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1 **Title:** Strong effect of *Penicillium roqueforti* populations on volatile and metabolic
2 compounds responsible for aromas, flavor and texture in blue cheeses

3

4 **Running title:** Impact of mold population on blue cheeses

5

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26

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38

39 **Abstract**

40 Studies of food microorganism domestication can provide important insight into adaptation
41 mechanisms and lead to commercial applications. *Penicillium roqueforti* is a fungus with four
42 genetically differentiated populations, two of which were independently domesticated for
43 blue cheese-making, with the other two populations thriving in other environments. Most
44 blue cheeses are made with strains from a single *P. roqueforti* population, whereas Roquefort
45 cheeses are inoculated with strains from a second population. We made blue cheeses in
46 accordance with the production specifications for Roquefort-type cheeses, inoculating each
47 cheese with a single *P. roqueforti* strain, using a total of three strains from each of the four
48 populations. We investigated differences between the cheeses made with the strains from the
49 four *P. roqueforti* populations, in terms of the induced flora, the proportion of blue color,
50 water activity and the identity and abundance of aqueous and organic metabolites as proxies
51 for proteolysis and lipolysis as well as volatile compounds responsible for flavor and aroma.
52 We found that the population-of-origin of the *P. roqueforti* strains used for inoculation had a
53 minor impact on bacterial diversity and no effect on the abundance of the main
54 microorganism. The cheeses produced with *P. roqueforti* strains from cheese populations had
55 a higher percentage of blue area and a higher abundance of the volatile compounds typical of
56 blue cheeses, such as methyl ketones and secondary alcohols. In particular, the Roquefort
57 strains produced higher amounts of these aromatic compounds, partly due to more efficient
58 proteolysis and lipolysis. The Roquefort strains also led to cheeses with a lower water
59 availability, an important feature for preventing spoilage in blue cheeses, which is subject to
60 controls for the sale of Roquefort cheese. The typical appearance and flavors of blue cheeses
61 thus result from human selection on *P. roqueforti*, leading to the acquisition of specific
62 features by the two cheese populations. These findings have important implications for our
63 understanding of adaptation and domestication, and for cheese improvement.

64 Domestication is an evolutionary process that has been studied by many biologists since
65 Darwin. Indeed, domestication is an excellent model for understanding adaptation, because it
66 results from strong and recent selection on traits that are often known and of interest to
67 humans (Larson *et al.*, 2014). Furthermore, studies of domestication frequently have
68 important implications for the improvement of cultivated organisms. Domesticated fungi
69 have been less studied than crops in this respect, despite being excellent models in this field
70 (Giraud *et al.*, 2017; Gladieux *et al.*, 2014). Most fungi can be cultured in Petri dishes, can
71 remain alive for decades when stored in freezers and are propagated asexually. All these
72 features facilitate experimentation. Fungal metabolism results in the production of various
73 compounds of interest, including fuel alcohols, enzymes and antibiotics (Bigelis, 2001). The
74 most ancient and frequent human use of fungi is for fermentation, to preserve and mature
75 food. For example, the yeast *Saccharomyces cerevisiae* is used in the production of bread,
76 wine and beer, and the filamentous fungus *Aspergillus oryzae* is used for the production of
77 soy sauce and sake (Dupont *et al.*, 2017). These models have provided important insight into
78 mechanisms of adaptation and domestication (Almeida *et al.*, 2014; Baker *et al.*, 2015;
79 Gallone *et al.*, 2016; Gibbons *et al.*, 2012; Gonçalves *et al.*, 2016; Libkind *et al.*, 2011; Sicard
80 *et al.*, 2011).

81

82 The *Penicillium* genus contains more than 300 species, several of which are used by humans.
83 For example, penicillin was discovered in *P. rubens*, and two other species, *P. nalgiovense*
84 and *P. salami*, are used in the production of dry-cured meat (Fleming, 1929; Ludemann *et al.*,
85 2010, Perrone *et al.*, 2015). For centuries, *Penicillium roqueforti* (Thom) has been used in the
86 maturation of all blue cheeses worldwide (Coton *et al.*, 2020a; Labbe *et al.*, 2004, 2009;
87 Vabre, 2015). This fungus generates cheeses with a blue-veined appearance, by producing
88 melanized conidia within cavities in the cheese in which oxygen is readily available (Moreau,

89 1980). *Penicillium roqueforti* is also found in non-cheese environments (Pitt *et al.*, 2009;
90 Ropars *et al.*, 2012), and four genetically differentiated clusters of individuals (i.e.,
91 populations) have been identified in *P. roqueforti*. Two populations are used for
92 cheesemaking, whereas the other two populations thrive in silage, lumber or spoiled food
93 (Dumas *et al.*, 2020; Gillot *et al.*, 2015; Ropars *et al.*, 2014). Genomic and experimental
94 approaches have provided compelling evidence for the domestication of cheese *P. roqueforti*
95 populations (Cheeseman *et al.*, 2014; Dumas *et al.*, 2020; Gillot *et al.*, 2015, 2017; Ropars *et*
96 *al.*, 2015, 2016b, 2017) which have been reviewed (Coton *et al.*, 2020b; Ropars *et al.*,
97 2020a). Indeed, the populations of *P. roqueforti* used to make blue cheeses display the
98 characteristic features of domesticated organisms: genetic and phenotypic differences relative
99 to non-cheese populations, with, in particular, traits beneficial for cheese production, such as
100 faster growth on cheese medium (Dumas *et al.*, 2020; Gillot *et al.*, 2015; Ropars *et al.*, 2014,
101 2016), but also lower fertility and lower fitness in nutrient-poor conditions (Ropars *et al.*,
102 2015, 2016). Both cheese populations have lower levels of genetic diversity than the two non-
103 cheese populations, indicating an occurrence of bottlenecks (Dumas *et al.*, 2020), which
104 typically occur during domestication. The two cheese populations are genetically and
105 phenotypically differentiated from each other, suggesting that they result from independent
106 domestication events (Dumas *et al.*, 2020). One of the cheese populations, the non-Roquefort
107 population, is a clonal lineage with a very low level of genetic diversity used to produce most
108 types of blue cheeses worldwide. The second cheese population, the Roquefort population, is
109 genetically more diverse and contains all the strains used to produce blue cheeses from the
110 emblematic Roquefort protected designation of origin (PDO) (Dumas *et al.*, 2020). *In vitro*
111 tests have shown that the non-Roquefort population displays faster tributyrin degradation (*i.e.*
112 a certain type of lipolysis) and higher salt tolerance, faster *in vitro* growth on cheese medium,
113 better exclusion of competitors than the Roquefort population (Dumas *et al.*, 2020; Ropars *et*

114 *al.*, 2014, 2015), and an absence of toxic mycophenolic acid production (Gillot *et al.*, 2017).
115 The specific features of the Roquefort population may result from the constraints of the PDO,
116 requiring the use of local strains and at least 90 days of maturation, and preventing the use of
117 strains from the non-Roquefort population better suited to modern modes of production
118 (Dumas *et al.*, 2020). Genomic footprints of domestication (i.e., of adaptive genetic changes)
119 have been identified in the two *P. roqueforti* populations used for cheesemaking. Indeed, it
120 has been suggested that horizontally transferred genes found only in the non-Roquefort
121 population are involved in the production of an antifungal peptide and in lactose catabolism
122 (Cheeseman *et al.*, 2014; Ropars *et al.*, 2014, 2015). The effects of positive selection have
123 been detected in genes with predicted functions in flavor compound production, in each of
124 the cheese populations (Dumas *et al.*, 2020).

125

126 Thus, the four *P. roqueforti* populations probably harbor multiple specific traits, leading to
127 the generation of cheeses with different physicochemical properties and flavors, although this
128 has yet to be tested. Assessments of the effect of the population-of-origin of the *P. roqueforti*
129 strain used on the features of the cheese will i) provide important fundamental knowledge
130 about the trait under selection for cheesemaking and adaptation to the cheese environment, ii)
131 provide a basis for the elucidation of other genomic changes and iii) be crucial to
132 improvements in strain use and improvement. *Penicillium roqueforti* is used as a secondary
133 starter for flavor production, mostly through proteolysis (*i.e.* casein catabolism) and lipolysis
134 during ripening (Moreau, 1980). The main characteristic feature of blue cheeses, and of
135 Roquefort PDO cheeses in particular, is their intense, spicy flavors (Kinsella *et al.*, 1976;
136 Rothe *et al.*, 1982). The specific volatile and metabolic compounds responsible for these
137 flavors are generated principally by lipolysis in blue cheeses (Cerning *et al.*, 1987; Collins *et*
138 *al.*, 2003) and their intensity varies among *P. roqueforti* strains (Dumas *et al.*, 2020; Gillot *et*

139 *al.*, 2017; Larsen *et al.*, 1999). The fatty acids released by lipolysis are the precursors of
140 aldehydes, alcohols, acids, lactones and methyl ketones, which provide the moldy aromas
141 typical of blue cheeses (Collins *et al.*, 2003). *Penicillium roqueforti* degrades most proteins,
142 but the efficiency of proteolysis differs between strains (Cerning *et al.*, 1987; Dumas *et al.*,
143 2020; Gillot *et al.*, 2017; Larsen *et al.*, 1998). The resulting peptides contribute to flavors,
144 and their degradation into amino acids further influences cheese aroma and the growth of
145 other microorganisms (McSweeney *et al.*, 2000; Williams *et al.*, 2004). *Penicillium*
146 *roqueforti* also contributes to lactate degradation, which is necessary for deacidification and
147 promotes the development of less acid-tolerant microorganisms (McSweeney *et al.*, 2017).
148 Through these effects, and by producing secondary metabolites with antimicrobial properties,
149 *P. roqueforti* may also affect the microbial composition of the cheese (Kopp *et al.*, 1979;
150 Vallone *et al.*, 2014). Another parameter potentially affected by *P. roqueforti* populations and
151 restricting the occurrence of spoiler microorganisms is the lack of free water, (i.e., low water
152 activity, A_w), which is subject to strong controls for the sale of Roquefort cheese and is
153 affected by the degree of proteolysis (Ardö *et al.*, 2017). The *P. roqueforti* population may,
154 thus, also have an indirect effect on the features of the cheese, through various effects on
155 beneficial or undesirable contaminants.

156

157 The differences between *P. roqueforti* populations have, to date, been studied only *in vitro* or
158 in very rudimentary cheese models. Here, our objective was to assess the effect of the *P.*
159 *roqueforti* population-of-origin of the strain used for inoculation on the features of blue
160 cheeses. We focused on several features considered important for cheese quality. Given the
161 evidence from previous studies that cheese *P. roqueforti* populations have been domesticated,
162 any differences between the cheeses produced with cheese and non-cheese populations,
163 and/or between the two cheese populations can probably be considered to reflect selection by

164 humans for the production of good cheeses, either on standing variation in the ancestral *P.*
165 *roqueforti* population or for *de novo* mutations. We therefore produced blue cheeses in
166 conditions very similar to those used in industrial Roquefort PDO production, using, in
167 particular, milk from the local “Lacaune” breed, with strains from the four *P. roqueforti*
168 populations. We compared several important cheese features between the four populations: i)
169 physicochemical features, relating to texture and biochemical composition, ii) cheese
170 microbiota composition and abundance, which may have effects on several cheese features,
171 iii) the blue area as a proportion of cheese slices, which is important for the blue-veined
172 appearance of the cheese and is dependent on the growth and sporulation of *P. roqueforti* in
173 cheese cavities, and iv) the types and abundance of the metabolic and volatile compounds
174 produced, which influence flavor and aroma. We investigated the differences in these features
175 between the cheeses produced with strains from the four *P. roqueforti* populations (Roquefort
176 cheese, non-Roquefort cheese, silage and lumber/food spoiler populations). We also
177 investigated the possible differences between cheeses made with cheese and non-cheese
178 populations, and between the Roquefort and non-Roquefort cheese populations. Assessments
179 of the traits differing between cheese and non-cheese *P. roqueforti* populations, and between
180 the two cheese populations, and investigations of whether the cheese populations are more
181 suitable for cheese-making, are of fundamental importance for understanding the
182 domestication of cheese fungi, through the identification of traits subjected to selection.
183 Furthermore, the identification of differences between *P. roqueforti* populations of relevance
184 for cheese making has many applied consequences for the cheese industry, in terms of strain
185 choice for different kinds of blue cheeses, paving the way for the improvement of mold
186 strains by generating progenies from crosses of the two cheese populations, and for the
187 choice of traits for measurement and selection in offspring.
188

189 **Materials and Methods**

190 The Materials and Methods are described in more details in the Supplementary Methods.

191

192 **Experimental design and fungal strains:** We made cheeses with three different strains from
193 each of the four *P. roqueforti* populations (Figure 1, a strain is defined here as a haploid
194 individual obtained by monospore isolation), according to population assignments obtained in
195 a previous study based on molecular markers (Dumas *et al.*, 2020). For cheese production
196 (Figure 1B), we used a single strain per cheese for inoculation, using a total of three different
197 strains from each of the four *P. roqueforti* populations (Figure 1A). Due to the limited
198 production capacity of the experimental facility, we were unable to make all the cheeses at
199 the same time. We therefore split production into three assays, each including one strain from
200 each of the four populations (Figure 1A). For each strain in each assay, we created three
201 production replicates, with two cheeses per strain in each replicate, to ensure the production
202 of sufficient material for sampling. In total, we produced 72 cheeses (4 strains * 2 cheeses * 3
203 replicates * 3 assays, Figure 1A). The assays were performed sequentially from February to
204 April. The effect of the seasonal change in milk composition was therefore confounded with
205 the strain effect within the population, hereafter referred to as the "assay effect". The three
206 replicates within each assay were also set up at different times, a few days apart, and thus
207 with different batches of raw milk (Figure 1A). LCP (*Laboratoire de Cryptogamie de Paris*)
208 strains were obtained from the *Muséum d'Histoire Naturelle de Paris*, France, and BRFM
209 (Biological Resource Fungi Marseille) strains were obtained from the *Centre International*
210 *de Ressources Microbiennes de Marseille*, France.

211 **Cheesemaking:** The cheesemaking protocol was typical of the procedures used by the main
212 producers of Roquefort cheese and complied with the Roquefort PDO specifications, except
213 that the ripening process took place in artificial cellars in the INRA facilities at Aurillac and

214 with strains from different *P. roqueforti* populations. Briefly, we slowly mixed about 35 L of
215 milk in each vat, and heated it to 32.5°C. We then added 20 mg.L⁻¹ mesophilic starter culture
216 containing *Leuconostoc* spp., *Lactococcus lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis*, and
217 *L. lactis* subsp. *lactis* biovar *diacetylactis* (FD-DVS CHN-11, CHR HANSEN, Saint-
218 Germain-lès-Arpajon, France), 25 mg.L⁻¹ gas-producing *Leuconostoc mesenteroides* subsp.
219 *mesenteroides* (CHOOZIT LM 57 LYO 20 DCU, DANISCO, Paris, France) and about 3.6 x
220 10⁶ CFU.L⁻¹ *P. roqueforti* spores (SAS LIP, Aurillac, France). The mixture was homogenized
221 for five minutes, and we then added 0.25 ml of active chymosin at a concentration of 520
222 mg.L⁻¹ (Laboratoires Humeau, La Chapelle-sur-Erdre, France). The resulting curd was cut
223 into 1.5 cm³ cubes, left to rest for five minutes, and allowed to ferment for 50 minutes. The
224 curd was drained and placed in perforated cylinders of 20x10 cm at 26°C/85% humidity. The
225 cheeses were turned five times after molding. On the second day, the cheeses were unmolded.
226 On the third day, the cheeses were rubbed with sterilized coarse salt and transferred to
227 ripening cellars at 11°C / 95% humidity. On the fifth day, the cheeses were resalted in the
228 same way. On the seventh day, the cheeses were pricked. They were left to ripen until day 20,
229 when they were wrapped in sterile aluminum foil and allowed to mature at -2°C until day
230 180.

231

232 Cheese samples were collected on days 0, 9, 20, 90 and 180. These time points are referred to
233 hereafter as “stages”. Day 0 corresponded to raw milk for microbiology analysis and sowed
234 curd for metabolic and volatile compound analyses. Days 9 and 20 corresponded to half-way
235 through and the end of the ripening period, respectively. Days 90 and 180 (i.e. 3 and 6
236 months) corresponded to the minimum maturation times for the Roquefort PDO and a typical
237 maturation period for sale, respectively.

238 **Microbial and metabarcoding analyses:** We estimated the concentrations of various
239 microbial communities in the initial unpasteurized milk and at various stages of cheese
240 maturation, to determine whether the *P. roqueforti* population affected the cheese microbiota
241 (for more information, see the Supplementary Methods). We counted the total number of
242 aerobic mesophilic bacteria on plate count agar (PCA; Nelson, 1940). Mesophilic lactic acid
243 bacteria were counted on Man Rogosa Sharpe agar (MRS; De Man *et al.*, 1960) and
244 thermophilic lactic acid bacteria were counted on M17 agar (Terzaghi, 1975). Dextran-
245 positive *Leuconostoc* spp. were counted on Mayeux, Sandine and Elliker medium (MSE;
246 Mayeux *et al.*, 1962). Molds and yeasts were counted on oxytetracycline gelose agar medium
247 (OGA; Mossel *et al.*, 1970). Gram-positive catalase-positive bacteria were counted on
248 cheese-ripening bacterial medium (CRBM; Denis *et al.*, 2001). Enterobacteria were counted
249 on violet red bile glucose (VRBG; Mossel *et al.*, 1978). We further investigated the possible
250 effects of the *P. roqueforti* population on the identity and relative abundance of other
251 microorganisms in cheeses, by performing metabarcoding analyses on our experimental
252 cheeses at 9 and 20 days (during the ripening period), by extracting DNA, and amplifying
253 and sequencing the V3-V4 region of the 16S rDNA gene. Amplicons were sequenced with
254 Illumina Miseq technology. The mean read depth of the 71 samples (36 at 9 and 20 days of
255 ripening minus one lost) was 28539 reads. Amplicon data from high-throughput sequencing
256 were analyzed with Find Rapidly OTUs in Galaxy Solution (FROGS) v3.0 (Escudié *et al.*,
257 2018). For each OTU, taxonomic assignment was determined with the Silva-132
258 (<https://www.arb-silva.de/>) and 16S rDNA RefSeq databases
259 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Four widely used diversity parameters were
260 calculated from OTU compositions: two for alpha diversity (Shannon index, Simpson index)
261 and the Bray-Curtis dissimilarity for beta diversity.

262 **Blue area:** We estimated the percentage area of the cheese that was blue, on fresh inner
263 cheese slices (Figure 3B). This percentage is dependent on the formation of cavities within
264 the cheese, the growth of *P. roqueforti* within the cavities and its sporulation, as the blue
265 color is due to the melanin present in *P. roqueforti* spores. We cut all the 20-, 90- and 180-
266 day cheeses in half and took three photographs of each fresh slice with a Canon PowerShot
267 SX410 IS (JPEG format, 5152 x 3864 pixels, 100 ISO, without flash). We analyzed these
268 images with imageJ 1.52n (Schneider *et al.*, 2012; Figure S2): (i) the brightness and contrast
269 of the raw images were standardized with a stack contrast adjustment plugin, using a
270 reference image (Figure S2A,B) (ii) rectangular selection was used to crop the images
271 manually to ensure that they contained only the cheese slices (Figure S2C), (iii) the red
272 channel was segmented with a grayscale threshold of 102 to distinguish colonized cavities
273 from the inner white parts of the cheese and empty cavities (Figure S2D), (iv) the number of
274 dark pixels in the cavities was counted and divided by the total number of pixels for the entire
275 cheese slice, to determine the percentage blue area of the slice. Within the dark patches in the
276 cheese cavities colonized by the fungus, all pixels were considered dark (script at
277 <https://gitlab.com/snippets/1945218>).

278 **Physicochemistry:** We performed standard physicochemical measurements on the cheeses.
279 We measured dry matter content, fat content as a proportion of dry matter, the moisture
280 content of the defatted cheese, total, soluble and non-protein nitrogen contents, chloride and
281 salt content, water activity and pH at various stages of maturation, according to reference
282 methods (for more information, see the Supplementary Methods). We measured glucose,
283 lactose, lactate, acetate and butyrate concentrations in the cheeses on days 9 and 20, by high-
284 performance liquid chromatography (HPLC, for more information, see the Supplementary
285 Methods).

286 **Metabolic and volatile compounds:** We investigated possible differences in proteolytic and
287 lipolytic activities between the four populations, by UHPLC-MS after two extraction
288 procedures (in water and an organic solvent). We analyzed the abundance of free fatty acids
289 and residual glycerides in 90-day cheeses, by coupling a global extraction (accelerated
290 solvent extraction with hexane-isobutanol) with UHPLC-MS analysis in the positive
291 (triglycerides) and negative (fatty acids) ionization modes (for more information, see the
292 Supplementary Methods). We investigated the identity and abundance of volatile flavor and
293 aroma compounds, using a dynamic headspace system (DHS) with a Gerstel MPS
294 autosampler (Mülheim an der Ruhr, Germany) and gas chromatography-mass spectrometry
295 analysis with an Agilent 7890B GC system coupled to an Agilent 5977B quadrupole mass
296 spectrometer (Santa Clara, United States). Statistical analyses were performed with R
297 software (<http://www.r-project.org/>). Further details about the materials and methods are
298 provided in the Supplementary Methods.

299

300 **Results**

301 ***Penicillium roqueforti* population-of-origin influences bacterial diversity slightly, but**
302 **has no effect on the abundance of the main microorganisms in the cheese.** We
303 investigated whether the population-of-origin of the *P. roqueforti* strains used for inoculation
304 affected the composition of the cheese microbiota, by estimating the densities of key
305 microbial communities through colony counts (CFU/g) on various specific culture media and
306 a metabarcoding approach based on 16S sequencing targeting the bacteria in cheeses at
307 several stages of maturation. Based on microbial counts on plate, we found no significant
308 effect of *P. roqueforti* population on the abundance of any of the counted microorganisms,
309 including molds (i.e. *P. roqueforti*), at any sampled stage of maturation (Supplementary
310 Figure 1; Supplementary Table 1A).

311 The metabarcoding approach targeting bacteria identified mostly sequences from the
312 *Lactococcus* and *Leuconostoc* spp. starters (about 58 and 40%, respectively, Figure 2A,
313 Supplementary Table 1B), which are responsible for acidification and cavity formation,
314 respectively, in the cheese. The remaining sequences corresponded to 12 bacterial genera
315 frequently found in raw milk cheeses, such as *Lactobacillus*, *Staphylococcus* and
316 *Arthrobacter* (<1%, Supplementary Table 1B). However, the large predominance of starters
317 made it impossible to obtain sufficient data for other bacteria to assess differences in the
318 abundance of particular bacteria between cheeses made with strains from the four *P.*
319 *roqueforti* populations (Supplementary Table 1C). We also targeted eukaryotes, based on ITS
320 sequences, in a few samples. Most sequences were assigned to *P. roqueforti* spp. (89%) and,
321 to a lesser extent, *Candida* spp. (10%, data not shown), so no further analyses were
322 performed on the remaining low-abundance sequences. We estimated three OTU (operational
323 taxonomic unit) diversity parameters based on bacterial barcode sequence abundances, to
324 measure OTU richness and/or evenness. Bray-Curtis dissimilarity showed that cheeses made
325 with strains from the same *P. roqueforti* population were no more similar than those made
326 with strains from different *P. roqueforti* populations. However, we found a significant effect
327 of *P. roqueforti* population, in addition to a stage effect, on the Shannon and Simpson
328 diversity indices (Figures 2B and 2C). Cheeses made with strains from the cheese *P.*
329 *roqueforti* populations tended to have a higher bacterial OTU diversity, particularly at nine
330 days of maturation and for the Roquefort population (Figures 2B and 2C), although the post-
331 hoc analyses were not powerful enough to detect significant pairwise differences
332 (Supplementary Table 1C). There may also be undetected differences at species level or for
333 low-abundance microorganisms that might nevertheless have substantial effects. However,
334 even if this were the case, it would constitute an indirect effect of the *P. roqueforti*
335 population, as this was the only difference during our cheesemaking process.

336

337 **Higher proportion of blue area in cheeses produced with cheese *P. roqueforti***
338 **populations.** The percentage blue area in cheese cavities was determined on freshly cut
339 cheese slices (Figure 3B). The blue veins result from the formation of cavities in the cheese,
340 and the growth and sporulation of *P. roqueforti* in these cavities. The percentage blue area
341 was significantly higher in cheeses produced with cheese population strains than in those
342 produced with non-cheese population strains (Figure 3A and 3B; Supplementary Table 1D).
343 We also found a significant decrease in the percentage blue area from 20 to 180 days of
344 maturation, for all populations except the Roquefort population, for which the percentage
345 blue area remained relatively constant (Figure 3A; Supplementary Table 1D).

346

347 **More efficient proteolysis and lipolysis by the Roquefort *P. roqueforti* population.** We
348 investigated the proteolysis and lipolysis efficiencies of the four populations in cheeses after
349 90 days of maturation. Both targeted and non-targeted chromatographic analyses showed that
350 proteolytic efficiency was highest in the Roquefort *P. roqueforti* population. We performed
351 the targeted analysis with standards for the principal 23 amino acids (Supplementary Table
352 2A). We found that eight amino acids discriminated significantly between cheeses made with
353 the different *P. roqueforti* populations (Supplementary Table 1E, mainly ornithine, leucine,
354 and alanine), 15 discriminated between the cheese and non-cheese populations (mainly
355 spermidine, isoleucine, methionine, glutamic acid, citrulline, serine and leucine) and 14
356 distinguished between the Roquefort and non-Roquefort populations (Supplementary Figure
357 3A, mainly valine, leucine, isoleucine, serine and threonine). The cheeses made with strains
358 from cheese populations, and from the Roquefort population, in particular, had a higher total
359 amino-acid concentration (Supplementary Tables 1E and 2B).

360 We also assessed proteolytic activity in a non-targeted analysis (fingerprint approach) on
361 whole chromatograms (8,364 signals), which provided much more powerful discrimination
362 between metabolites. Each metabolite generates a signal specific to its mass-to-charge (m/z)
363 ratio at a given retention time. We obtained the largest number of aqueous signals, indicating
364 the most efficient proteolysis, for cheeses inoculated with strains from the Roquefort
365 population, followed by the lumber and non-Roquefort cheese populations, which were not
366 significantly different from each other, and proteolysis was least efficient for the silage
367 population (Figure 4; Supplementary Table 1F).

368

369 Lipolysis was also more efficient for the Roquefort population than for the other populations.
370 We investigated whether the *P. roqueforti* population influenced the abundance of free fatty
371 acids and residual glycerides, as a proxy for the efficiency of lipolysis, in 90-day cheeses,
372 with targeted and non-targeted chromatographic analyses in the positive and negative
373 ionization modes. We specifically targeted glycerides and free fatty acids. In the targeted
374 analysis, we identified seven free fatty acids and 20 triglycerides, and found that three free
375 fatty acids (stearic, oleic and linoleic acids) were significantly more concentrated in cheeses
376 made with Roquefort strains than in those made with strains from non-Roquefort populations
377 (Supplementary Table 1G). In the non-targeted analysis, we obtained 3,094 signals and
378 observed a higher abundance for organic signals specific to free fatty acids, indicating the
379 most efficient lipolysis, in cheeses made with strains from the Roquefort population, followed
380 by the lumber and non-Roquefort cheese populations, which were very similar to each other,
381 with lipolytic efficiency lowest for cheeses made with strains from the silage population
382 (Figure 5; Supplementary Table 1H). For residual glycerides, we obtained 8,472 signals, with
383 no significant difference between the populations (Supplementary Figure 4; Supplementary
384 Table 1I).

385

386 We performed the most relevant standard physicochemical measurements on raw milk and on
387 the cheeses at 9, 20, 90 and 180 days of maturation. As expected, we observed a maturation
388 stage effect for 11 of the 16 physicochemical parameters (Supplementary Table 1J). Non-
389 protein nitrogenous content was significantly higher in cheeses inoculated with strains from
390 cheese *P. roqueforti* populations than in cheeses inoculated with strains from the other
391 populations, consistent with the greater efficiency of proteolysis associated with these strains
392 (Supplementary Figure 5A). Cheese water activity differed significantly between the cheeses
393 made with strains from the four *P. roqueforti* populations (Supplementary figure 5B): it was
394 significantly lower for the Roquefort cheese population than for the non-Roquefort cheese
395 and silage populations (see statistics in the Supplementary Table 1J).

396

397 **Strong influence of *P. roqueforti* population on volatile compound production.** We
398 investigated the effect of *P. roqueforti* population on cheese volatile compounds in 90-day
399 cheeses. We focused on the GC-MS data for the 40 principal volatile compounds considered
400 to be most important for the aromatic quality of blue cheeses (Rothe *et al.*, 1982): 11 acids,
401 12 ketones, 10 esters, six alcohols and one aldehyde (Supplementary Table 3). We found that
402 *P. roqueforti* population strongly influenced the relative abundance of the compounds from
403 these aromatic families in the cheeses (Supplementary Table 1K; Figures 6 and 7, and see
404 below). In fact, the odors of the cheeses differed considerably (pers. obs.): the cheeses made
405 with strains from the cheese *P. roqueforti* populations smelled as good as typical ripened blue
406 cheeses, whereas those made with strains from non-cheese *P. roqueforti* populations had
407 unpleasant odors, similar to those of a wet mop (Supplementary Figure 6; personal
408 observation).

409

410 The abundance of acids, methyl ketones and secondary alcohols resulting from proteolysis
411 and lipolysis, and contributing to the typical flavor of blue cheese, was higher in cheeses
412 produced with strains from cheese populations than in those produced with strains from non-
413 cheese populations (Figure 6). These compounds were present at a particularly high
414 abundance in cheeses made with strains from the Roquefort population. Four of the 40
415 compounds analyzed were proteolysis by-products (primary alcohols: 3-methyl-butanal, 3-
416 methyl-butanol and isopropyl-alcohol, hereafter referred to as alcohols I, and 3-methyl-
417 butanoic acid, hereafter referred to as acid I; Supplementary Table 3). The abundance of
418 alcohols I was significantly higher in cheeses made with strains from cheese *P. roqueforti*
419 populations than in those made with strains from non-cheese populations, and the highest
420 values were obtained for the Roquefort population (mainly 3-methyl-butanol; Figure 6;
421 Supplementary Table 1K). Acid I (i.e. 3-methyl-butanoic acid) was also present in higher
422 abundance in cheeses made with strains from the Roquefort population than in other cheeses
423 (Figure 6). Two acids, by-products of glycolysis (acetic and propionic acid, hereafter referred
424 to as acids II), were present at higher abundance in cheeses made with strains from the
425 Roquefort and lumber/food spoiler *P. roqueforti* populations than in other cheeses (Figure 6;
426 Supplementary Tables 1K and 3). The other 35 aromatic compounds (i.e. acids from beta-
427 oxidation, hereafter referred to as acids III, ketones, secondary alcohols hereafter referred to
428 as alcohols II, and esters) were almost all direct or indirect by-products of lipolysis
429 (Supplementary Table 3). The abundance of acids III was higher in cheeses made with strains
430 from the Roquefort and lumber/food spoiler populations than in cheeses made with strains
431 from the non-Roquefort cheese population (mainly butanoic, pentanoic, hexanoic and
432 octanoic acid; Figure 6; Supplementary Table 1K). The levels of these compounds were
433 lowest in cheeses made with strains from the silage population. Esters and methyl ketones
434 (especially 2-pentanone and 2-heptanone) were present at higher abundance in cheeses made

435 with strains from cheese *P. roqueforti* populations (Supplementary Table 1K). Cheeses made
436 with strains from the Roquefort population contained the highest abundance of methyl
437 ketones, and these compounds were barely detectable in cheeses made from silage population
438 strains (mainly 2-heptanone, 2-pentanone and 2-nonanone; Figure 7A). The abundance of
439 alcohols II, particularly 2-heptanol, was also much higher in cheeses made with Roquefort
440 population strains than in other cheeses (Supplementary Table 1K; Figure 7B).

441

442

443 **Discussion**

444 **Cheese *P. roqueforti* populations have been selected to produce better blue cheeses.**

445 Measurements of multiple features of blue cheeses made under conditions resembling those
446 typically used in commercial Roquefort production revealed a strong influence of the
447 differentiated *P. roqueforti* populations on several aspects of cheese quality. The cheese
448 populations appeared the best adapted to cheesemaking, in terms of both the appearance and
449 volatile compound content of the resulting cheese. The differences between the four *P.*
450 *roqueforti* populations and the more appealing cheeses produced with strains from the cheese
451 populations suggest that humans have exerted selection for the production of better cheeses,
452 and this corresponds to domestication. Indeed, we found that cheese *P. roqueforti* strains
453 produced a higher percentage blue area on cheese slices, an important visual aspect of blue
454 cheeses. We also found that proteolysis and lipolysis were more efficient in cheeses made
455 with Roquefort population strains than in cheeses made with strains from the other *P.*
456 *roqueforti* populations, resulting in a higher abundance of desirable volatile compounds,
457 including alcohols and associated acids. Cheese water activity was lower in cheeses made
458 with strains from the Roquefort population, probably due to more efficient proteolysis (Ardö
459 *et al.*, 2017). We found no significant difference in the identities and abundances of

460 microorganisms between the cheeses made with strains from the four *P. roqueforti*
461 populations. Some minor differences in bacterial alpha diversity were observed, however, and
462 the differences in all other measurements than diversity between cheeses probably reflected a
463 direct effect of the specific features of the *P. roqueforti* population, although minor indirect
464 effects involving the induction of more diverse bacterial communities by cheese *P. roqueforti*
465 strains may also have occurred. Overall, our findings strongly support the view that cheese *P.*
466 *roqueforti* populations have been selected by humans for better appearance and aroma. This
467 selection may have involved the choice of the most beneficial strains for making good
468 cheeses from standing variation, and/or the selection of *de novo* genetic changes. Previous
469 studies found footprints of genomic changes in cheese populations in the form of beneficial
470 horizontal gene transfers and positive selection (Dumas *et al.*, 2020; Ropars *et al.*, 2015).

471

472 Previous studies reported differences between *P. roqueforti* populations, in terms of growth,
473 lipolysis and proteolysis, but on synthetic media (Dumas *et al.*, 2020; Ropars *et al.*, 2015).
474 Here, using experimental cheeses made in commercial cheese production conditions, we
475 reveal important features specific to cheese *P. roqueforti* populations, and to the Roquefort
476 and non-Roquefort cheese populations. These findings are important in the context of
477 domestication, for understanding rapid adaptation and diversification, and future studies
478 based on quantitative trait mapping may be able to identify further genomic changes
479 responsible for the specific features of the populations, according to the contrasting
480 phenotypes revealed here. Progenies can indeed be obtained from crosses between strains
481 from different populations of *P. roqueforti* (Ropars *et al.*, 2015), and this could facilitate
482 strain improvement through recombination between the different populations. Our results are,
483 therefore, also important for improving blue cheese production.

484

485 **The four *P. roqueforti* populations induce similar microbiotas, but water availability is**
486 **lower with cheese population strains, restricting the occurrence of spoiler**
487 **microorganisms.** Based on microbiological counts, we found no significant differences in
488 abundance for any of the species monitored between cheeses made with strains from the four
489 populations of *P. roqueforti*. In particular, we found no significant difference in the
490 abundance of molds on Petri dishes. However, microbiological counts are known to provide
491 poor estimates of fungal biomass, especially for mycelium growth (Schnurer, 1993).

492

493 The abundance and identity of the microorganisms studied were globally similar to those in
494 four commercial Roquefort cheeses (personal information from C. Callon; Devoyod *et al.*,
495 1968) and closely related blue cheeses (Diezhandino *et al.*, 2015). The metabarcoding
496 approach suggested that the different *P. roqueforti* populations induced bacterial
497 communities of different levels of diversity. The cheese populations, and the Roquefort
498 population in particular, were associated with the highest level of alpha diversity. The
499 differences in cheese bacterial diversity, although minor, suggest that the differences between
500 cheeses made with strains from the four *P. roqueforti* populations may be due not only to a
501 direct effect of *P. roqueforti* population, but also to an indirect effect mediated by the
502 induction of bacterial communities of different diversities. The large predominance of
503 bacterial starters made it impossible to collect sufficient data for an assessment of the
504 differences in relative abundance between subdominant bacterial species on the basis of
505 metabarcoding. We also found a significant difference in water activity between cheeses
506 made with strains from different *P. roqueforti* populations, the lowest value obtained being
507 that for the Roquefort population. This may also reflect human selection, as low water
508 activity restricts the occurrence of spoiler microorganisms. This characteristic is, therefore,
509 subject to tight control in Roquefort cheeses for sale, particularly those for export.

510

511 **Cheese *P. roqueforti* populations produce bluer cheeses.** We found significantly higher
512 percentage blue areas in cheese slices from cheeses made with cheese *P. roqueforti* strains
513 than in those made from non-cheese strains, potentially reflecting greater *P. roqueforti*
514 growth in cheese and/or a higher sporulation efficiency in cavities. The percentage blue area
515 in cheese slices also depends on the formation of cavities in the cheese, as *P. roqueforti* can
516 only sporulate in cavities in which oxygen is available. The cavities are mostly generated by
517 the gas-producing bacterium *Leuconostoc mesenteroides*, the abundance of which did not
518 differ between the cheeses made with strains from different *P. roqueforti* populations,
519 suggesting a direct effect of *P. roqueforti* populations on the blueness of cheese slices. The
520 significantly higher percentage blue area in slices of cheeses made with cheese *P. roqueforti*
521 strains than in those made with non-cheese strains therefore probably reflects better cheese
522 and cavity colonization and sporulation, probably due to selection on the basis of appearance.
523 The percentage blue area decreased by the end of maturation, perhaps due to the death of the
524 fungus. Only cheeses made with Roquefort strains retained a high percentage blue area at 90
525 days of maturation, again potentially reflecting selection in pre-industrial times, when
526 Roquefort cheeses had to be stored for several months at cave temperature before sale. The
527 minimum maturation time for Roquefort PDO remains 90 days, which is longer than for other
528 blue cheeses. These findings contrast with a previous study showing that a non-Roquefort
529 population colonized the cavities of model cheeses better than other populations (Dumas *et*
530 *al.*, 2020); this discrepancy may reflect differences between studies in terms of the
531 measurements used (total percentage blue area versus percentage blue area within cavities),
532 the type of milk (ewe versus goat) or the mode of cheesemaking (rudimentary models versus
533 commercial-like cheeses). Our findings are consistent with the presence of horizontally

534 transferred genes in cheese populations with predicted functions in fungal development,
535 including sporulation and hyphal growth (Dumas *et al.*, 2020).

536

537 **Proteolysis and lipolysis are more efficient in the Roquefort *P. roqueforti* population.**

538 Based on chemical analyses and powerful chromatographic discrimination methods, we
539 showed that the abundance of amino acids and small peptides (i.e., residual products of
540 proteolysis) was highest in cheeses made with Roquefort *P. roqueforti* strains. Thus, these
541 strains had the highest capacity for proteolysis, which is an important process in
542 cheesemaking. Indeed, proteolysis contributes to the development of cheese texture, flavors
543 and aromas (Andersen *et al.*, 2010; Ardö, 2006, 2017; McSweeney, 1997; Roudot-Algaron,
544 1996). Previous measurements of proteolytic activity in synthetic media detected significant
545 differences between *P. roqueforti* populations, but not between the two cheese populations
546 (Dumas *et al.*, 2020). We show here that experimental cheeses made with strains from the
547 Roquefort population have a higher content of residual products of proteolysis, a sign of more
548 advanced ripening.

549

550 We also found that lipolysis was more efficient in the cheeses made with strains from the
551 Roquefort *P. roqueforti* population. By contrast, previous studies in synthetic media found
552 that lipolysis was most efficient in the non-Roquefort population (Dumas *et al.*, 2020). The
553 discrepancy between these studies demonstrates the need for measurements in real cheeses
554 for the reliable assessment of metabolic activities. Lipolytic activity is known to affect cheese
555 texture and the production of volatile compounds affecting pungency (Alonso *et al.*, 1987;
556 González De Llano *et al.*, 1990, 1992; Martín *et al.*, 2016; Thierry *et al.*, 2017; Woo *et al.*,
557 1984). The more efficient proteolysis and lipolysis in the Roquefort *P. roqueforti* population

558 should have a strong impact on cheese texture and flavor. It therefore probably results from
559 selection to obtain better cheeses, i.e. from a domestication process, as previously reported
560 for other fungi (Almeida *et al.*, 2014; Baker *et al.*, 2015; Gallone *et al.*, 2016; Gibbons *et al.*,
561 2012; Gonçalves *et al.*, 2016; Libkind *et al.*, 2011; Sicard *et al.*, 2011). Roquefort cheeses are
562 widely considered to be the blue cheeses with the strongest aromas and flavors. The less
563 efficient lipolysis and proteolysis in the non-Roquefort population may result from more
564 recent selection for milder cheeses.

565

566 **Cheese *P. roqueforti* populations produce cheeses with more abundant and diverse**
567 **volatile compounds.** We found major differences between the cheeses made with strains
568 from different *P. roqueforti* populations, in terms of the volatile compounds resulting from
569 lipolysis and, to a lesser extent, also from proteolysis. Only four of the aromatic compounds
570 detected in our cheeses (3-methyl-butanal, 3-methyl-butanol, isopropyl-alcohol and 3-methyl-
571 butanoic acid) were by-products of casein proteolysis (McSweeney *et al.*, 2000), and the
572 concentrations of these molecules were significantly higher in cheeses made with Roquefort
573 *P. roqueforti* strains, consistent with the higher proteolysis efficiency and amino-acid
574 precursor (i.e. valine, leucine and isoleucine) concentrations of these strains. These
575 compounds produce fruity (banana), cheesy and alcoholic notes, which were probably
576 important selection criteria during the domestication of the Roquefort *P. roqueforti*
577 population. For the products of metabolic pathways leading from amino acids to alcohols
578 (Ehrlich pathway with aldehyde reduction) or acids (aldehyde oxidation; Ganesan *et al.*,
579 2017), the higher concentration of alcohols than of acids observed for all populations is
580 consistent with the general micro-aerobic conditions of blue cheese cavities.

581 Most of the aromatic compounds identified were direct or indirect by-products of lipolysis,
582 consistent with the known key role of lipolysis in the generation of typical blue cheese aroma

583 (Cerning *et al.*, 1987; Collins *et al.*, 2003). The aromatic compounds resulting from lipolysis
584 belonged to four chemical families (acids, methyl ketones, secondary alcohols and esters).
585 Methyl ketones were the most diverse and abundant for cheese *P. roqueforti* populations,
586 particularly for the Roquefort population, in which abundance was highest for 2-pentanone
587 and 2-heptanone; 2-heptanone underlies the characteristic “blue cheese” sensory descriptor
588 (Anderson *et al.*, 1966; González De Llano *et al.*, 1990, 1992; Moio *et al.*, 2000). In *P.*
589 *roqueforti*, methyl ketones with odd numbers of carbons are mostly produced by fatty-acid
590 beta-oxidation, whereas those with even numbers of carbons may be produced by the beta-
591 oxidation or autoxidation of fatty acids (Spinnler, 2011). These compounds are produced by
592 the decarboxylation of hexanoic acid and octanoic acid, respectively, which were the most
593 abundant acids found in our cheeses. This reaction is considered to be a form of
594 detoxification, because methyl ketones are less toxic than acids (Kinderlerer, 1993; Spinnler,
595 2011). Interestingly, this pathway appeared to be more active in the cheese *P. roqueforti*
596 populations, as methyl ketone levels were lower in cheeses made with lumber (four-fold
597 difference) and silage (10-fold lower) strains than in cheeses made with cheese population
598 strains. Methyl ketone concentrations were not directly associated with the concentrations of
599 their precursors (acids), the highest concentrations being found in the lumber and Roquefort
600 populations. The biosynthesis pathway producing methyl ketones must, therefore, be more
601 efficient in cheese populations, particularly the non-Roquefort population. The cheese *P.*
602 *roqueforti* populations were probably selected for their higher acid detoxification capacity, as
603 this produces aromatic compounds with a very positive impact on flavor (Spinnler, 2011).

604 The concentrations of secondary alcohols (resulting from the reduction of methyl ketones)
605 were also higher in cheeses produced by cheese *P. roqueforti* strains, particularly those of the
606 Roquefort population, for which they were seven times higher than for the non-Roquefort
607 cheese population and 20 times higher than for the silage/lumber populations; 2-heptanol was

608 the major alcoholic compound produced. The reduction of 2-heptanone to 2-heptanol occurs
609 specifically in anaerobic conditions and is much stronger in the Roquefort population; aerobic
610 conditions were similar for all the populations. The Roquefort *P. roqueforti* population may
611 also have been selected for this feature, as secondary alcohols provide “fruity notes”, which
612 are associated with better aromatic quality (Spinnler, 2011). Methyl ketones may be reduced
613 to alcohols by an alcohol dehydrogenase, as occurs when aldehyde is reduced to alcohol via
614 the Ehrlich pathway. Alcohol dehydrogenase genes may thus have been targets of selection in
615 the Roquefort *P. roqueforti* population, although they were not detected as evolving under
616 positive selection in a previous study (Dumas *et al.*, 2020).

617 We also found higher levels of esters in cheeses made with cheese *P. roqueforti* populations.
618 Esters are produced principally by the esterification of ethanol with acids generated by beta-
619 oxidation. *Leuconostoc* starters can produce ethanol, and ester synthesis has also been
620 described as a detoxification mechanism (Mason *et al.*, 2000). These results further indicate
621 that cheese *P. roqueforti* populations, particularly the Roquefort population, have been
622 selected for acid detoxification capacity, leading to a large variety of less toxic aromatic
623 compounds with strong aromas and flavors.

624 Overall, the aromas of cheeses made with cheese *P. roqueforti* strains had more appealing
625 aromas, and this was particularly true for cheeses made with Roquefort strains. These aroma
626 properties probably reflect selection by humans. The cheeses made with silage and lumber
627 populations had a mildly unpleasant smell, whereas those made with cheese strains smelled
628 like typical blue cheeses, with cheeses made with Roquefort strains having the strongest
629 smell. This may reflect previously reported horizontal gene transfers in cheese populations,
630 involving genes with predicted functions in lipolysis or amino-acid catabolism, and the
631 positive selection of genes involved in aroma production (Dumas *et al.*, 2020). We compared
632 *P. roqueforti* populations between cheeses made following commercial modes of production,

633 which represents a major advance relative to previous studies based on experimental models
634 or synthetic media (Dumas *et al.*, 2020; Gillot *et al.*, 2017). We used unpasteurized ewe's
635 milk, in accordance with the requirements for Roquefort PDO production, which also affects
636 cheese aromas. In future studies, it would be interesting to determine whether the use of
637 pasteurized or unpasteurized ewe's milk or cow's milk leads to similar specific features of the
638 Roquefort versus non-Roquefort cheese *P. roqueforti* populations, as there may have been
639 selection during domestication, leading to an adaptation of the Roquefort population for the
640 catabolism of unpasteurized ewe's milk.

641

642 **Conclusion.** We show that the *P. roqueforti* population has a strong impact on cheese
643 quality, appearance and aroma. The populations used for cheesemaking led to bluer cheeses,
644 with better aromas, probably due to domestication involving the selection of multiple fungal
645 traits by humans seeking to make the best possible cheeses. French cheese producers have
646 been inoculating cheeses with *P. roqueforti* spores from moldy rye bread since the end of the
647 19th century (Labbe *et al.*, 2004, 2009; Vabre, 2015). This process made it possible for them
648 to re-inoculate with the strains producing the best cheeses, thereby applying a strong selection
649 pressure. The two cheese populations displayed a number of specific features, with the
650 Roquefort population notably producing more intense and specific aromas and flavors. The
651 selection of different fungal varieties for different usages has also been reported in the
652 fermenting yeast *Saccharomyces cerevisiae* (Gallone *et al.*, 2016; Legras *et al.*, 2018).
653 Previous studies on *P. roqueforti* detected recurrent changes in amino acids and horizontal
654 gene transfers in cheese populations, both of which facilitated rapid adaptation (Dumas *et al.*,
655 2020; Ropars *et al.*, 2015). Our findings provide greater insight into *P. roqueforti*
656 domestication and pave the way for strain improvement through the targeting of relevant
657 traits. A protocol inducing sexual selection has been developed in *P. roqueforti* (Ropars *et al.*,

658 2014), making it possible to perform crosses between strains from the two cheese
659 populations, each of which harbors very little genetic diversity (Dumas *et al.*, 2020), to
660 generate variability and to identify strains with high levels of performance. The results of this
661 study will facilitate the choice of the parental strains for crossing and of the most important
662 phenotypes to be measured in the offspring. Parental strains with strongly contrasting
663 phenotypes for the traits important for cheesemaking that we found to be differentiated
664 between populations (such as volatile compound production, lipolysis and proteolysis) should
665 be used, to maximize variability in the progeny. Other traits may also be worth investigating
666 in the future, to understand the changes that have occurred during the domestication of the
667 two cheese *P. roqueforti* populations, particularly as concerns toxin production in cheeses.
668 Previous *in vitro* studies have shown a lack of production of the toxic mycophenolic acid by
669 the non-Roquefort population due to a deletion in one of the genes of the synthesis pathway
670 (Gillot *et al.*, 2017). In the *P. camemberti* fungus, domestication has also led to a lack of
671 production of a mycotoxin due to a deletion in a gene (Ropars *et al.*, 2020b).

672

673

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968 **Figure legends**

969

970 **Figure 1:** Experimental cheesemaking. (A) Experimental design for cheesemaking, using one
971 strain per cheese, and three different strains from each of the four *Penicillium roqueforti*
972 populations (non-Roquefort cheese in blue, Roquefort cheese in purple, silage/food spoiler in
973 orange, lumber/food spoiler in green, the lineages of which are shown on the left). Each assay
974 (February, March, April) corresponded to a single strain from each of the four populations,
975 with three production replicates at different times, different batches of unpasteurized milk
976 and with two cheeses produced per strain in each replicate. The identities of the strains used
977 are indicated on the left of each assay, for each of the four *P. roqueforti* populations. (B)
978 Photograph of the experimental cheeses after 20 days of maturation.

979

980 **Figure 2:** (A) Relative abundance of the six main bacterial operational taxonomic units in
981 cheeses made with strains from the four *Penicillium roqueforti* populations (non-Roquefort
982 cheese in blue, Roquefort cheese in purple, silage/food spoiler in orange and lumber/food
983 spoiler in green) in cheeses at 9 (left) and 20 (right) days of maturation. (B & C) Mean
984 bacterial genus diversity; A: Shannon index, B: Inverse of Simpson index (= $1 - \text{Simpson}$
985 index) for the operational taxonomic units detected by metabarcoding in 9-day cheeses (left)
986 and 20-day cheeses (right) made with strains from the four *Penicillium roqueforti* populations
987 (lumber/food spoiler in green, non-Roquefort cheese in blue, Roquefort cheese in purple and
988 silage/food spoiler in orange).

989

990 **Figure 3:** (A) Mean percentage blue area per cheese slice at 20, 90 and 180 days of
991 maturation, for cheeses made with strains from the four *Penicillium roqueforti* populations
992 (non-Roquefort cheese in blue, Roquefort cheese in purple, silage/food spoiler in orange and
993 lumber/food spoiler in green). Error bars indicate 95% confidence intervals. (B) Illustration

994 of the differences in mean percentage blue area per cheese slice at 180 days of maturation
995 between the four *Penicillium roqueforti* populations (non-Roquefort cheese in blue,
996 Roquefort cheese in purple, silage/food spoiler in orange and lumber/food spoiler in green).
997 Contrast and brightness have been standardized and the edges cropped.

998

999 **Figure 4:** Violin plot depicting the distribution of the sums of 3,864 non-targeted aqueous
1000 signal peak areas, weighted by their mass-to-charge ratios (“m/z”), obtained in positive
1001 ionization mode for 90-day cheeses made with strains from the four *Penicillium roqueforti*
1002 populations (lumber/food spoiler in green, non-Roquefort cheese in blue, Roquefort cheese in
1003 purple and silage/food spoiler in orange). Boxplots within violin plots represent the median
1004 (center line), the 25th and 75th percentiles (box bounds), the 5th and 95th percentiles
1005 (whiskers), the points being the outliers from these 95th and 5th percentiles. The red dots
1006 depict the mean values.

1007

1008 **Figure 5:** Violin plot depicting the distribution of the sums of 3,094 non-targeted organic
1009 signal peak areas, weighted by their mass-to-charge ratios (“m/z”), obtained in negative
1010 ionization mode for 90-day cheeses made with strains from the four *Penicillium roqueforti*
1011 populations (lumber/food spoiler in green, non-Roquefort cheese in blue, Roquefort cheese in
1012 purple and silage/food spoiler in orange). Boxplots within violin plots represent the median
1013 (center line), the 25th and 75th percentiles (box bounds), the 5th and 95th percentiles
1014 (whiskers), and the red dots depict the mean values.

1015

1016 **Figure 6:** Volatile compound production (integrated peak areas from chromatograms in
1017 arbitrary units) in 90-day cheeses inoculated with strains from the four *Penicillium roqueforti*
1018 populations (non-Roquefort cheese in blue, Roquefort cheese in purple, silage/food spoiler in

1019 orange and lumber/food spoiler in green). The areas for each family of compounds are the
1020 sum of the integrated areas of the compounds belonging to the family concerned. Alcohols I
1021 and II are derived from proteolysis and lipolysis, respectively. Acids I, II and III are derived
1022 from proteolysis, glycolysis and lipolysis, respectively (Supplementary Table 3). The color of
1023 the titles indicates the affiliation of the compounds to their families, as in Figure S6.

1024

1025 **Figure 7:** Integrated surface area (from chromatograms in arbitrary units) of the three most
1026 discriminant methyl ketones (A) and four secondary alcohols (B) for each assay (February,
1027 March, April) for the three strains of each *Penicillium roqueforti* population (lumber/food
1028 spoiler in green, non-Roquefort cheese in blue, Roquefort cheese in purple, silage/food
1029 spoiler in orange). Error bars represent standard deviations across cheese replicates.

1030

1031 **Figure legends for the Supplementary Material**

1032

1033 **Figure S1:** Abundance of microorganisms (in log colony-forming units/g) of the eight types
1034 monitored at various stages of cheese maturation (i.e. unpasteurized milk, 9, 20, 90 and 180
1035 days), for each of the four *Penicillium roqueforti* populations used to inoculate the cheeses
1036 (non-Roquefort cheese in blue, Roquefort cheese in purple, silage/food spoiler in orange and
1037 lumber/food spoiler in green). Error bars represent standard deviations across assays.

1038 **Figure S2:** Illustration of image processing for estimation of the percentage blue area on
1039 cheese slices: (a) example of an unprocessed image of a cheese slice; (b) image after
1040 brightness and contrast standardization; (c) image after cropping; (d) corresponding image
1041 binarization with a grayscale of 102 on the red channel. White and black correspond to pixel
1042 classification: in white, the inner part of the cheese and empty cavities; in black, cavities
1043 filled with the fungus.

1044

1045 **Figure S3:** Differences in aminoacid content between cheeses according to the population-of-
1046 origin of the *Penicillium roqueforti* strains. A. Discrimination between 90-day cheeses made
1047 with cheese (blue) and non-cheese (green) *P. roqueforti* populations (left), or Roquefort
1048 cheese (purple) and non-Roquefort cheese (blue) *P. roqueforti* populations (right), based on
1049 the abundance of the 23 identified amino acids present, according to an orthogonal signal-
1050 corrected partial least squares (PLS) discriminant analysis. Vertical and horizontal axes
1051 represent PLS1 and PLS 2 scores and gray arrows represent the relative contribution of
1052 loadings of signals significantly discriminating the group considered in a *t*-test with jackknife
1053 resampling. B. Abundance of molecules from particular classes detected in cheeses: mean
1054 integrated peak area from chromatograms in arbitrary units (bars, left axis) and cumulative
1055 percentage (line with dots, right axis) of aqueous extracts across all 90-day cheeses.

1056

1057 **Figure S4:** Violin plot depicting the distribution of the sums of 8,472 non-targeted organic
1058 signal peak areas, weighted by their mass-to-charge ratios (“m/z”), obtained in positive
1059 ionization mode for 90-day cheeses made with strains from the four *Penicillium roqueforti*
1060 populations (lumber/food spoiler in green, non-Roquefort cheese in blue, Roquefort cheese in
1061 purple and silage/food spoiler in orange). Boxplots within violin plots represent the median
1062 (center line), the 25th and 75th percentiles (box bounds), the 5th and 95th percentiles
1063 (whiskers), and the red dots depict the mean values.

1064

1065 **Figure S5:** Non-protein nitrogen levels at 20, 90 and 180 days of maturation, and water
1066 activity at 90 and 180 days of maturation. Comparison of cheeses made with strains from
1067 different *Penicillium roqueforti* populations (non-Roquefort cheese in blue, Roquefort cheese
1068 in purple, silage/food spoiler in orange and lumber/food spoiler in green). Error bars indicate

1069 95% confidence intervals.

1070

1071 **Figure S6:** Discrimination between 90-day cheeses inoculated with strains from the four
1072 *Penicillium roqueforti* populations (non-Roquefort cheese in blue, Roquefort cheese in
1073 purple, silage/food spoiler in yellow and lumber/food spoiler in green), based on the
1074 abundance of 41 volatile compounds in an orthogonal signal-corrected partial least squares
1075 (PLS) discriminant analysis. Vertical and horizontal axes represent the PLS1 and PLS2
1076 variances, and arrows represent the relative contributions of compound odor loadings
1077 significantly discriminating the group considered (according to
1078 www.thegoodscentscompany.com) in a *t*-test with jackknife resampling. The odor colors
1079 indicate the families in Figure 6 to which the associated compounds belong.

1080

1081 **Conflict of interest:** TC, MLP, SB, DR and MP were employed by SAS LIP, which
1082 produces starters for fermented food products, during the course of the study and therefore
1083 declare a competing financial interest. None of the other authors has any conflict of interest to
1084 declare. With the exception of TC employed by the LIP, the funders had no role in decisions
1085 concerning design, data analysis and interpretation, or in the decision to submit the work for
1086 publication.

Figure 1

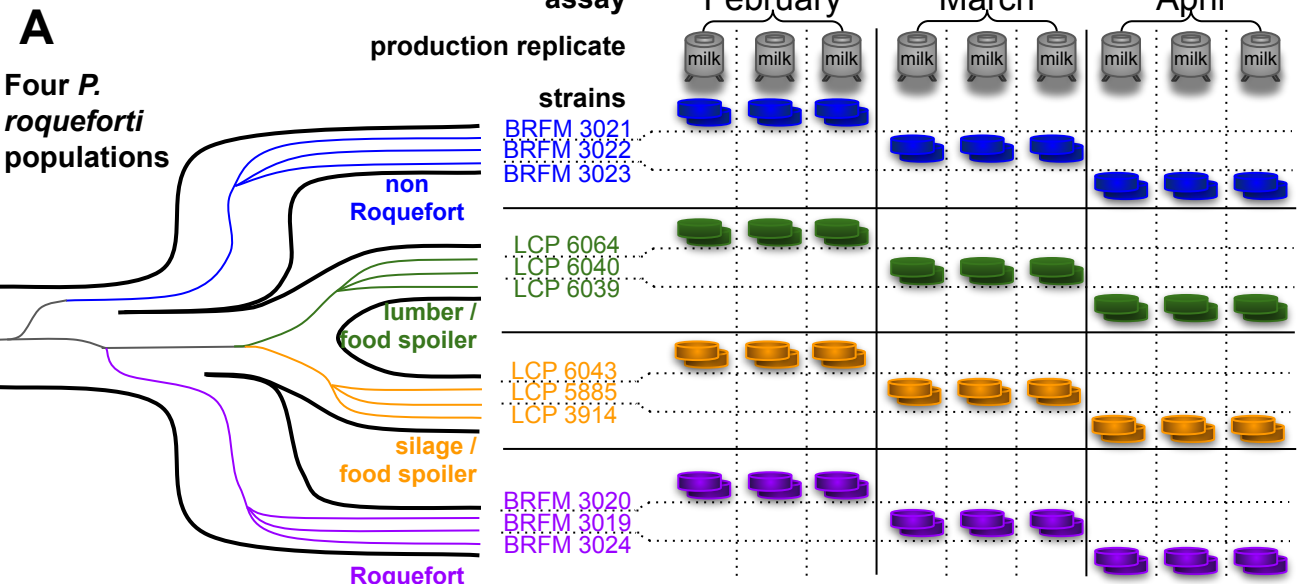


Figure 2A

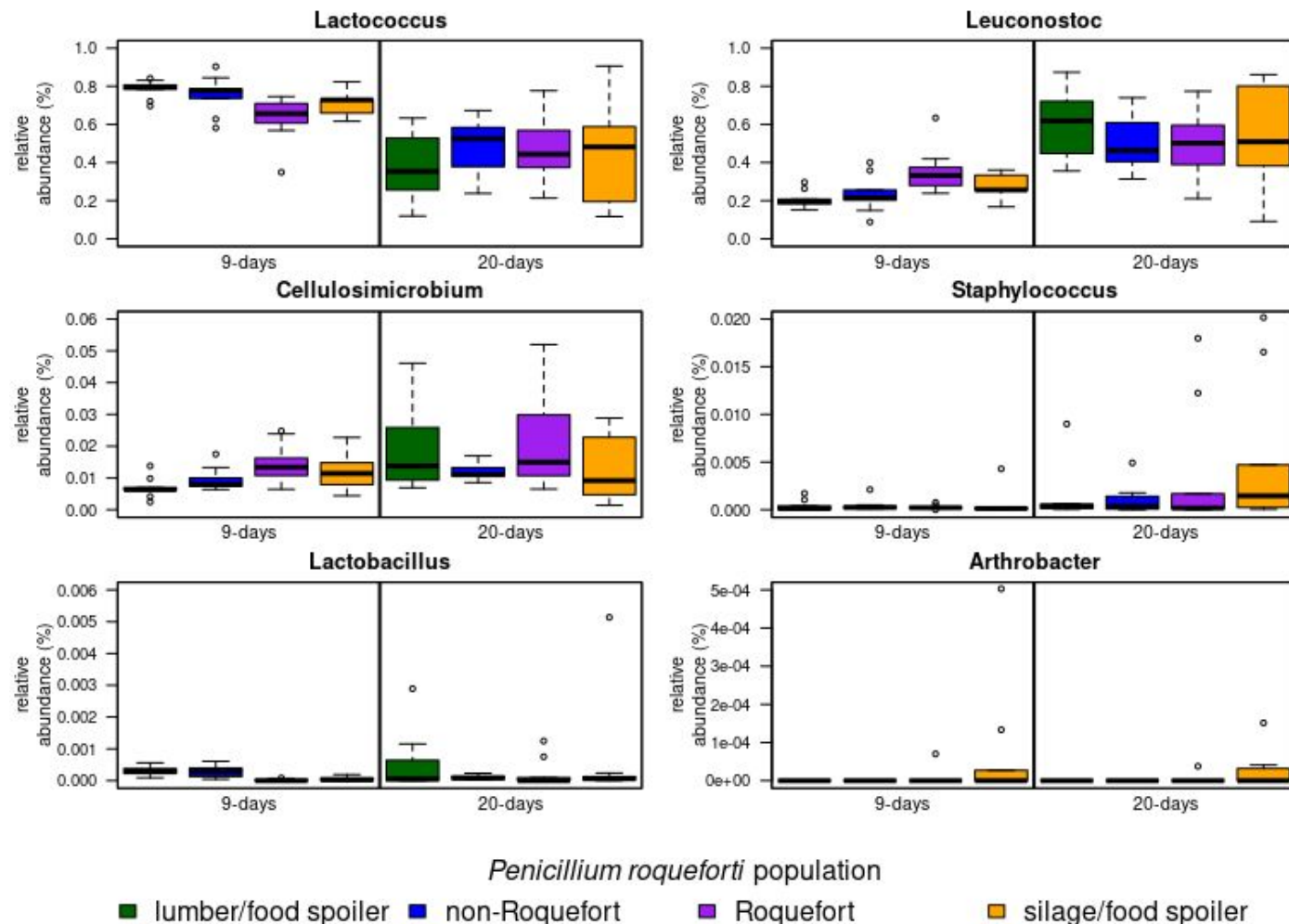


Figure 2B

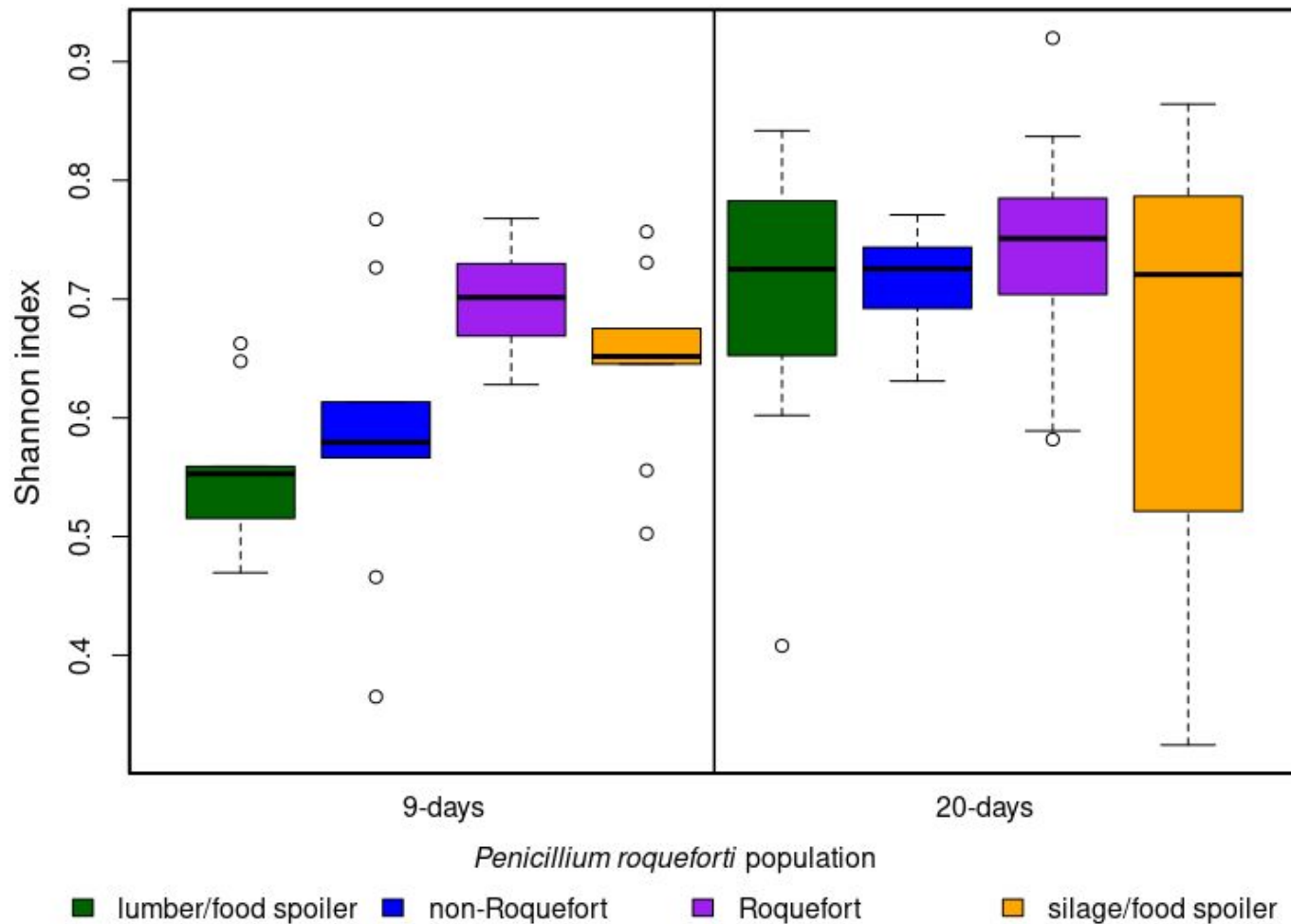


Figure 2C

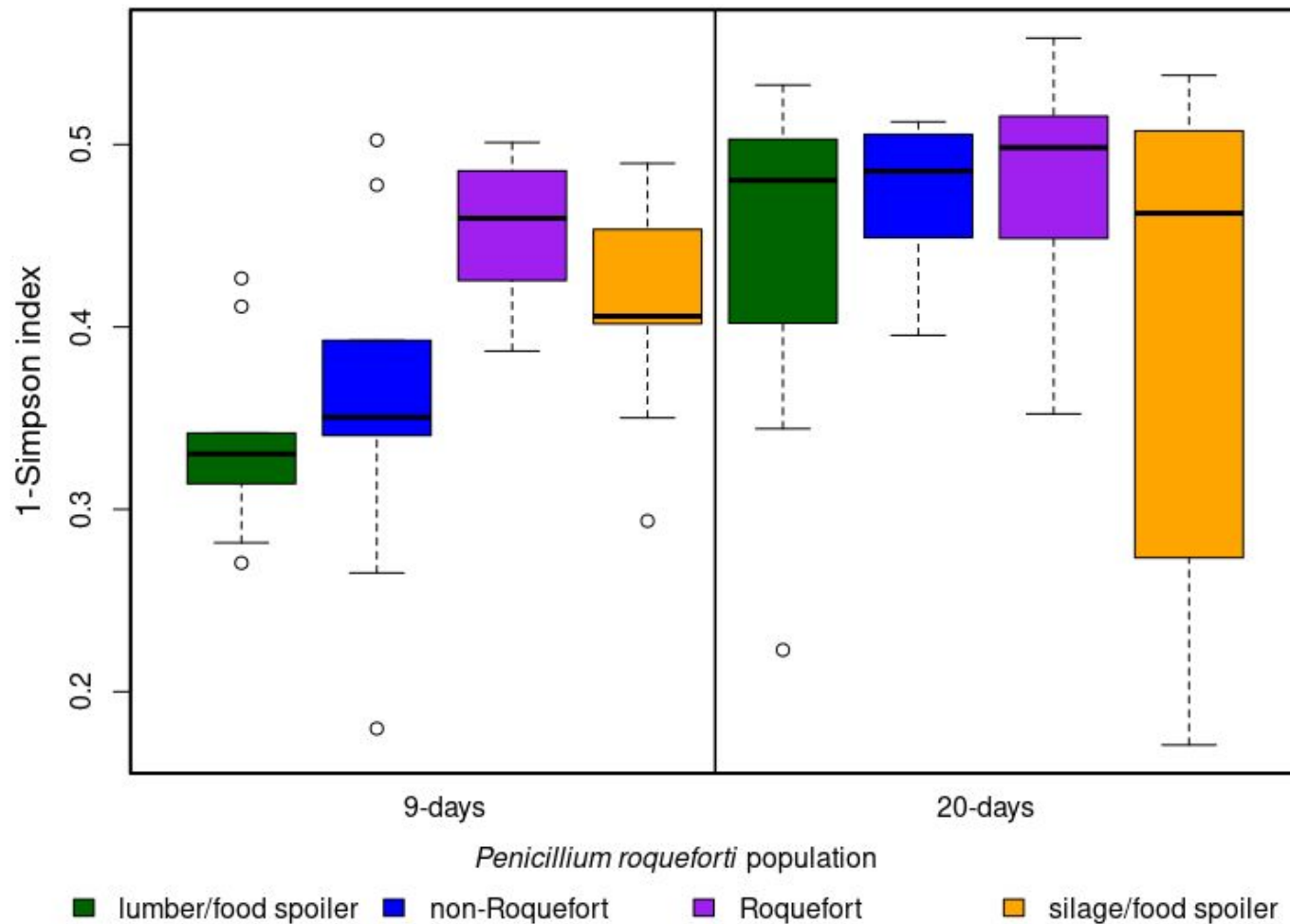


Figure 3A

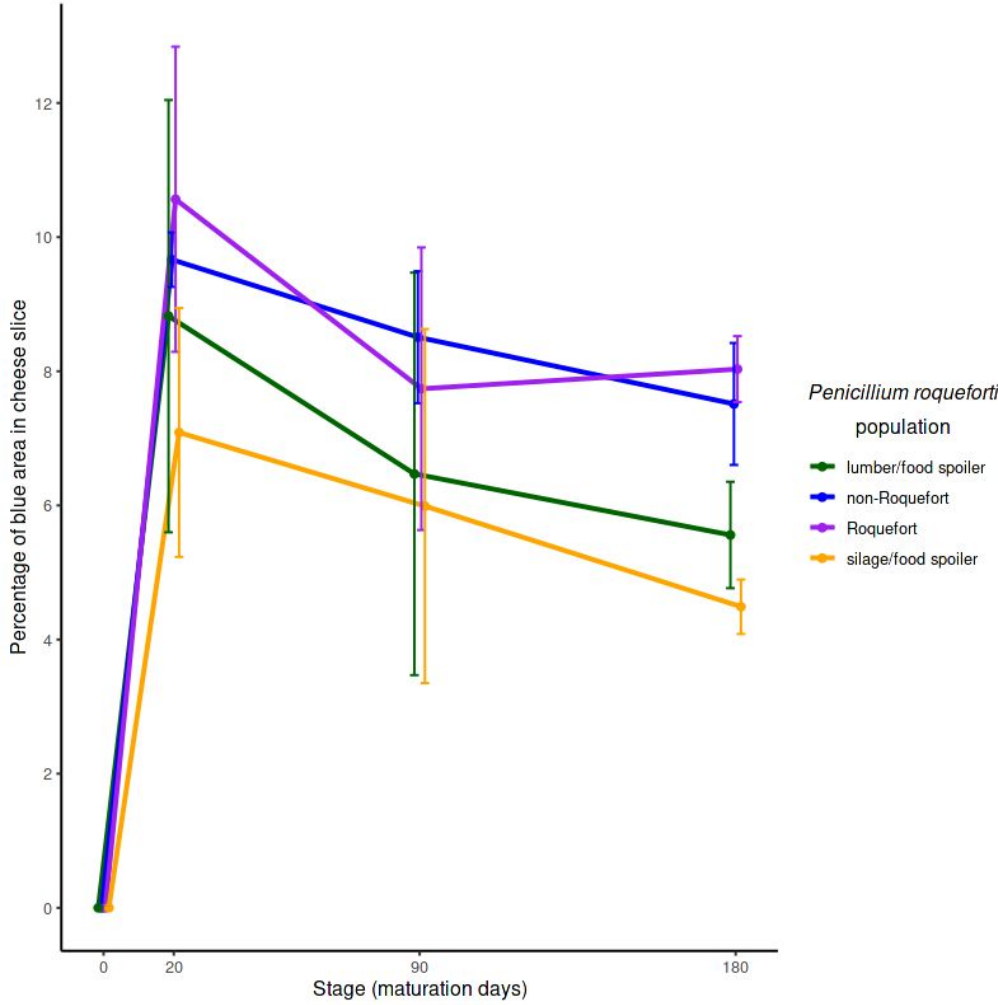


Figure 3B


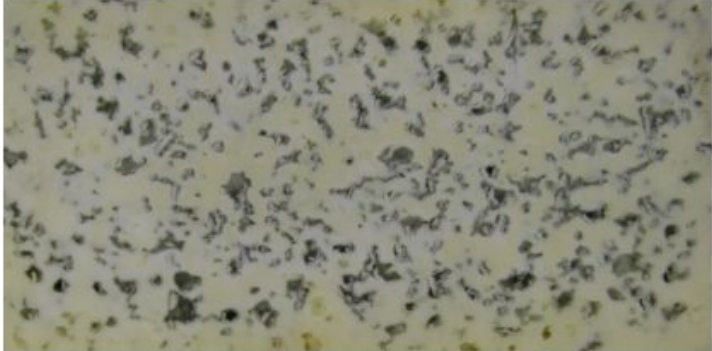


Non-cheese <i>P. roqueforti</i> populations	Cheese <i>P. roqueforti</i> populations
<p data-bbox="285 158 788 202">lumber/food spoiler (5.2 %)</p> 	<p data-bbox="1033 158 1468 202">non-Roquefort (10.2 %)</p> 
<p data-bbox="285 616 757 660">silage/food spoiler (5.5%)</p> 	<p data-bbox="1033 616 1381 660">Roquefort (11.8 %)</p> 

Figure 4

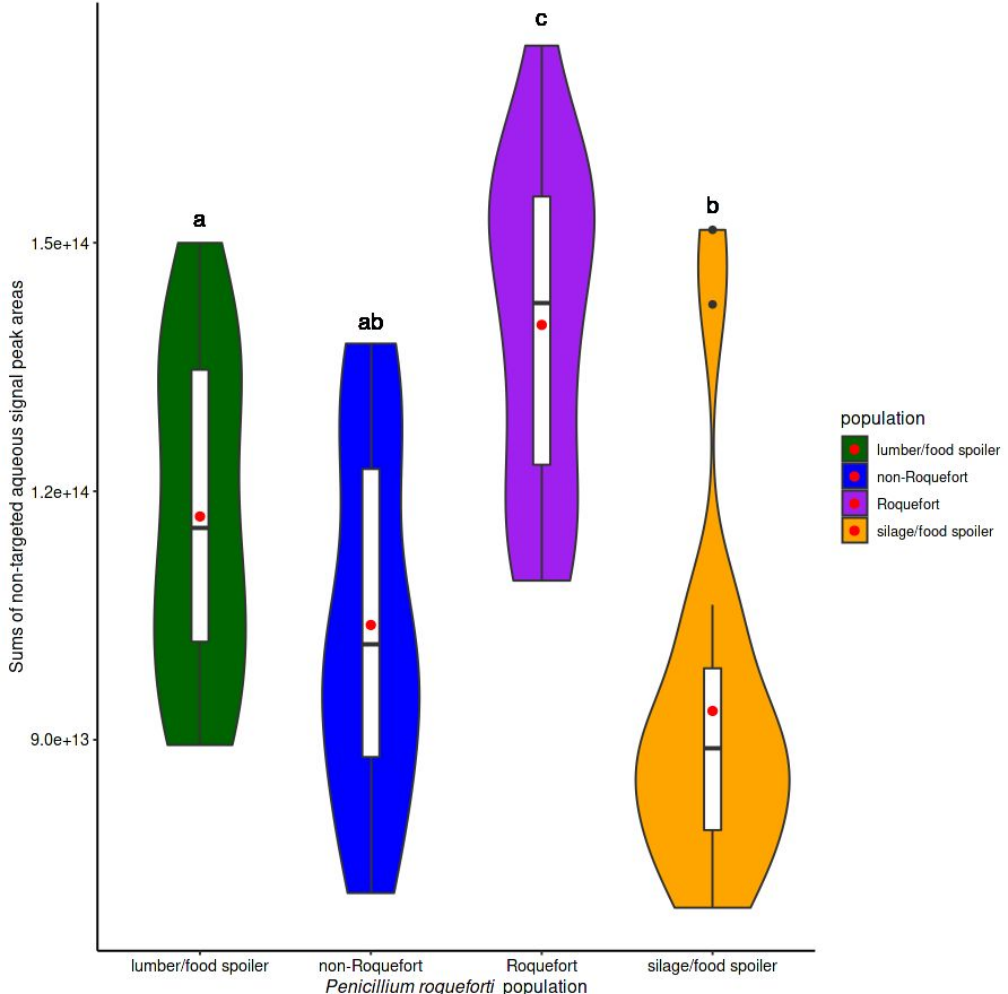


Figure 5

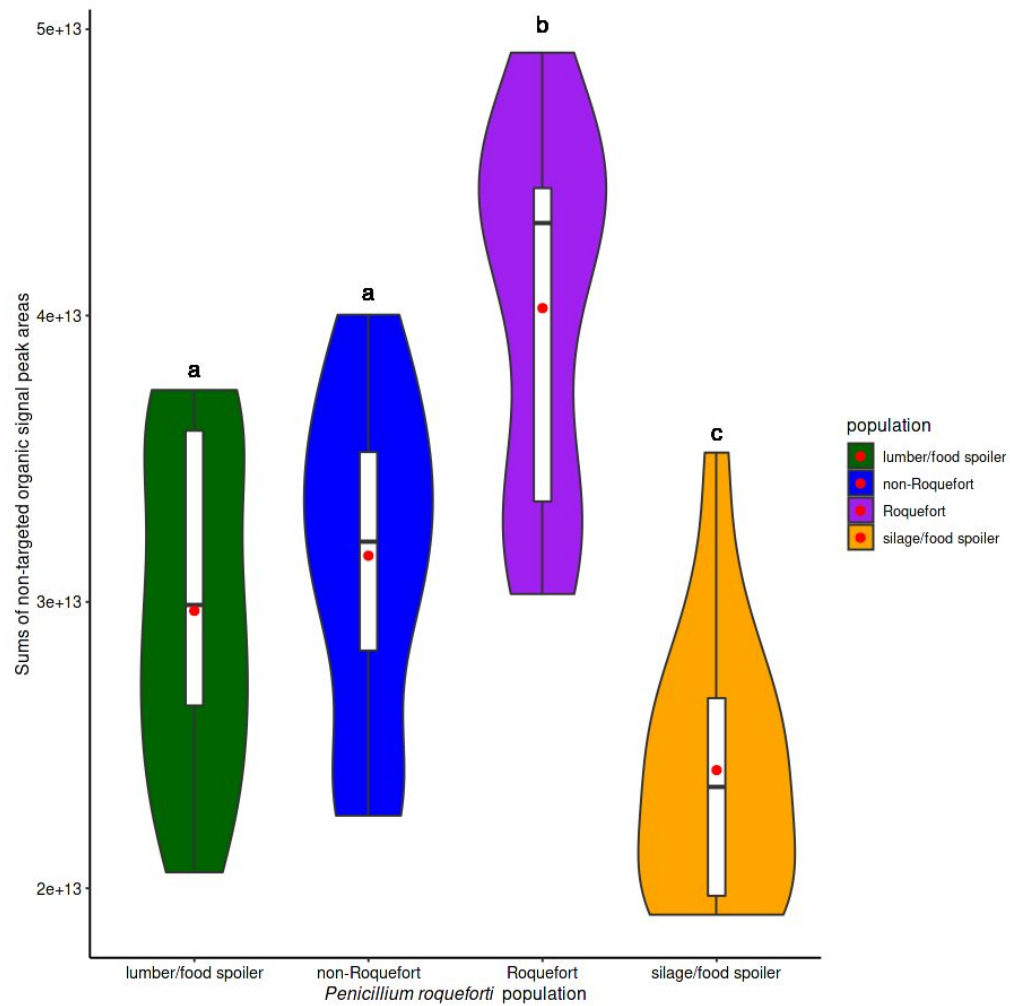


Figure 6

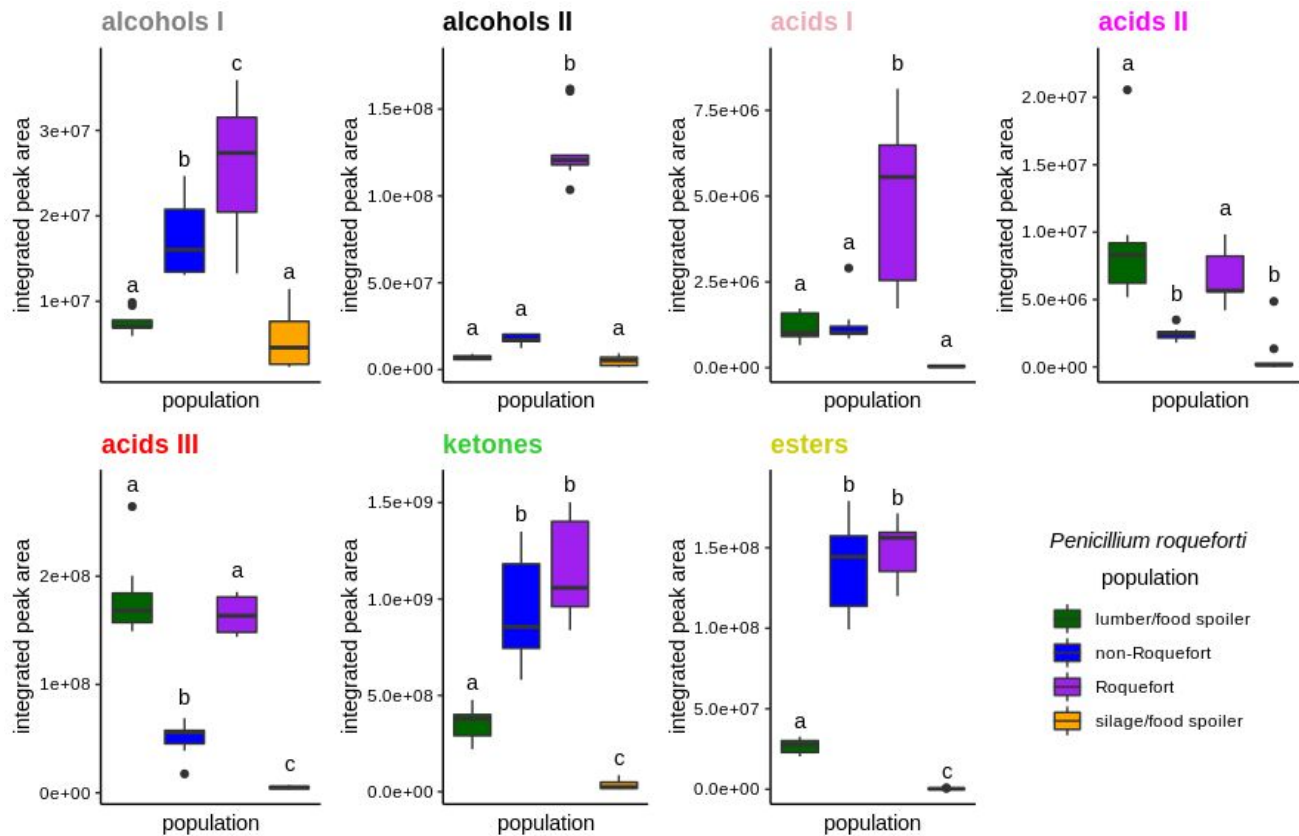
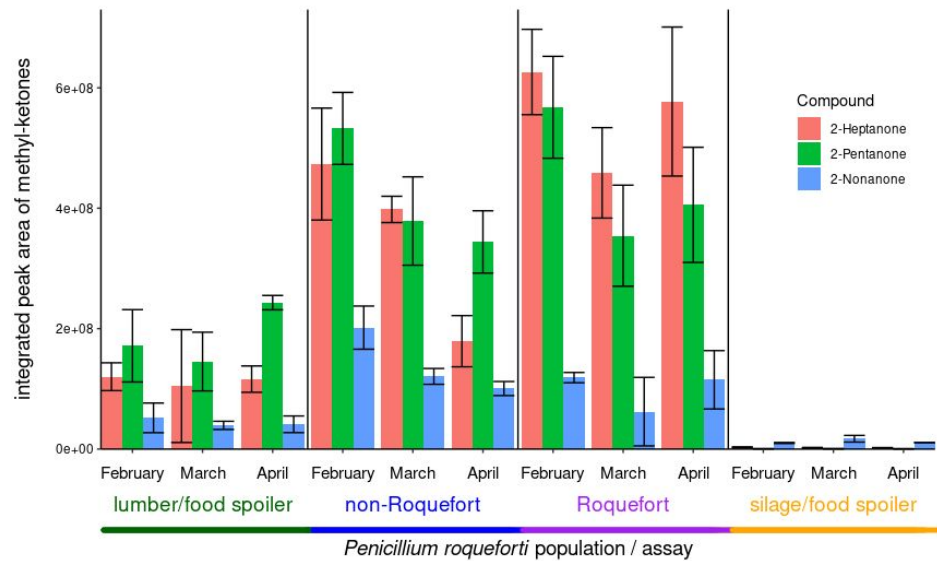


Figure 7

A



B

