459 and the short branched-chain acids iC4 and iC5, derived from valine and leucine, respectively  
460 (Smid & Kleerebezem, 2014). 2,3-Pentanedione (PD) is synthesized from pyruvate and  
461 threonine (Ott, Germond, & Chaintreau, 2000). Free fatty acids result from milk fat  
462 hydrolysis by LAB, although LAB are known to be slightly lipolytic compared to other dairy  
463 microorganisms (Thierry et al., 2017). Benzoic acid (BZA) results from the breakdown of  
464 hippuric acid, present in milk (Urbach, 1995). Four 3-hydroxylated fatty acids, including  
465 3HC12 and HC14 identified in the present study, were produced by *L. plantarum* MiLAB 14  
466 supernatant and could result from hydroxylation of unsaturated fatty acids by LAB (Sjogren  
467 et al., 2003). Many of the volatiles cited above are known to contribute to cheese flavor  
468 (Curioni & Bosset, 2002) and could thus be involved in flavor changes in biopreserved dairy  
469 products.

470 The amounts of antifungal compounds produced increased with the population reached by  
471 antifungal cultures, but not proportionally. For example, when the inoculation level of culture  
472 A1 in sour cream was increased by factors of 5 and 20, its population after fermentation  
473 increased by factors of 7 and 17 (Leyva Salas et al., 2018), but even in the latter case, most  
474 organic acids increased by less than 2-fold. The highest factors of increase between low and  
475 high inoculation levels were observed for volatiles (~7-fold for diacetyl) (Supplementary Fig.  
476 2). The present results show that many antifungal compounds increased in concentration  
477 during ripening in cheese containing antifungal cultures and in the control cheeses  
478 (Supplementary Fig. 1). This results shows that a significant proportion of LAB kept their  
479 antifungal compound-producing activity while they did not grow anymore (for antifungal  
480 lactobacilli cultures) or even showed decrease in cultivability (for the lactococci used as  
481 starters) (Leyva Salas et al. 2018). Similarly, the production of diacetyl by *L. paracasei*  
482 DGCC 2132 in yogurt continued to increase with longer fermentation time and further storage  
483 at 4 °C (Aunbjerg et al. 2015).

484 To identify the compounds that are most likely responsible for the antifungal activity  
485 observed (Leyva Salas et al., 2018), we used a supervised multivariate analysis, namely PLS–  
486 DA, for each dairy product. More than thirty compounds were associated with antifungal  
487 activity (Table 3). Out of them, nine ‘key’ compounds were associated with the antifungal  
488 activity in 3 or the 4 dairy products studied, including acetic acid, 5 aromatic acids (PPA,  
489 HPLA, HPPA, PCA and PLA) and three volatiles, namely diacetyl, acetoin and VX (Table 3).  
490 Overall, these compounds roughly corresponded with the ones found at significantly higher  
491 amounts in products with antifungal cultures (Table 1). These results are consistent with the  
492 fact that culture A1, which was previously shown to exhibit the greatest inhibitory activity  
493 (Leyva Salas et al. 2018), was here identified as a better producer of antifungal compounds,  
494 both qualitatively and quantitatively, compared to cultures A2 and A3 (Fig. 3). Culture A1  
495 was also reported to have a larger spectrum of inhibition compared to A3 in yogurt and cheese  
496 model (5 of the 8 other fungal targets tested inhibited for a longer time; Leyva Salas et al.  
497 2018), which could be explained by the greater amounts of free fatty acids released by A1.  
498 Only *Yarrowia lipolytica* was more inhibited in yogurt by antifungal culture A3 compared to  
499 A1 (Leyva Salas et al., 2018), which could be explained by the high number of compounds  
500 produced by A3 in yogurt (Fig. 3).

501 The comparison of antifungal compound concentrations with MIC data available in the  
502 literature shows that all compounds identified in this study were present at concentrations far  
503 below their MIC. Concerning organic acids, the antifungal activity of lactic and acetic acids is  
504 well documented (Bian et al., 2016; Gerez, Torino, Obregozo, & Font de Valdez, 2010).  
505 However, even if present as the most abundant fermentation products, lactic and acetic acids  
506 were at concentrations 6 to 20 times below their MIC, *i.e.* 180 and 12 g/kg against *P.*  
507 *commune* and *M. racemosus* in potato dextrose agar at pH 5 (Mounier, personal  
508 communication). Three 2-hydroxyacids, PLA, HPLA and HiC6, were produced at

509 concentrations 100 to 1000 times their MIC of 5–10 g/kg determined against two  
510 *Penicillium* strains at pH 4.5 (Honoré et al., 2016). 2-Hydroxyacids were reported as  
511 correlated with the antifungal activity against *Penicillium* spp. (Honoré et al., 2016). Other  
512 organic acids, such as benzoic acid (BZA), methylcinnamic acid (MCA) and phenylpropanoic  
513 acid (PPA), were also present at concentrations far below their MIC, which ranges from 0.1 to  
514 1 g/kg (Broberg et al., 2007; Ryan et al., 2011; Yoon, 2016). Concerning fatty acids, they  
515 have been reported to inhibit different fungi, including *Penicillium*, *Aspergillus*, *Rhodotorula*  
516 and *Candida*, at low MIC. For example, the MIC reported for capric acid varied according to  
517 the fungal target and the conditions: 100–500 mg/kg in glycerol agar, pH 5 (Huang, Wilson,  
518 Chapman, & Hocking, 2010), 5–100 mg/kg (Sjogren et al., 2003), and 20–30 mg/kg in  
519 Sabouraud agar, pH 5.6 (Clément, Tremblay, Lange, Thibodeau, & Belhumeur, 2008). In the  
520 dairy products studied here, capric acid was present at concentrations of 3–15 mg/kg, *i.e.* 3 to  
521 100 times below its MIC. Regarding hydroxylated fatty acids, the concentrations of HC10,  
522 3HC12 and HC14 determined in the present study (2–4 mg/kg in yogurt) were closer to their  
523 MICs, which ranged from 10 to >100 mg/L in malt extract broth (Sjogren et al. 2003).  
524 Hydroxylated fatty acids are more likely to disrupt the fungal membrane than the non-  
525 hydroxylated compounds (Pohl, Kock, & Thibane, 2011). Fatty and hydroxylated fatty acids  
526 could be involved in particular in the inhibition observed in yogurt (Table 3). Regarding  
527 volatile compounds, they have been highlighted for their antifungal activity, e.g., diacetyl  
528 against *Penicillium solitum* (Aunbjerg et al., 2015). In the present study, diacetyl was at  
529 concentrations 2 to 6 times higher in the presence of antifungal cultures compared to the  
530 controls. The unidentified VX volatile compound was detected in 3 dairy products (yogurt,  
531 cheese model and sour cream) at 3, 4, and approximately 100 times higher amounts in the  
532 presence of the antifungal culture A1 compared to the controls. This compound was present at  
533 trace amounts and despite our efforts to overproduce it, we did not succeed in identifying it

534 (Supplementary Fig. 4). It is likely that the inhibition observed in the present study results  
535 from a range of antifungal products that act in synergy, as previously described (Dagnas et al.,  
536 2015; Honoré et al., 2016).

537 Many acids were found associated with antifungal activity (Table 3). The antifungal  
538 activity of weak organic acids depends on their  $pK_a$  and on the pH of the medium. The  
539 antifungal activity of organic acids is due to their undissociated form, which is able to diffuse  
540 through the membrane of fungi (Coton & Leguerinel, 2014; Theron & Lues, 2011). The  $pK_a$   
541 values of acetic, lactic, succinic, and pyruvic acids are 4.75, 4.21, 3.90, and 2.45, respectively,  
542 explaining why acetic acid has generally a major contribution to antifungal activity. The pH  
543 of three of the dairy products studied was 4.7 to 5 (cheese model, yogurt and semi-hard  
544 cheese), while in sour cream, which had a pH of 4.2, the inhibitory effect of acids is expected  
545 to be far more pronounced, in agreement with the inhibition effectively observed. For  
546 example, the greatest number of days of inhibition reached > 24 in sour cream containing  
547 antifungal culture A1, while it ranged from 1 to 10 days in the three other dairy products with  
548 the same culture. Apart from pH, other physicochemical properties of the dairy products  
549 could have an impact on the antifungal activity observed. The four dairy products differed in  
550 their moisture (85%, 79%, 59% and 47% in Y, CM, SC and Chi, respectively), fat (2.5%, 7.5,  
551 35%, and 25% respectively) and NaCl-in-moisture (0%, 0.7%, 0%, and 0.74.0%,  
552 respectively) contents. The composition of the product and its production conditions (time of  
553 incubation, temperature) directly affect the growth and metabolism of antifungal cultures, as  
554 previously discussed (Leyva Salas et al. 2018). However, product composition could also  
555 directly impact the actual efficiency of the produced antifungal molecules. For example, fat is  
556 a reservoir of hydrophobic molecules, such as fatty acids, PPA and PLA, and could thus  
557 modulate the interactions between antifungal molecules and fungal targets.



558 Despite the overall consistency of the results, the presence of other antifungal compounds  
559 cannot be excluded. For example, antifungal proteinaceous compounds can be produced by  
560 lactobacilli, e. g., *L. plantarum* IS10, ALAC-4 and YML007, *L. brevis* NCDC 02, *L.*  
561 *paracasei* M3 (Crowley et al., 2013). Further studies would be needed to determine whether  
562 proteinaceous compounds may also be involved with the observed antifungal activity, but this  
563 is complicated by the huge amounts of peptides released from casein hydrolysis in dairy  
564 products. Another approach would be to investigate the antifungal activity of a mixture of the  
565 (main) identified compounds, as performed by Le Lay et al. 2016 but in a dairy model.  
566 Preliminary experiments in this regard showed the complexity of such an approach, due to the  
567 necessity to accurately control the pH and composition of the dairy products and due to the  
568 occurrence of precipitation and syneresis phenomena. Finally, volatile VX should be further  
569 investigated, to identify it and to evaluate its actual antifungal activity.

570

#### 571 **Main chemical compounds studied in this article**

572 Acetic acid (PubChem CID: 176); 3-phenylpropanoic acid (PubChem CID: 107); 5-  
573 oxopyrrolidine-2-carboxylic acid (PubChem CID: 499); 2-hydroxy-3-(4-  
574 hydroxyphenyl)propanoic acid (PubChem CID: 9378); 3-(4-hydroxyphenyl)propanoic acid  
575 (PubChem CID: 10394); 2,3-butanedione (PubChem CID: 650)

576

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586

587 **Figure Captions**

588 **Fig. 1.** Means of organic and free fatty acid concentrations in mg/kg of (CM) cheese model,  
589 (Y) yogurt, (SC) sour cream and semi-hard cheese (Chi) before and (Chf) after 4 weeks  
590 ripening. (A), compounds at concentrations above 5% of all detected compounds in at least  
591 one dairy product; (B), compounds above 2% in at least one dairy product among the “others”  
592 shown in (A); (C) detail of the “others” shown in (B). See Table 1 for abbreviations

593

594 **Fig. 2.** Correlation circle and Individual factor map of the first two dimensions of multiple  
595 factor analysis performed using the concentration of three groups of antifungal compounds  
596 (21 organic acids, 12 fatty acids, 11 volatiles) in 4 dairy products (CM, cheese model; Y,  
597 yogurt; SC, sour cream; Chi, semi-hard cheeses before ripening and Chf after 4 weeks of  
598 ripening) inoculated with different antifungal cultures (A1, A2, A3), with (CC) commercial  
599 antifungal cultures and in control products (C) without antifungal cultures. For compounds  
600 abbreviation see Tables 1 and 2. The pH and variables of inhibition (in italics, dotted lines)  
601 are projected as supplementary variables. Fungal inhibition was evaluated by score intensity  
602 (*Muc\_score*, *Pen\_score*) and number of days of inhibition (*Muc\_day*, *Pen\_day*) against two  
603 fungal targets *Mucor racemosus*, and *Penicillium commune*.

604

605 **Fig. 3.** Venn diagrams show the compounds present at significantly greater amounts in the  
606 presence of an antifungal culture (A1, A2, A3, and commercial antifungal cultures X1 and  
607 CC3) compared to the corresponding controls without antifungal cultures, in four dairy

608 products (A), cheese model, (B) yogurt, (C) sour cream, and (D) semi-hard cheese before  
609 ripening. Antifungal cultures were inoculated at  $2 \times 10^7$  CFU/mL except in semi-hard cheese  
610 ( $5 \times 10^6$  CFU/mL). Bar graphs illustrate the concentrations of some selected antifungal  
611 compounds in the presence or the absence (controls; C) of antifungal cultures. For compound  
612 abbreviations see Tables 1 and 2. Compounds produced by the reference culture X2 are not  
613 shown because of its low antifungal activity (Leyva Salas et al. 2018).

614

### 615 **Supplementary Figure Captions**

616 **Supplementary Fig. 1.** Increase in concentration of some antifungal compounds during the  
617 ripening of semi-hard cheese, illustrated for six out of the 20 compounds produced by  
618 antifungal culture A1. Initial (i) and final (f) concentrations before and after 4 weeks of  
619 ripening are shown in light colours and dark colours, respectively. Cheeses were inoculated  
620 with antifungal cultures A1 or A3 or with commercial antifungal culture CC3 or without  
621 antifungal cultures (controls C). (A) acetoin, (B) acetic acid, (C) 2-pyrrolidone-2-carboxylic  
622 acid, (D) hydroxyphenyllactic acid, (E) phenyllactic acid and (F) succinic acid. Values and  
623 bars are means and standard deviation of two biological replicates.

624

625 **Supplementary Fig. 2.** Concentrations of 6 compounds which varied in concentration  
626 according to the inoculum level of the antifungal cultures A1 and A3 in sour cream. A1 and  
627 A3 were inoculated at  $2 \cdot 10^7$ ,  $5 \cdot 10^6$  and  $10^6$  CFU/mL in sour cream, X1 is a commercial  
628 antifungal culture inoculated at  $10^6$  CFU/mL, C is a control sour cream without antifungal  
629 cultures.

630 Compound names: C2, acetic acid, HPLA, hydroxyphenyllactic acid; PCA, 3-pyrrolidone-5-  
631 carboxylic acid; D, diacetyl; AC, acetoin; VX. The volatiles D, AC and VX are expressed in  
632 arbitrary units (au).

633

634 **Supplementary Fig. 3.** Score and observation plots from the partial least squares  
635 discriminant analysis (PLS-DA) performed to identify the compounds that discriminated the  
636 best the sour cream samples according to the level of inhibition of *Penicillium commune*.  
637 Samples were classified into three groups of inhibition: (0) no-inhibition, [1;2], slight  
638 inhibition, and (> 2) strong inhibition according to the number of days of total inhibition. For  
639 compounds abbreviations see Table 1 and 2.

640

641 **Supplementary Fig. 4.** Mass spectral and linear retention index data concerning the  
642 unidentified VX peak detected in significantly greater amounts in dairy products inoculated  
643 with the A1 antifungal culture (combination of *Lactobacillus harbinensis* L172 and  
644 *Lactobacillus. plantarum* L244

645

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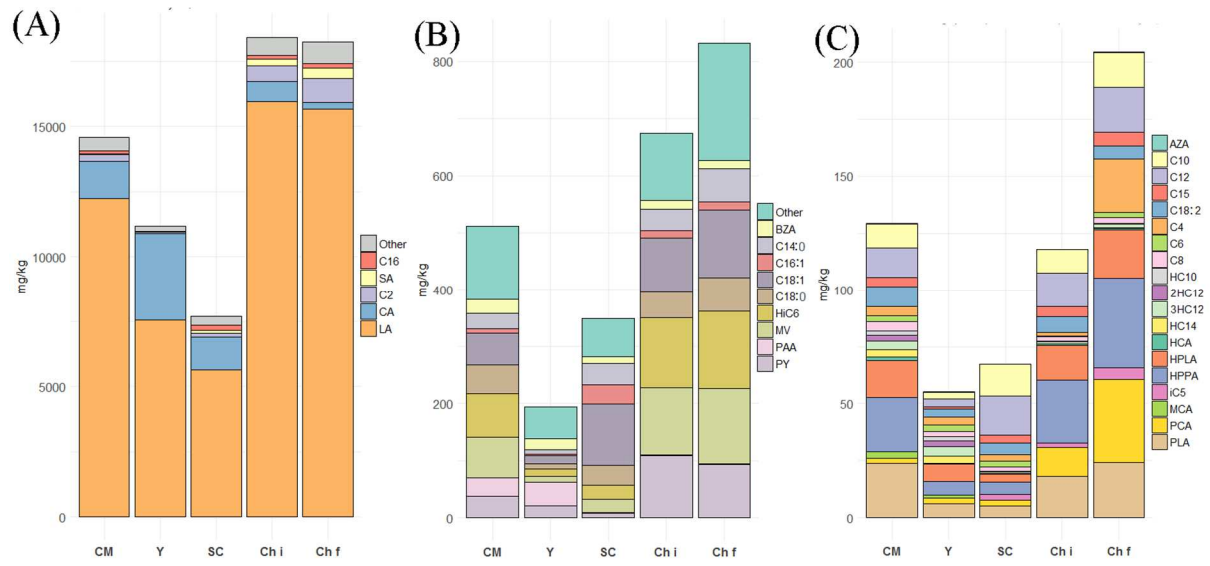
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**Figure 1.**

**Table 1. Targeted antifungal compounds and corresponding detection methods used.**

Ab.	Compound IUPAC name (common name)	CAS	Or.	Method	CM	Y	SC	Chi	Chf
C2	ethanoic acid (acetic acid)	64-19-7	C	1*, 3	•	•	•	•	+
C3	propanoic acid (propionic acid)	79-09-4	C	1, 3	+	-	-	+	+
C4:0	butanoic acid (butyric acid)	107-92-6	L	1, 3*	+	+	+	+	•
C6:0	hexanoic acid (caproic acid)	142-62-1	L	1, 3*	+	+	+	+	+
C7:0	heptanoic acid	111-14-8	L	3	-	-	-	-	-
C8:0	octanoic acid (caprylic acid)	124-07-2	L	3	+	•	+	+	+
C9:0	nonanoic acid	112-05-0	L	3	-	-	-	-	-
C10:0	decanoic acid (capric acid)	334-48-5	L	2, 3*	+	•	+	+	+
C11:0	undecanoic acid	112-37-8	L	3	-	-	-	-	-
C12:0	dodecanoic acid	143-07-7	L	3	+	•	+	+	+
C14:0	tetradecanoic acid (myristic acid)	544-63-8	L	3	+	+	+	+	+
C15:0	pentadecanoic acid	1002-84-2	L	3	•	+	+	+	+
C16:0	hexadecanoic acid (palmitic acid)	57-10-3	L	3	+	•	+	+	+
C16:1	9-hexadecenoic acid (palmitoleic acid)	373-49-9	L	3	•	•	+	+	+
C18:0	octadecanoic acid (stearic acid)	57-11-4	L	3	•	•	+	+	+
C18:1	(9Z)-9-octadecenoic acid (oleic acid)	112-80-1	L	3	•	•	+	+	+
C18:2	(9Z,12Z)-9,12-octadecadienoic acid (linoleic acid)	60-33-3	L	3	•	•	+	+	+
C18:3	(9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid (linolenic acid)	463-40-1	L	3	-	-	-	-	-
C19:0	nonadecanoic acid	646-30-0	L	3	-	-	-	-	-
C20:0	eicosanoic acid (arachidic acid)	506-30-9	L	3	-	-	-	-	-
AGA	<i>N</i> -acetyl-D-glucosamine	7512-17-6	AA	2	-	-	-	-	-
AZA	nonanedioic acid (azelaic acid)	64-19-7	C	2	+	+	+	+	+
BPH	2,4- <i>di-tert</i> -butylphenol	96-76-4	AA	2	-	-	-	-	-
BZA	benzoic acid (benzenecarboxylic acid)	65-85-0	M	1, 2 *	+	+	+	+	•
CA	2-hydroxypropane-1,2,3-tricarboxylic acid (citric acid)	77-92-9	M	1	+	+	+	+	+
CY	cytidine	65-46-3	AA	2	-	-	-	-	-
DCY	2'-deoxycytidine	951-77-9	AA	2	-	-	-	-	-
DHB	1,2-dihydroxybenzene (catechol)	120-80-9	AA	2	-	-	-	-	-
DHCA	3,4-dihydroxy-3-phenylpropenoic acid (caffeic acid)	331-39-5	AA	2	-	-	-	-	-
DHPPA	3-(3,4-dihydroxyphenyl)propanoic acid	1078-61-1	AA	2	-	-	-	-	-
FA	( <i>E</i> )-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoic acid (ferulic acid)	1135-24-6	AA	2	-	-	-	-	-
GA	D-glucuronic acid	6556-12-3	AA	2	-	-	-	-	-
HBZA	4-hydroxybenzoic acid	99-96-7	AA	2	-	-	-	-	-
HC10	3-hydroxydecanoic acid	5561-87-5	L	2	•	+	+	+	•
2HC12	2-hydroxydodecanoic acid (2-hydroxy lauric acid)	2984-55-6	L	2	+	+	-	+	-
3HC12	3-hydroxydodecanoic acid (3-hydroxy lauric acid)	1883-13-2	L	2	+	+	+	+	+
HC14	3-hydroxytetradecanoic acid (3-hydroxymyristic acid)	3422-31-9	L	2	+	+	-	-	-
4HCA	4-hydroxy-3-phenylpropenoic acid (4-hydroxycinnamic acid, <i>p</i> -coumaric acid)	501-98-4	AA	2	-	-	-	-	-
HiC6	2-hydroxy-4-methylpentanoic acid (hydroxyisocaproic acid)	13748-90-8	AA	2	+	•	+	•	•
HMPPA	3-(4-hydroxy-3-methoxyphenyl)propanoic acid	1135-23-5	AA	2	-	-	-	-	-
HPLA	2-hydroxy-3-(4-hydroxyphenyl)propanoic acid (hydroxyphenyllactic acid)	6482-98-0	AA	1, 2 *	•	•	•	•	•
HPPA	3-(4-hydroxyphenyl)propanoic acid	501-97-3	AA	2	•	•	•	•	•
LA	2-hydroxypropanoic acid (DL-lactic acid)	50-21-5	C	1	+	+	+	+	•
iC4	2-methylpropanoic acid (isobutyric acid)	79-31-2	AA	1, 3	-	-	-	-	-
iC5	3-methylbutanoic acid (isovaleric acid)	503-74-2	AA	3*	-	-	+	+	+
MCA	( <i>2E</i> )-2-methyl-3-phenylpropenoic acid ( $\alpha$ -methylcinnamic acid)	1199-77-5	AA	2	+	•	-	-	-
MV	4-hydroxy-4-methylloxan-2-one (4-hydroxy-4-methyltetrahydro-2H-pyran-2-one, mevalonolactone)	19115-49-2	C	2	+	•	+	•	•
PA	phenyl ethanoate (phenyl acetate)	122-79-2	AA	1, 2*	+	•	+	+	+
PCA	5-oxopyrrolidine-2-carboxylic acid (DL-pyrroglutamic acid, 5-oxoproline, pidolic acid)	149-87-1	AA	1	•	•	•	+	•
PLA	( <i>S</i> )-2-hydroxy-3-phenylpropanoic acid (phenyllactic acid)	828-01-3	AA	1, 2*	•	•	+	•	+
PPA	3-phenylpropanoic acid (hydrocinnamic acid)	501-52-0	AA	1, 2*	•	•	•	•	•
PyA	2-oxopropanoic acid (pyruvic acid)	127-17-3	C	1	•	•	+	•	•
RA	( <i>R,Z</i> )-12-hydroxyoctadec-9-enoic acid (ricinoleic acid)	141-22-0	FFA	2	-	-	-	-	-
SA	butanedioic acid (succinic acid)	110-15-6	C	1	+	•	•	+	•
SaA	2-hydroxybenzoic acid (salicylic acid)	69-72-7	AA	1, 2	-	-	-	-	-
VA	4-hydroxy-3-methoxybenzoic acid (vanillic acid)	121-34-6	AA	2	-	-	-	-	-

(Ab.) = abbreviations. Compound name according to IUPAC (International Union of Pure and Applied Chemistry) nomenclature followed by trivial name(s). Identification and quantification method: (1) HPLC coupled to UV or refractometer detectors; (2) LC-Q-ToF; (3) GC-FID. (\*) chosen quantification for statistical analysis. Probable pathway of origin (Or.): (L) lipolysis and free fatty acid metabolism, (C) carbohydrate metabolism, (AA) amino acid metabolism, M miscellaneous (or unknown). Compounds: (+) detected (•) present at significantly higher concentrations in each product with A1, A2, A3, X1, X2 and/or CC3 compared to controls, (-) not detected or at concentrations < LOD in cheese model (CM), yogurt (Y), sour cream (SC) and semi-hard cheese before ripening (Chi) and after 4 weeks of ripening (Chf).

**Table 1. Volatile compounds and their corresponding retention index, quantification ion and CAS number**

Ab.	Compound (trivial name)	m/z	Identification	LRI	CAS	Or.	CM	Y	SC	Chi	Chf
AC	3,2-hydroxybutanone (acetoin)	88	S, LRI, DB	1274	513-86-0	C	+	•	•	•	+
ACTN	2-propanone (acetone)	58	LRI, DB	811	67-64-1	C	•	+	+	+	•
BTN	2-butanone	72	LRI, DB	895	78-93-3	C	+	+	+	+	-
BZH	benzaldehyde	106	S, LRI, DB	1511	100-52-7	AA	•	•	+	+	+
C2	acetic acid	60	S, LRI, DB	1451	64-19-7	C	•	•	•	•	+
C4:0	butanoic acid	60	S, LRI, DB	1624	107-92-6	L	+	+	+	+	•
C6:0	hexanoic acid	60	S, DB	nd	142-62-1	L	+	+	+	+	+
C7:0	heptanoic acid	87	S, DB	nd	111-14-8	L	+	+	-	-	-
C8:0	octanoic acid	101	S, DB	nd	124-07-2	L	+	+	+	+	+
C9:0	nonanoic acid	115	S, DB	nd	112-05-0	L	+	+	-	+	+
D	2,3-butanedione (diacetyl)	86	S, LRI, DB	970	431-03-8	C	•	•	•	•	+
DMSO2	methylsulfonylmethane (dimethyl sulfone)	79	LRI, DB	nd	67-71-0	AA	+	+	+	+	+
HTN	2-heptanone	58	S, LRI, DB	1178	110-43-0	FFA	•	+	+	•	•
iC4	2-methylpropanoic acid	73	S, LRI, DB	1563	79-31-2	AA	-	-	-	+	+
iC5	3-methylbutanoic acid	60	S, LRI, DB	1664	503-74-2	AA	-	-	-	+	+
NN	2-nonanone	58	S, LRI, DB	1378	821-55-6	FFA	•	+	+	•	•
PD	2,3-pentanedione	100	LRI, DB	1042	600-14-6	C	+	•	-	-	-
UDN	2-undecanone	58	LRI, DB	1588	112-12-9	FFA	•	+	-	•	•
VX	unidentified	87	DB	1372	-	ukn	•	•	•	-	-

(Ab.) = abbreviations. Compound name according to IUPAC (International Union of Pure and Applied Chemistry) nomenclature, followed by trivial name. Compounds identified based on: (S), Retention time and mass spectrum from standard; (LRI), linear retention index; (DB), mass spectral data library NIST; (CAS), Chemical Abstract Service registry number. (Or.), probable pathway of origin: (L) lipolysis, (C) carbohydrate metabolism, (AA), amino acid metabolism, (FFA) free fatty acid metabolism, (ukn) unknown. (+) Detected compounds; (•) compound present at significantly higher concentration in the products with A1, A2, A3, X1, X2 and/or CC3 compared to controls, (-) not detected in (CM), cheese model; (Y), yogurt; (SC), sour cream; and (Chi) semi-hard cheese before ripening and (Chf) after 4 weeks of ripening. (nd), not determined.

**Table 3. Most probable antifungal compounds according to the results of partial least square discriminant analyses (PLS–DA) discriminating samples according to the intensity of inhibition (no/weak/strong, see material and methods) observed for two fungal targets, *Penicillium commune* and *Mucor racemosus*. PLS–DA were performed on a dataset constituted of the concentrations of 45 antifungal compounds (11 volatiles, 21 organic acids and other compounds, and 12 free fatty acids), for each of the four dairy products. The coefficient of correlation between concentrations and the number of days of inhibition compared to the control are also given.**

	Cheese model		Yogurt <sup>1</sup>		Sour cream		Unripened semi-hard cheese <sup>1</sup>					
	<i>P. commune</i>	<i>M. racemosus</i>	<i>P. commune</i>	<i>P. commune</i>	<i>M. racemosus</i>	<i>P. commune</i>	<i>P. commune</i>					
Organic acids <sup>2</sup>	<b>C2<sup>3</sup></b>	0.68	<b>C2</b>	0.94	BZA	0.49	<b>C2</b>	0.58	<b>C2</b>	0.77	<b>C2</b>	0.25
	<b>HC10</b>	0.73	<b>HC10</b>	0.77	<b>C2</b>	0.88	<b>PPA</b>	0.52	<b>PPA</b>	0.56	<b>HiC6</b>	0.47
	<b>HPLA</b>	0.59	2HC12	0.73	HC10	0.55	<b>HPLA</b>	0.40	<b>HPLA</b>	0.34	<b>PPA</b>	0.37
	<b>HPPA</b>	0.33	<b>PPA</b>	0.48	3HC12	0.48	<b>PAA</b>	0.42	<b>PAA</b>	0.41	<b>HPLA</b>	0.22
	MCA	0.83	<b>HPLA</b>	0.72	<b>PPA</b>	0.60	PyA	0.30	<b>PCA</b>	0.54	<b>HPPA</b>	0.34
	<b>PLA</b>	0.31	<b>HPPA</b>	0.68	<b>HPLA</b>	0.34			PyA	0.47	iC5	0.65
	<b>PyA</b>	0.91	<b>PLA</b>	0.69	<b>HPPA</b>	0.26					<b>MV</b>	0.44
			<b>PyA</b>	0.91	<b>PAA</b>	0.39					<b>PCA</b>	0.33
					<b>PCA</b>	0.81					<b>PLA</b>	0.45
					<b>PLA</b>	0.32						
				<b>SA</b>	0.82							
Free fatty acids <sup>4</sup>			C16:0	0.45	<b>C10:0</b>	0.86					C6:0	0.60
			<b>C18:1</b>	0.48	<b>C12:0</b>	0.81						
					<b>C16:0</b>	0.82						
					<b>C18:1</b>	0.81						
Volatiles	<b>D</b>	0.86	<b>D</b>	0.64	<b>AC</b>	0.54	<b>AC</b>	0.64	<b>AC</b>	0.94	<b>AC</b>	0.63
	<b>NN</b>	0.78	<b>NN</b>	0.47	<b>D</b>	0.80	<b>D</b>	0.78	<b>D</b>	0.88	<b>D</b>	0.41
	<b>PD</b>	0.75	<b>PD</b>	0.60	<b>VX</b>	0.73	<b>VX</b>	0.58	<b>VX</b>	0.78		
	<b>VX</b>	0.86	<b>UDN</b>	0.76								
		<b>VX</b>	0.37									

For compound abbreviations, see Tables 1 & 2.

<sup>1</sup>, no inhibition of *M. racemosus* was observed in yogurt and semi-hard cheese.

<sup>2</sup>, mevalonolactone (MV) was classified in this group, as well as hydroxyfatty acids (which do not result from milk fat lipolysis).

<sup>3</sup>, Compounds in bold are those for which concentrations were significantly higher in the presence of an antifungal culture compared to controls.

<sup>4</sup>, for yogurt, only 4 fatty acids with the highest correlation coefficient (*r*) are listed, but other fatty acids were also discriminant: C6:0, C8:0, C14:0, C15:0, C16:1, C:18:0, C18:2. (*r* = 0.60–0.79)