

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

3

4 **Running title:** Impact of mold population impact 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. The *Penicillium roqueforti* fungus consists  
42 of four genetically differentiated populations, two of which have been domesticated for blue  
43 cheese-making, the other two thriving in other environments. Most blue cheeses are made  
44 with strains from a single *P. roqueforti* population, whereas Roquefort cheeses are inoculated  
45 with strains from a second population. We made blue cheeses in accordance with the  
46 production specifications for Roquefort-type cheeses, inoculating each cheese with a single  
47 *P. roqueforti* strain and using three strains from each of the four populations. The strain  
48 population-of-origin had a minor impact on bacterial diversity and none on the main  
49 microorganism abundance. The strains from cheese populations produced cheeses with  
50 higher percentages of blue area and larger amounts of desired volatile compounds. In  
51 particular, the Roquefort strains produced larger amounts of appealing aromatic compounds,  
52 in part due to their greater efficiency of proteolysis and lipolysis. The typical appearance and  
53 flavors of blue cheeses thus result from human selection on *P. roqueforti*, and the two cheese  
54 populations have acquired specific features. This has important implications for our  
55 understanding of adaptation and domestication, and for cheese improvement.

56 Domestication is an evolutionary process that has been studied by many biologists since  
57 Darwin. Indeed, domestication is an excellent model for understanding adaptation, being the  
58 result of strong and recent selection on traits that are often known and of interest for humans  
59 (Larson *et al.*, 2014). In addition, studies of domestication frequently have important  
60 implications for the improvement of cultivated organisms. However, domesticated fungi have  
61 been much less studied than crops, despite being excellent models in this field (Gladieux *et*  
62 *al.*, 2014; Giraud *et al.*, 2017). Most fungi can be cultured in Petri dishes, can remain alive  
63 for decades when stored in freezers and are propagated asexually. All these features facilitate  
64 experiments. Fungal metabolism produces various compounds of interest, including fuels,  
65 enzymes and antibiotics (Bigelis, 2001). The most ancient and frequent use of fungi by  
66 humans is for fermentation, to preserve and mature food. For example, the yeast  
67 *Saccharomyces cerevisiae* is used for bread, wine and beer fermentation, and the filamentous  
68 fungus *Aspergillus oryzae* is used for soy sauce and sake fermentation (Dupont *et al.*, 2017).  
69 These models have provided important insight into mechanisms of adaptation and  
70 domestication (Almeida *et al.*, 2014; Baker *et al.*, 2015; Gallone *et al.*, 2016; Gibbons *et al.*,  
71 2012; Gonçalves *et al.*, 2016; Libkind *et al.*, 2011; Sicard *et al.*, 2011).

72

73 The *Penicillium* genus contains more than 300 species, several of which are used by humans.  
74 For example, penicillin was discovered in *P. rubens*, and *P. nalgiovense* and *P. salamii* are  
75 used for the production of dry-cured meat (Fleming, 1929; Ludemann *et al.*, 2010, Perrone *et*  
76 *al.*, 2015). For centuries, *Penicillium roqueforti* (Thom) has been used in the maturation of all  
77 the many varieties of blue cheese worldwide (Labbe *et al.*, 2004, 2009; Vabre, 2015). This  
78 fungus generates the blue-veined appearance of these cheeses, by producing melanized spores  
79 in cavities within the cheese in which oxygen is available (Moreau, 1980). *Penicillium*  
80 *roqueforti* is also found in non-cheese environments (Pitt *et al.*, 2009; Ropars *et al.*, 2012),

81 and four genetically differentiated clusters of individuals (i.e., populations) have been  
82 identified in *P. roqueforti*. Two populations are used for cheesemaking, whereas the other  
83 two populations thrive in silage, lumber or spoiled food (Ropars *et al.*, 2014; Gillot *et al.*,  
84 2015; Dumas *et al.*, 2020). Genomic and experimental approaches have provided compelling  
85 evidence for the domestication of cheese *P. roqueforti* populations (Cheeseman *et al.*, 2014;  
86 Ropars *et al.*, 2015, 2016 a & b, 2017; Gillot *et al.*, 2015, 2017; Dumas *et al.*, 2020). Indeed,  
87 the populations of *P. roqueforti* used to make blue cheeses display the characteristic features  
88 of domesticated organisms: genetic and phenotypic differences relative to non-cheese  
89 populations, with, in particular, traits beneficial for cheese production, such as faster growth  
90 on cheese medium (Ropars *et al.*, 2014, 2016; Gillot *et al.*, 2015; Dumas *et al.*, 2020), but  
91 also lower fertility and lower fitness in nutrient-poor conditions (Ropars *et al.*, 2015, 2016).  
92 Both cheese populations have lower levels of genetic diversity than the two non-cheese  
93 populations, indicating an occurrence of bottlenecks (Dumas *et al.*, 2020), which typically  
94 occur during domestication. The two cheese populations are genetically and phenotypically  
95 differentiated from each other, suggesting that they result from independent domestication  
96 events (Dumas *et al.*, 2020). One of the cheese populations, the non-Roquefort population, is  
97 a clonal lineage with a very low level of genetic diversity, used to produce most types of blue  
98 cheeses worldwide. The second cheese population, the Roquefort population, is genetically  
99 more diverse and contains all the strains used to produce blue cheeses from the emblematic  
100 Roquefort protected designation of origin (PDO) (Dumas *et al.*, 2020). *In vitro* tests showed  
101 that the non-Roquefort population displayed faster tributyrin degradation (*i.e.* a certain type  
102 of lipolysis) and a higher salt tolerance, faster *in vitro* growth on cheese medium and better  
103 exclusion of competitors than the Roquefort population (Ropars *et al.*, 2014, 2015; Dumas *et*  
104 *al.*, 2020). The specific features of the Roquefort population may result from the constraints  
105 of the PDO, requiring the use of local strains and at least 90 days of maturation, and

106 preventing the use of strains from the non-Roquefort population better suited to modern  
107 modes of production (Dumas *et al.*, 2020). Genomic footprints of domestication (i.e., of  
108 adaptive genetic changes) have also been identified in the two *P. roqueforti* populations used  
109 for cheesemaking. Indeed, it has been suggested that horizontally transferred genes found  
110 only in the non-Roquefort population are involved in the production of an antifungal peptide  
111 and in lactose catabolism (Ropars *et al.*, 2014, 2015; Cheeseman *et al.*, 2014). The effects of  
112 positive selection have been detected in genes with predicted functions in flavor compound  
113 production, in each of the cheese populations (Dumas *et al.*, 2020).

114

115 Thus, the four *P. roqueforti* populations probably harbor multiple specific traits, leading to  
116 the generation of cheeses with different physicochemical properties and flavors, although this  
117 has yet to be tested. Assessments of the effect of the population-of-origin of the *P. roqueforti*  
118 strain used on the features of the cheese will i) provide important fundamental knowledge  
119 about the trait under selection for cheesemaking and adaptation to the cheese environment, ii)  
120 provide a basis for the elucidation of other genomic changes and iii) be of crucial applied  
121 importance for governing strain use and strain improvement. *Penicillium roqueforti* is used as  
122 a secondary starter for flavor production, mostly through proteolysis (*i.e.* casein catabolism)  
123 and lipolysis during ripening (Moreau, 1980). The main characteristic feature of blue cheeses,  
124 and of Roquefort PDO cheeses in particular, is their intense, spicy flavors (Kinsella *et al.*,  
125 1976; Rothe *et al.*, 1982). The specific volatile and metabolic compounds responsible for  
126 these flavors are generated principally by lipolysis in blue cheeses (Cerning *et al.*, 1987;  
127 Collins *et al.*, 2003), but their intensity varies between *P. roqueforti* strains (Larsen *et al.*,  
128 1999; Dumas *et al.*, 2020). The fatty acids released by lipolysis are the precursors of  
129 aldehydes, alcohols, acids, lactones and methyl ketones, which provide the moldy aromas  
130 typical of blue cheeses (Collins *et al.*, 2003). *Penicillium roqueforti* degrades most proteins,

131 but proteolysis efficiency varies between strains (Cerning *et al.*, 1987; Larsen *et al.*, 1998;  
132 Dumas *et al.*, 2020). The resulting peptides contribute to flavors, and their degradation into  
133 amino acids further influences cheese aroma and the growth of other microorganisms  
134 (Williams *et al.*, 2004; McSweeney *et al.*, 2000). *Penicillium roqueforti* also contributes to  
135 lactate degradation, which is necessary for deacidification and promotes the development of  
136 less acid-tolerant microorganisms (McSweeney *et al.*, 2017). Through these effects, and by  
137 producing secondary metabolites with antimicrobial properties, *P. roqueforti* may also affect  
138 the microbial composition of the cheese (Kopp *et al.*, 1979; Vallone *et al.*, 2014). Another  
139 parameter potentially affected by *P. roqueforti* populations and restricting the occurrence of  
140 spoiler microorganisms is the lack of free water, (i.e., a low water activity *aka*  $A_w$ ), which is  
141 heavily controlled for Roquefort cheese sales and is affected by the degree of proteolysis  
142 (Ardö *et al.*, 2017). The *P. roqueforti* population may thus also have an indirect effect on the  
143 features of the cheese, through various effects on beneficial or undesirable contaminants.

144

145 The differences between *P. roqueforti* populations have, to date, been studied only *in vitro* or  
146 in very rudimentary cheese models. Here, our objective was to assess the effect of the *P.*  
147 *roqueforti* population-of-origin of the inoculated strains on the features of blue cheeses  
148 produced in conditions closely mimicking those of commercial Roquefort PDO cheese  
149 production. We focused on several features considered important for cheese quality. Given  
150 the evidence from previous studies that cheese *P. roqueforti* populations have been  
151 domesticated, any differences between the cheeses produced with cheese and non-cheese  
152 populations, and/or between the two cheese populations would probably reflect human  
153 selection for the production of good cheeses, either on standing variation in the ancestral *P.*  
154 *roqueforti* population or for *de novo* mutations. Identifying the differences between *P.*  
155 *roqueforti* populations in terms of their properties for cheesemaking (e.g., ripening dynamics

156 and specific flavors) would improve our understanding of domestication and adaptation  
157 processes, and might drive important applications and developments. We therefore produced  
158 blue cheeses in conditions very similar to those used in industrial Roquefort PDO production,  
159 using, in particular, milk from the local “Lacaune” breed, with strains from the four *P.*  
160 *roqueforti* populations. We compared several important cheese features between the four  
161 populations: i) physicochemical features, relating to texture and biochemical composition, ii)  
162 cheese microbiota composition and abundance, which may have effects on several cheese  
163 features, iii) the proportion of the blue area in cheese slices, which is important for the blue-  
164 veined appearance of the cheese and is dependent on the growth and sporulation of *P.*  
165 *roqueforti* in cheese cavities, and iv) the metabolic and volatile compounds produced and  
166 their amounts, which influence flavor and aroma. We investigated the differences in these  
167 features between the cheeses produced with strains from the four *P. roqueforti* populations  
168 (Roquefort cheese, non-Roquefort cheese, silage, and lumber/food spoiler populations). We  
169 also investigated the possible differences between cheeses made with cheese and non-cheese  
170 populations, and between the Roquefort and non-Roquefort cheese populations. Assessments  
171 of the traits differing between cheese and non-cheese *P. roqueforti* populations, and between  
172 the two cheese populations, and investigations of whether the cheese populations are more  
173 suitable for cheese-making, i) is of fundamental importance for understanding the  
174 domestication of cheese fungi, through the identification of traits subjected to selection, and  
175 ii) has many applied consequences for the cheese industry, in terms of strain choice for  
176 different kinds of blue cheeses, paving the way for the improvement of mold strains by  
177 generating progenies from crosses of the two cheese populations, and for the choice of traits  
178 for measurement and selection in offsprings.

179

180 **Materials and Methods**

181 More details about the Materials and Methods are provided in the Supplementary Methods.

182 **Cheesemaking:** The cheesemaking protocol was typical of the procedures used by the main  
183 producers of Roquefort cheese and complied with the Roquefort PDO specifications, except  
184 that the ripening process took place in artificial cellars in the INRA facilities at Aurillac, with  
185 strains from different *P. roqueforti* populations (Figure 1A; a strain is defined here as a  
186 haploid individual obtained by monospore isolation). We made cheeses by inoculating a  
187 single strain per cheese, using in total three different strains from each of the four *P.*  
188 *roqueforti* populations (Figure 1A). The strains were assigned to these populations in a  
189 previous study, on the basis of molecular markers (Dumas *et al.*, 2020). Due to the limited  
190 production capacity of the experimental facility, it was not possible to make all the cheeses at  
191 the same time. We therefore split cheese production into three assays, each including one  
192 strain from each of the four populations (Figure 1A). For each strain in each assay, we  
193 created three production replicates, with two cheeses per strain in each replicate, to ensure  
194 that enough material was produced for sampling. In total, we produced 72 cheeses (4 strains \*  
195 2 cheeses \* 3 replicates \* 3 assays; figure 1B). The assays were performed sequentially from  
196 February to April. The effect of the seasonal change in milk composition was therefore  
197 confounded with the strain effect within the population, hereafter referred to as the "assay  
198 effect". The three replicates within each assay were also set up at different times, a few days  
199 apart, and thus with different batches of unpasteurized milk (Figure 1A).

200 **Microbial analyses:** We estimated the concentrations of various microorganism  
201 communities in the initial unpasteurized milk and at various stages of cheese maturation (for  
202 more information see the Supplementary Methods). We performed a metabarcoding analysis  
203 on the experimental cheeses at 9 and 20 days of maturation, by sequencing the 16S DNA  
204 fragment with Illumina Miseq technology and analyzing sequences with Find Rapidly OTUs  
205 in Galaxy Solution (FROGS), v3.0 (Escudié *et al.*, 2018). For each OTU, taxonomic



206 assignment was determined with the Silva-132 (<https://www.arb-silva.de/>) and 16S rDNA  
207 RefSeq databases (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

208 **Blue area:** We estimated the percentage area of the cheese that was blue, on fresh inner  
209 cheese slices, by analyzing images of the slices with ImageJ software and counting the  
210 number of dark pixels.

211 **Physicochemistry:** We performed standard physicochemical measurements on the cheeses.  
212 We measured dry matter content, fat over dry matter content, the moisture content of the  
213 defatted cheese, total, soluble and non-protein nitrogen contents, chloride and salt content,  
214 water activity and pH at various stages of maturation, according to reference methods (for  
215 more information, see the Supplementary Methods). We measured glucose, lactose, lactate,  
216 acetate and butyrate concentrations in the cheeses on days 9 and 20, by high-performance  
217 liquid chromatography (HPLC, for more information, see the Supplementary Methods).

218 **Metabolic and volatile compounds:** We investigated possible differences in proteolytic and  
219 lipolytic activities between the four populations, by UHPLC-MS after two extraction  
220 procedures (water and an organic solvent). We analyzed the amounts of free fatty acids and  
221 residual glycerides in 90-day cheeses, by coupling a global extraction (accelerated solvent  
222 extraction with hexane-isobutanol) with UHPLC-MS analysis in the positive (triglycerides)  
223 and negative (fatty acids) ionization modes (for more information, see the Supplementary  
224 Methods). We investigated the identity and abundance of volatile flavor and aroma  
225 compounds, using a dynamic headspace system (DHS) with a Gerstel MPS autosampler  
226 (Mülheim an der Ruhr, Germany) and gas chromatography-mass spectrometry analysis with a  
227 7890B Agilent GC system coupled to an Agilent 5977B quadrupole mass spectrometer (Santa  
228 Clara, United States). Statistical analyses were performed with R software ([http://www.r-](http://www.r-project.org/)  
229 [project.org/](http://www.r-project.org/)). Further details about the materials and methods are provided in the  
230 Supplementary Methods.

231

## 232 **Results**

233 **Design and cheesemaking.** We made cheeses by inoculating a single strain per cheese and  
234 using in total three different strains (biological replicates) from each of the four *P. roqueforti*  
235 populations (Figure 1A). We divided the production into three assays, each including one  
236 strain from each of the four populations (Figure 1A). For each strain in each assay, we  
237 generated three production replicates at different times, with different batches of  
238 unpasteurized milk, each production replicate encompassing two cheeses per strain. The  
239 assays were performed sequentially from February to April. The effect of the seasonal change  
240 in milk composition was therefore confounded with the strain effect, hereafter referred to as  
241 the "assay effect". The experimental design did not, therefore, allow for the testing of a strain  
242 effect, but it was possible to test for a population effect (the replicates being the three strains  
243 used per population), which was our goal. The seasonal effect, if any, would blur the  
244 population effect, so any differences between populations detected in this study can be  
245 considered to be robust.

246

247 ***Penicillium roqueforti* population-of-origin influences the bacterial diversity of the**  
248 **cheese, but not the abundance of the main microorganisms.** We investigated whether *P.*  
249 *roqueforti* population-of-origin affected the composition of the cheese microbiota, by  
250 estimating the densities of key microbial communities with colony counts (cfu/g) on various  
251 specific culture media (for total aerobic mesophilic bacteria, mesophilic lactic acid bacteria,  
252 thermophilic lactic acid bacteria, dextran-producing *Leuconostoc* spp., molds and yeasts,  
253 Gram-positive catalase-positive bacteria and enterobacteria) and with a metabarcoding  
254 approach based on 16S sequencing targeting the bacteria in cheeses at several stages of

255 maturation. The abundance and identity of the microorganisms studied (Supplementary  
256 Figures 1A and 1B) were similar to those in four commercial Roquefort cheeses (Devoyod *et*  
257 *al.*, 1968; personal information from C. Callon) and closely related blue cheeses  
258 (Diezhandino *et al.*, 2015). Based on microbial counts, we found no significant effect of *P.*  
259 *roqueforti* population on the abundance of any of the counted microorganisms, including  
260 molds (i.e., mainly *P. roqueforti*), at any stage of maturation (Supplementary Table 1A).

261 The metabarcoding approach targeting bacteria identified mostly sequences from the  
262 *Lactococcus* and *Leuconostoc spp.* starters, which are responsible for acidification and cavity  
263 formation in the cheese, respectively. The remaining sequences corresponded to 12 bacterial  
264 genera frequently found in unpasteurized milk cheeses, such as *Lactobacillus*,  
265 *Staphylococcus* and *Arthrobacter*. However, the large predominance of starters made it  
266 impossible to obtain sufficient data for other bacteria to assess differences in the abundance  
267 of particular bacteria between cheeses made with strains from the four *P. roqueforti*  
268 populations (Supplementary Table 1B). We estimated three OTU (operational taxonomic  
269 unit) diversity parameters based on bacterial barcode sequence abundances, to measure OTU  
270 richness and/or evenness. Bray-Curtis dissimilarity showed that cheeses made with strains  
271 from the same *P. roqueforti* population were no more similar than those made with strains  
272 from different *P. roqueforti* populations. However, we found a significant effect of *P.*  
273 *roqueforti* population, in addition to a stage effect, on the Shannon and Simpson diversity  
274 indices. Cheeses made with strains from the cheese *P. roqueforti* populations tended to have a  
275 higher bacterial OTU diversity, particularly at nine days of maturation and for the Roquefort  
276 population (Supplementary figure 2A and 2B), although the post-hoc analyses were not  
277 powerful enough to detect significant pairwise differences (Supplementary Table 1B). The  
278 differences in cheese bacterial diversity, although minor, suggest that the differences between  
279 cheeses made with strains from the four *P. roqueforti* populations may be due not only to a

280 direct effect of *P. roqueforti* population, but also to an indirect effect mediated by the  
281 induction of bacterial communities of different diversities. There may also be undetected  
282 differences at species level or for low-abundance microorganisms that might nevertheless  
283 have substantial effects. However, even if this were the case, it would constitute an indirect  
284 effect of the *P. roqueforti* population, as this was the only difference during our  
285 cheesemaking process.

286

287 **Higher proportion of blue area in cheeses produced with cheese *P. roqueforti***  
288 **populations.** We estimated the percentage of the cheese area that was blue on fresh inner  
289 cheese slices. The blue veins depend on the formation of cavities in the cheese, and the  
290 growth and sporulation of *P. roqueforti* in these cavities. The percentage blue area was  
291 significantly larger in cheeses produced with cheese population strains than in those produced  
292 with non-cheese population strains (Figure 3; Supplementary Table 1C). We also found a  
293 significant decrease in blue area from 20 to 180 days of maturation, for all populations except  
294 the Roquefort population, for which the percentage blue area remained approximately  
295 constant (Figure 3; Supplementary Table 1C).

296

297 **More efficient proteolysis and lipolysis by the Roquefort *P. roqueforti* population.** The *P.*  
298 *roqueforti* strains used for cheesemaking are known to have high proteolytic and lipolytic  
299 activities, which play a key role in cheese ripening. We therefore investigated the proteolysis  
300 and lipolysis efficiencies of the four populations. Both targeted and non-targeted  
301 chromatographic analyses showed that proteolysis efficiency was highest in the Roquefort *P.*  
302 *roqueforti* population. We performed the targeted analysis with standards for the principal 23  
303 amino acids (Supplementary Table 2A). We found that eight amino acids discriminated

304 significantly between cheeses made with the different *P. roqueforti* populations  
305 (Supplementary Table 1D), 15 discriminated between the cheese and non-cheese populations  
306 and 14 distinguished between the Roquefort and non-Roquefort populations (Supplementary  
307 Figure 4A). The cheeses made with strains from cheese populations, and from the Roquefort  
308 population, in particular, had a higher total amino-acid concentration (Supplementary Tables  
309 1D and 2B).

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

318

319 Lipolysis was also more efficient for the Roquefort population than for the other populations.  
320 We investigated whether the *P. roqueforti* population influenced the amounts of free fatty  
321 acids and residual glycerides, as a proxy for lipolysis efficiency, in 90-day cheeses, with  
322 targeted and non-targeted chromatographic analyses in the positive and negative ionization  
323 modes. We specifically targeted glycerides and free fatty acids. In the targeted analysis, we  
324 identified seven free fatty acids and 20 triglycerides, and found that three free fatty acids  
325 were significantly more concentrated in cheeses made with Roquefort strains than in those  
326 made with strains from non-Roquefort populations (Supplementary Table 1F). In the non-  
327 targeted analysis, we obtained 3,094 signals and observed higher amounts of organic signals  
328 specific to free fatty acids, indicating the most efficient lipolysis, in cheeses made with strains

329 from the Roquefort population, followed by the lumber and non-Roquefort cheese  
330 populations, which were very similar to each other, with lipolysis efficiency lowest for  
331 cheeses made with strains from the silage population (Figure 5; Supplementary Table 1G).  
332 For residual glycerides, we obtained 8,472 signals, with no significant difference between the  
333 populations (Supplementary Figure 5; Supplementary Table 1H).

334

335 As expected, we observed a maturation stage effect for 11 of the 16 physicochemical  
336 parameters (Supplementary Table 1I). Non-protein nitrogenous content was significantly  
337 higher in cheeses inoculated with strains from cheese *P. roqueforti* populations than in  
338 cheeses inoculated with strains from the other populations, consistent with the greater  
339 efficiency of proteolysis associated with these strains (Supplementary Figure 6A). Cheese  
340 water activity differed significantly between the cheeses made with strains from the four *P.*  
341 *roqueforti* populations (Supplementary figure 6B): it was significantly lower for the  
342 Roquefort cheese population than for the non-Roquefort cheese and silage populations  
343 (Supplementary Table 1I).

344

345 **Strong influence of *P. roqueforti* population on volatile compound production.** We  
346 investigated the effect of *P. roqueforti* population on cheese aroma and flavor, by  
347 determining the relative abundance of the most relevant volatile compounds in 90-day  
348 cheeses. We focused on the GC-MS data for the 40 principal volatile compounds considered  
349 to be markers of the aromatic quality of blue cheeses (Rothe *et al.*, 1982): 11 acids, 12  
350 ketones, 10 esters, six alcohols and one aldehyde (Supplementary Table 3). We found that *P.*  
351 *roqueforti* population strongly influenced the amounts of the compounds from these aromatic  
352 families in the cheeses (Supplementary Table 1J; Figures 6 and 7). Indeed, the odors of the  
353 cheeses differed considerably: the cheeses made with strains from the cheese *P. roqueforti*

354 populations smelled as good as typical ripened blue cheeses, whereas those made with strains  
355 from non-cheese *P. roqueforti* populations had unpleasant odors, similar to those of a wet  
356 swab (Supplementary Figure 7; personal observation).

357

358 The amounts of acids, methyl ketones and secondary alcohols resulting from proteolysis and  
359 lipolysis, and contributing to the typical flavor of blue cheese, were larger in cheeses  
360 produced with strains from cheese populations than in those produced with strains from non-  
361 cheese populations. These compounds were present in particularly large amounts in cheeses  
362 made with strains from the Roquefort population. Four of the 40 compounds analyzed were  
363 proteolysis by-products (primary alcohols: 3-methyl-butanal, 3-methyl-butanol and  
364 isopropyl-alcohol, named hereafter alcohols I, and 3-methyl-butanoic acid, named hereafter  
365 acid I; Supplementary Table 3). The abundance of alcohols I was significantly higher in  
366 cheeses made with strains from cheese *P. roqueforti* populations than in those made with  
367 strains from non-cheese populations, and the highest values were obtained for the Roquefort  
368 population (Supplementary Table 1J). Acid I was also present in larger amounts in cheeses  
369 made with strains from the Roquefort population than in other cheeses. Two acids, by-  
370 products of glycolysis (named hereafter acids II), were present in larger amounts in cheeses  
371 made with strains from the Roquefort and lumber/food spoiler *P. roqueforti* populations than  
372 in other cheeses (Supplementary Tables 1J and 3). The other 35 aromatic compounds (i.e.  
373 acids from beta-oxidation, named hereafter acids III, ketones, secondary alcohols named  
374 hereafter alcohols II, and esters) were almost all direct or indirect by-products of lipolysis  
375 (Supplementary Table 3). The abundance of acids III was higher in cheeses made with strains  
376 from the Roquefort and lumber/food spoiler populations than in cheeses made with strains  
377 from the non-Roquefort cheese population. The levels of these compounds were lowest in  
378 cheeses made with strains from the silage population. Larger amounts of esters and methyl

379 ketones (especially 2-pentanone and 2-heptanone) were found in cheeses made with strains  
380 from cheese *P. roqueforti* populations (Supplementary Table 1J). Cheeses made with strains  
381 from the Roquefort population contained the largest amounts of methyl ketones, and these  
382 compounds were barely detectable in cheeses made from silage population strains (Figure  
383 7A). The levels of alcohols II, particularly 2-heptanol, were also much higher in cheeses  
384 made with Roquefort population strains than in other cheeses (Supplementary Table 1J;  
385 Figure 7B).

386

387

## 388 **Discussion**

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

390 Measurements of multiple features of blue cheeses made under conditions resembling those  
391 typically used in commercial Roquefort production revealed a strong influence of the  
392 differentiated *P. roqueforti* populations on cheese quality, with the cheese populations  
393 appearing the best adapted to cheesemaking, in terms of both the appearance and aromatic  
394 quality of the resulting cheese. The differences between the four *P. roqueforti* populations  
395 and the more appealing cheeses produced with strains from the cheese populations suggest  
396 that humans have exerted selection for the production of better cheeses, either on standing  
397 variation or on *de novo* mutations, and this corresponds to domestication. Indeed, we found  
398 that cheese *P. roqueforti* strains produced a larger percentage blue area on cheese slices, an  
399 important visual aspect of blue cheeses. We also found that proteolysis and lipolysis were  
400 more efficient in cheeses made with Roquefort population strains than in cheeses made with  
401 strains from the other *P. roqueforti* populations, resulting in the production of larger amounts  
402 of desirable volatile compounds, including alcohols and associated acids. Cheese water  
403 activity was lower in cheeses made with strains from the Roquefort population, probably due



404 to more efficient proteolysis (Ardö *et al.*, 2017). We found no significant difference in the  
405 identities and abundances of microorganisms between the cheeses made with strains from the  
406 four *P. roqueforti* populations. Some minor differences in species diversity were observed,  
407 however, and the differences between cheeses probably reflected a direct effect of the  
408 specific features of the *P. roqueforti* population, although minor indirect effects involving the  
409 induction of more diverse bacterial communities by cheese *P. roqueforti* strains may also  
410 have occurred. Overall, our findings strongly support the view that cheese *P. roqueforti*  
411 populations have been selected by humans for better appearance and aroma. This selection  
412 may have involved the choice of the most beneficial strains for making good cheeses from  
413 standing variation, and/or the selection of *de novo* genetic changes. Previous studies found  
414 footprints of genomic changes in cheese populations in the form of beneficial horizontal gene  
415 transfers and positive selection (Dumas *et al.*, 2020; Ropars *et al.*, 2015).

416

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

429

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

437

438 The metabarcoding approach suggested that the different *P. roqueforti* populations induced  
439 bacterial communities of different levels of diversity. The cheese populations, and the  
440 Roquefort population in particular, were associated with the highest level of diversity. The  
441 large predominance of bacterial starters made it impossible to collect sufficient data for an  
442 assessment of the differences in relative abundance between subdominant bacterial species on  
443 the basis of metabarcoding. We also found a significant difference in water activity between  
444 cheeses made with strains from the four *P. roqueforti* populations, the lowest value obtained  
445 being that for the Roquefort population. This may also reflect human selection, as low water  
446 activity restricts the occurrence of spoiler microorganisms, and is therefore highly controlled  
447 for Roquefort cheese sales, particularly those for export.

448

449 **Cheese *P. roqueforti* populations produce bluer cheeses.** We found significantly higher  
450 percentage blue areas in cheese slices from cheeses made with cheese *P. roqueforti* strains  
451 than in those made from non-cheese strains, potentially reflecting greater *P. roqueforti*  
452 growth in cheese and/or a higher sporulation efficiency in cavities. The percentage blue area  
453 in cheese slices also depends on the formation of cavities in the cheese, as *P. roqueforti* can

454 only sporulate in cavities in which oxygen is available. The cavities are mostly generated by  
455 the gas-producing bacterium *Leuconostoc mesenteroides*, the abundance of which did not  
456 differ between the cheeses made with strains from different *P. roqueforti* populations,  
457 suggesting a direct effect of *P. roqueforti* populations on the blueness of cheese slices. The  
458 significantly higher percentage blue area in slices of cheese made with cheese *P. roqueforti*  
459 strains than in those made with non-cheese strains therefore probably reflects better cheese  
460 and cavity colonization and sporulation, probably due to selection on the basis of appearance.  
461 The percentage blue area decreased by the end of maturation, perhaps due to the death of the  
462 fungus. Only cheeses made with Roquefort strains retained a high percentage blue area at 90  
463 days of maturation, again potentially reflecting selection in pre-industrial times, when  
464 Roquefort cheeses had to be stored for several months at cave temperature before sale. The  
465 minimum maturation time for Roquefort PDO remains 90 days, which is longer than for other  
466 blue cheeses. These findings contrast with a previous study showing that non-Roquefort  
467 population colonized the cavities of model cheeses better than other populations (Dumas *et*  
468 *al.*, 2020); this discrepancy may reflect differences between studies in terms of the  
469 measurements used (total percentage blue area versus percentage blue area within cavities),  
470 the type of milk (ewe versus goat) or the mode of cheesemaking (rudimentary models versus  
471 commercial-like cheeses). Our findings are consistent with the presence of horizontally  
472 transferred genes in cheese populations with predicted functions in fungal development,  
473 including sporulation and hyphal growth (Dumas *et al.*, 2020).

474

475 **Proteolysis and lipolysis are more efficient in the Roquefort *P. roqueforti* population.**  
476 Based on chemical analyses and powerful chromatographic discrimination methods, we  
477 showed that the abundance of amino acids and small peptides (i.e., residual products of

478 proteolysis) was highest in cheeses made with Roquefort *P. roqueforti* strains. Thus, these  
479 strains had the highest capacity for proteolysis, which is an important process in  
480 cheesemaking. Indeed, proteolysis contributes to the development of cheese texture, flavors  
481 and aromas (Ardö *et al.*, 2017; Andersen *et al.*, 2010; McSweeney, 1997; Roudot-Algaron,  
482 1996; Ardö, 2006). Previous measurements of proteolytic activity in synthetic media detected  
483 significant differences between *P. roqueforti* populations, but not between the two cheese  
484 populations (Dumas *et al.*, 2020). We show here that experimental cheeses made with strains  
485 from the Roquefort population have a higher content of residual products of proteolysis, a  
486 sign of more advanced ripening.

487

488 We also found that lipolysis was more efficient in the cheeses made with strains from the  
489 Roquefort *P. roqueforti* population. By contrast, previous studies in synthetic media found  
490 that lipolysis was most efficient in the non-Roquefort population (Dumas *et al.*, 2020). The  
491 discrepancy between these studies demonstrates the need for measurements in real cheeses  
492 for the reliable assessment of metabolic activities. Lipolytic activity is known to affect cheese  
493 texture and the production of volatile compounds affecting pungency (Alonso *et al.*, 1987;  
494 González De Llano *et al.*, 1990, 1992; Martín *et al.*, 2016; Thierry *et al.*, 2017; Woo *et al.*,  
495 1984). The more efficient proteolysis and lipolysis in the Roquefort *P. roqueforti* population  
496 should have a strong impact on cheese texture and flavor. It therefore probably results from  
497 selection to obtain better cheeses, i.e. from a domestication process, as previously reported  
498 for other fungi (Almeida *et al.*, 2014; Baker *et al.*, 2015; Gallone *et al.*, 2016; Gibbons *et al.*,  
499 2012; Gonçalves *et al.*, 2016; Libkind *et al.*, 2011; Sicard *et al.*, 2011). Roquefort cheeses are  
500 widely considered to be the blue cheeses with the strongest aromas and flavours; the less  
501 efficient lipolysis and proteolysis in the non-Roquefort population may result from more  
502 recent selection for milder cheeses.

503

504 **Cheese *P. roqueforti* populations produce cheeses with better flavor and aromas.** We  
505 found major differences between the cheeses made with strains from different *P. roqueforti*  
506 populations, in terms of the volatile compounds resulting from lipolysis and, to a lesser  
507 extent, also from proteolysis. Only four of the aromatic compounds detected in our cheeses  
508 (3-methyl-butanal, 3-methyl-butanol, isopropyl-alcohol and 3-methyl-butanoic acid) were by-  
509 products of casein proteolysis (McSweeney *et al.*, 2000), and the concentrations of these  
510 molecules were significantly higher in cheeses made with Roquefort *P. roqueforti* strains,  
511 consistent with the higher proteolysis efficiency and amino-acid precursor (i.e. valine, leucine  
512 and isoleucine) concentrations of these strains. These compounds produce fruity (banana),  
513 cheesy and alcoholic notes, which were probably important selection criteria during the  
514 domestication of the Roquefort *P. roqueforti* population. For the products of metabolic  
515 pathways leading from amino acids to alcohols (Ehrlich pathway with aldehyde reduction) or  
516 acids (aldehyde oxidation; Ganesan *et al.*, 2017), the higher concentration of alcohols than of  
517 acids observed for all populations is consistent with the general micro-aerobic conditions of  
518 blue cheese cavities.

519 Most of the aromatic compounds identified were direct or indirect by-products of lipolysis,  
520 consistent with the known key role of lipolysis in the generation of typical blue cheese aroma  
521 (Cerning *et al.*, 1987; Collins *et al.*, 2003). The aromatic compounds resulting from lipolysis  
522 belonged to four chemical families (acids, methyl ketones, secondary alcohols and esters).  
523 Methyl ketones were the most diverse and abundant for cheese *P. roqueforti* populations,  
524 particularly for the Roquefort population, in which 2-pentanone and 2-heptanone were  
525 present in the largest amounts; 2-heptanone underlies the characteristic “blue cheese” sensory  
526 descriptor (González De Llano *et al.*, 1990, 1992; Moio *et al.*, 2000; Anderson *et al.*, 1966).  
527 In *P. roqueforti*, methyl ketones with odd numbers of carbons are mostly produced by fatty-

528 acid beta-oxidation, whereas those with even numbers of carbons may be produced by the  
529 beta-oxidation or autoxidation of fatty acids (Spinnler, 2011). These compounds are produced  
530 by the decarboxylation of hexanoic acid and octanoic acid, respectively, which were the most  
531 abundant acids found in our cheeses. This reaction is considered to be a form of  
532 detoxification, because methyl ketones are less toxic than acids (Kinderlerer, 1993; Spinnler,  
533 2011). Interestingly, this pathway appeared to be more active in the cheese *P. roqueforti*  
534 populations, as methyl ketone levels were lower in cheeses made with lumber (four-fold  
535 difference) and silage (10-fold lower) strains than in cheeses made with cheese population  
536 strains. Methyl ketone concentrations were not directly associated with the concentrations of  
537 their precursors (acids), the highest concentrations being found in the lumber and Roquefort  
538 populations. The biosynthesis pathway producing methyl ketones must, therefore, be more  
539 efficient in cheese populations, particularly the non-Roquefort population. The cheese *P.*  
540 *roqueforti* populations were probably selected for their higher acid detoxification capacity, as  
541 this produces aromatic compounds with a very positive impact on flavour (Spinnler, 2011).

542 The concentrations of secondary alcohols (resulting from the reduction of methyl ketones)  
543 were also higher in cheeses produced by cheese *P. roqueforti* strains, particularly those of the  
544 Roquefort population, for which they were seven times higher than for the non-Roquefort  
545 cheese population and 20 times higher than for the silage/lumber populations; 2-heptanol was  
546 the major alcoholic compound produced. The reduction of 2-heptanone to 2-heptanol occurs  
547 specifically in anaerobic conditions and is much stronger in the Roquefort population; aerobic  
548 conditions were similar for all the populations. The Roquefort *P. roqueforti* population may  
549 also have been selected for this feature, as secondary alcohols provide “fruity notes”, which  
550 are associated with better aromatic quality (Spinnler, 2011). Methyl ketones may be reduced  
551 to alcohols by an alcohol dehydrogenase, as occurs when aldehyde is reduced to alcohol via  
552 the Ehrlich pathway. Alcohol dehydrogenase genes may thus have been targets of selection in

553 the Roquefort *P. roqueforti* population, although they were not detected as evolving under  
554 positive selection in a previous study (Dumas *et al.*, 2020).

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

562 Overall, the aromas of cheeses made with cheese *P. roqueforti* strains had more appealing  
563 aromas, and this was particularly true for cheeses made with Roquefort strains. These aroma  
564 properties probably reflect selection by humans. The cheeses made with silage and lumber  
565 populations had a mild unpleasant smell, whereas those made with cheese strains smelled like  
566 typical blue cheeses, with cheeses made with Roquefort strains having the strongest smell.  
567 This may reflect previously reported horizontal gene transfers in cheese populations,  
568 involving genes with predicted functions in lipolysis or amino-acid catabolism, and the  
569 positive selection of genes involved in aroma production (Dumas *et al.*, 2020). We compared  
570 *P. roqueforti* populations between cheeses made following commercial modes of production,  
571 which represents a major advance relative to previous studies based on experimental models  
572 or synthetic media (Gillot *et al.*, 2017; Dumas *et al.*, 2020). We used unpasteurized ewe's  
573 milk, in accordance with the requirements for Roquefort PDO production, which also affects  
574 cheese aromas. In future studies, it would be interesting to determine whether the use of  
575 pasteurized or unpasteurized ewe's milk or cow's milk leads to similar specific features of the  
576 Roquefort versus non-Roquefort cheese *P. roqueforti* populations, as there may have been

577 selection during domestication, leading to an adaptation of the Roquefort population for the  
578 catabolism of unpasteurized ewe's milk.

579

580 **Conclusion.** We showed that the *P. roqueforti* population had a strong impact on cheese  
581 quality, appearance and aroma. The populations used for cheesemaking led to bluer cheeses,  
582 with better aromas, probably due to domestication involving the selection of multiple fungal  
583 traits by humans seeking to make the best possible cheeses. French cheese producers have  
584 been inoculating cheeses with *P. roqueforti* spores from moldy rye bread since the end of the  
585 19<sup>th</sup> century (Labbe *et al.*, 2004, 2009; Vabre, 2015). This process made it possible for them  
586 to re-inoculate with the strains producing the best cheeses, thereby applying a strong selection  
587 pressure. The two cheese populations displayed a number of specific features, with the  
588 Roquefort population notably producing more intense and specific aromas and flavors. The  
589 selection of different fungal varieties for different usages has also been reported in the  
590 fermenting yeast *Saccharomyces cerevisiae* (Gallone *et al.*, 2016; Legras *et al.*, 2018).  
591 Previous studies on *P. roqueforti* detected recurrent changes in amino acids and horizontal  
592 gene transfers in cheese populations, both of which facilitated rapid adaptation (Dumas *et al.*,  
593 2020; Ropars *et al.*, 2015). Our findings provide greater insight into *P. roqueforti*  
594 domestication and pave the way for strain improvement through the targeting of relevant  
595 traits. A protocol inducing sexual selection has been developed in *P. roqueforti* (Ropars *et al.*,  
596 2014), making it possible to perform crosses between strains from the two cheese  
597 populations, each of which harbors very little genetic diversity (Dumas *et al.*, 2020), to  
598 generate variability and to identify strains with high levels of performance; the results of this  
599 study will facilitate the choice of the parental strains for crossing and of the most important  
600 phenotypes to be measured in the offspring. Parental strains with strongly contrasting  
601 phenotypes for the traits important for cheesemaking that we found to be differentiated



602 between populations (such as volatile compound production, lipolysis and proteolysis) should  
603 be used, to maximize variability in the progeny.

604

605

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892 **Figure legends**

893

894 **Figure 1:** Experimental cheesemaking. (A) Experimental design for cheesemaking, using one  
895 strain per cheese, and three different strains from each of the four *Penicillium roqueforti*  
896 populations (non-Roquefort cheese in blue, Roquefort cheese in purple, silage/food spoiler in  
897 orange, lumber/food spoiler in green, the lineages of which are shown on the left). Each assay  
898 (February, March, April) corresponded to a single strain from each of the four populations,  
899 with three production replicates at different times, different batches of unpasteurized milk  
900 and with two cheeses produced per strain in each replicate. The identities of the strains used  
901 are indicated on the left of each assay, for each of the four *P. roqueforti* populations. (B)  
902 Picture of the experimental cheeses at 20 days of maturation.

903

904 **Figure 2:** Mean percentage blue area per cheese slice at 20, 90 and 180 days of maturation,  
905 for cheeses made with strains from the four *Penicillium roqueforti* populations (non-  
906 Roquefort cheese in blue, Roquefort cheese in purple, silage/food spoiler in orange and  
907 lumber/food spoiler in green). Error bars indicate 95% confidence intervals.

908

909 **Figure 3:** Illustration of the differences in the mean percentage blue area per cheese slice at  
910 180 days of maturation between the four *Penicillium roqueforti* populations (non-Roquefort  
911 cheese in blue, Roquefort cheese in purple, silage/food spoiler in orange and lumber/food  
912 spoiler in green). Contrast and brightness have been standardized and the edges cropped.

913

914 **Figure 4:** Sums of 3,864 non-targeted aqueous signal peak areas, weighted by their mass-to-  
915 charge ratios (“m/z”), obtained in positive ionization mode in 90-day cheeses made with  
916 strains from the four *Penicillium roqueforti* populations (lumber/food spoiler in green, non-  
917 Roquefort cheese in blue, Roquefort cheese in purple and silage/food spoiler in orange).

918

919 **Figure 5:** Sums of 3,094 non-targeted organic signal peak areas, weighted by their mass-to-  
920 charge ratios (“m/z”), obtained in negative ionization mode in 90-day cheeses made with  
921 strains from the four *Penicillium roqueforti* populations (lumber/food spoiler in green, non-  
922 Roquefort cheese in blue, Roquefort cheese in purple and silage/food spoiler in orange).

923

924 **Figure 6:** Volatile compound production (integrated peak areas from chromatograms in  
925 arbitrary units) in 90-day cheeses inoculated with strains from the four *Penicillium roqueforti*  
926 populations (non-Roquefort cheese in blue, Roquefort cheese in purple, silage/food spoiler in  
927 orange and lumber/food spoiler in green). The areas for each family of compounds are the  
928 sum of the integrated areas of the compounds belonging to the family concerned. Alcohols I  
929 and II are derived from proteolysis and lipolysis, respectively. Acids I, II and III are derived  
930 from proteolysis, glycolysis and lipolysis, respectively (Supplementary Table 3). The color of  
931 the titles indicates the affiliation of the compounds to their families, as in Figure S7.

932

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

938

### 939 **Figure legends for the Supplementary Material**

940

941 **Figure S1:** Abundance of microorganisms in experimental cheeses. A. Abundance (in log  
942 colony-forming units/g) of the eight types of microorganisms monitored at various stages of

943 cheese maturation (i.e. unpasteurized milk, 9, 20, 90 and 180 days), for each of the four  
944 *Penicillium roqueforti* populations used to inoculate the cheeses (non-Roquefort cheese in  
945 blue, Roquefort cheese in purple, silage/food spoiler in orange and lumber/food spoiler in  
946 green). Error bars represent standard deviations across assays. B. Relative abundance of the  
947 six main bacterial operational taxonomic units in cheeses made with strains from the four  
948 *Penicillium roqueforti* populations (non-Roquefort cheese in blue, Roquefort cheese in  
949 purple, silage/food spoiler in orange and lumber/food spoiler in green) in each assay  
950 (February in light gray, March in mid-gray and April in dark gray) in cheeses at nine (45°  
951 hatching) and 20 (135° hatching) days of maturation.

952

953 **Figure S2:** Mean bacterial genus diversity. A: Shannon index, B: Inverse of Simpson index=  
954  $1 - \text{Simpson index}$ ) for the operational taxonomic units detected by metabarcoding in 9-day  
955 cheeses (left) and 20-day cheeses (right) made with strains from the four *Penicillium*  
956 *roqueforti* populations (lumber/food spoiler in green, non-Roquefort cheese in blue,  
957 Roquefort cheese in purple and silage/food spoiler in orange).

958

959 **Figure S3:** Illustration of image processing for estimation of the percentage blue area on  
960 cheese slices: (a) example of an unprocessed image of a cheese slice; (b) image after  
961 brightness and contrast standardization; (c) image after cropping; (d) corresponding image  
962 binarization with a grayscale of 102 on the red channel. White and black correspond to pixel  
963 classification: in white, the inner part of the cheese and empty cavities; in black, cavities  
964 filled with the fungus.

965

966 **Figure S4:** Differences in amino acid content between cheeses according to the population-  
967 of-origin of the *Penicillium roqueforti* strains. A. Discrimination between 90-day cheeses

968 made with cheese (blue) and non-cheese (green) *P. roqueforti* populations (left), or Roquefort  
969 cheese (purple) and non-Roquefort cheese (blue) *P. roqueforti* populations (right), based on  
970 the amounts of the 23 identified amino acids present, according to an orthogonal signal-  
971 corrected partial least squares (PLS) discriminant analysis. Vertical and horizontal axes  
972 represent PLS1 and PLS 2 scores and gray arrows represent the relative contribution of  
973 loadings of signals significantly discriminating the group considered in a *t*-test with jackknife  
974 resampling. B. Amounts of molecules from particular classes detected in cheeses: mean  
975 integrated peak area from chromatograms in arbitrary units (bars, left axis) and cumulative  
976 percentage (line with dots, right axis) of aqueous extracts across all 90-day cheeses.

977

978 **Figure S5:** Sums of 8,472 non-targeted organic signal peak areas, weighted by their mass-to-  
979 charge ratios (“m/z”), obtained in positive ionization mode in 90-day cheeses made with  
980 strains from the four *Penicillium roqueforti* populations (lumber/food spoiler in green, non-  
981 Roquefort cheese in blue, Roquefort cheese in purple and silage/food spoiler in orange).

982

983 **Figure S6:** Non-protein nitrogen levels at 20, 90 and 180 days of maturation, and water  
984 activity at 90 and 180 days of maturation. Comparison of cheeses made with strains from  
985 different *Penicillium roqueforti* populations (non-Roquefort cheese in blue, Roquefort cheese  
986 in purple, silage/food spoiler in orange and lumber/food spoiler in green). Error bars indicate  
987 95% confidence intervals.

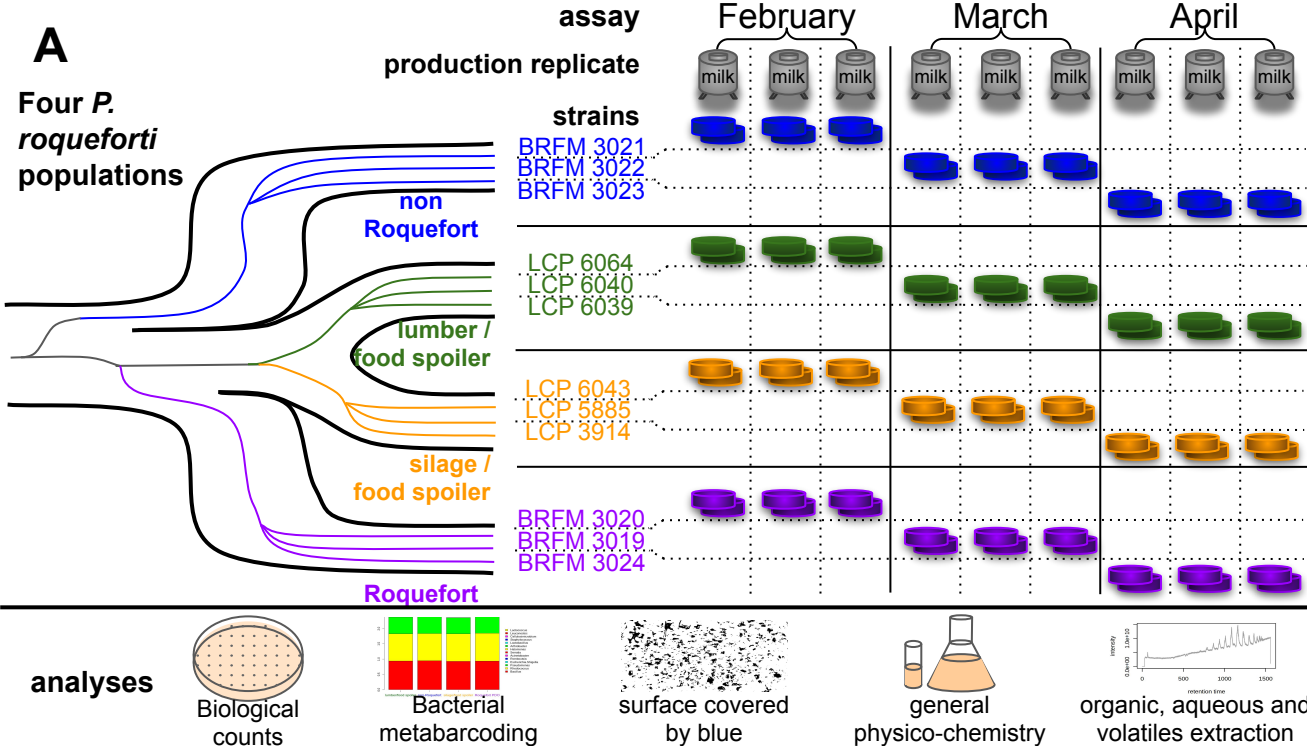
988

989 **Figure S7:** Discrimination between 90-day cheeses inoculated with strains from the four  
990 *Penicillium roqueforti* populations (non-Roquefort cheese in blue, Roquefort cheese in  
991 purple, silage/food spoiler in yellow and lumber/food spoiler in green), based on the amounts  
992 of 41 volatile compounds in an orthogonal signal-corrected partial least squares (PLS)

993 discriminant analysis. Vertical and horizontal axes represent the PLS1 and PLS2 variances,  
994 and arrows represent the relative contributions of compound odor loadings significantly  
995 discriminating the group considered (according to [www.thegoodscentscompany.com](http://www.thegoodscentscompany.com)) in a *t*-  
996 test with jackknife resampling. The odor colors indicate the families in Figure 6 to which the  
997 associated compounds belong.

998

999 **Featured image:** Roquefort cheese slice with symbols for two methyl ketones (2-heptanone  
1000 and 2-pentanone).



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

Cheese colonization

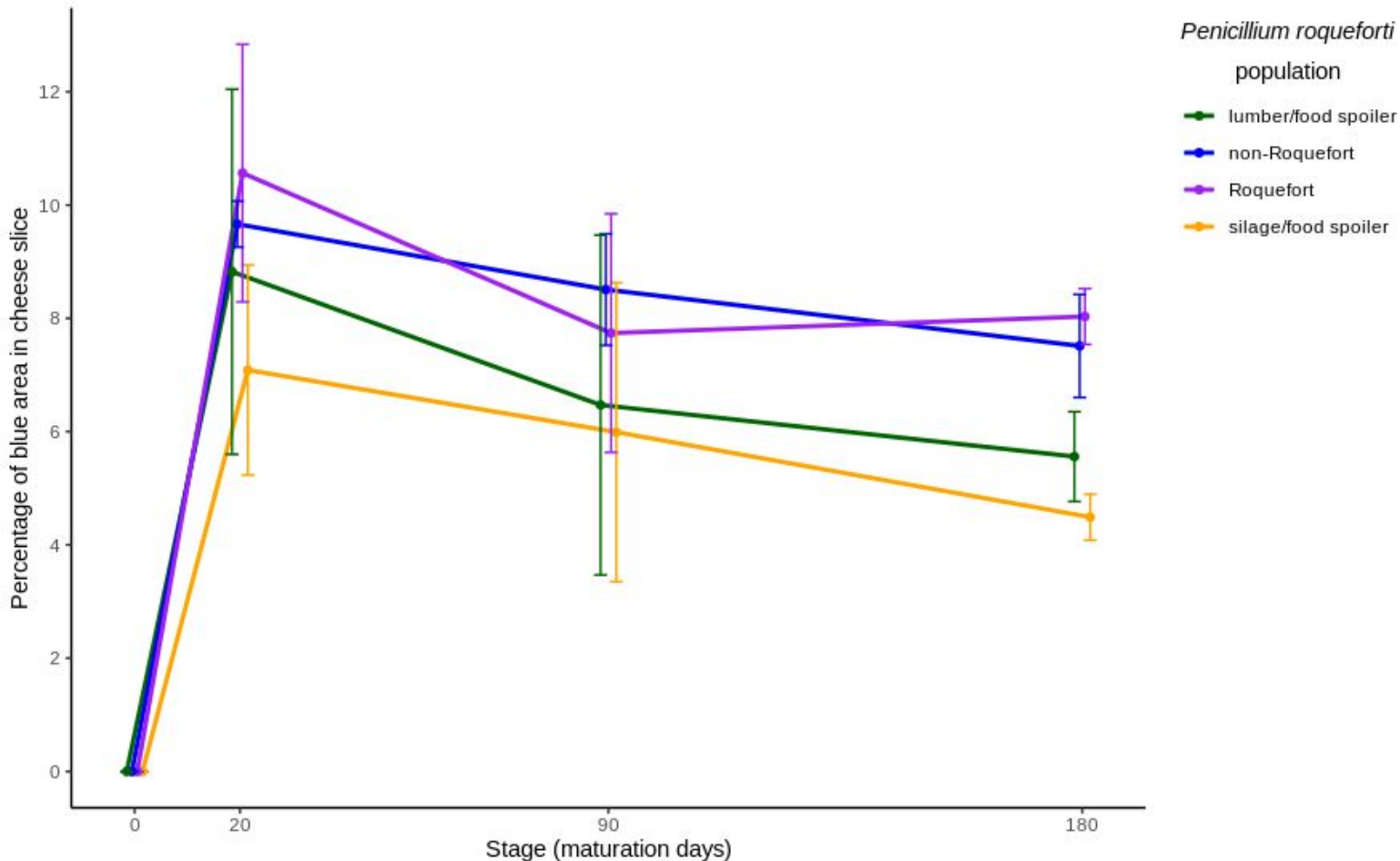


Figure 2: Mean percentage blue area per cheese slice at 20, 90 and 180 days of maturation, for cheeses made with strains from the four *Penicillium roqueforti* populations (non-Roquefort cheese in blue, Roquefort cheese in purple, silage/food spoiler in orange and lumber/food spoiler in green). Error bars indicate 95% confidence intervals.



Non-cheese *P. roqueforti* populations

lumber/food spoiler (5.2 %)

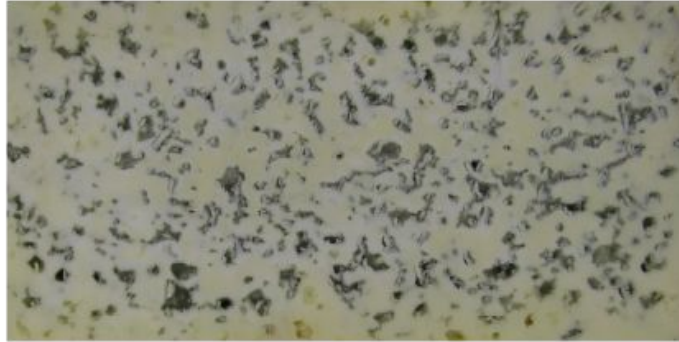


silage/food spoiler (5.5%)



Cheese *P. roqueforti* populations

non-Roquefort (10.2 %)



Roquefort (11.8 %)



Figure 3: Illustration of the differences in the mean percentage blue area per cheese slice at 180 days of maturation between the four *Penicillium roqueforti* populations (non-Roquefort cheese in blue, Roquefort cheese in purple, silage/food spoiler in orange and lumber/food spoiler in green). Contrast and brightness have been standardized and the edges cropped.

Sums of non-targeted aqueous signal peak areas

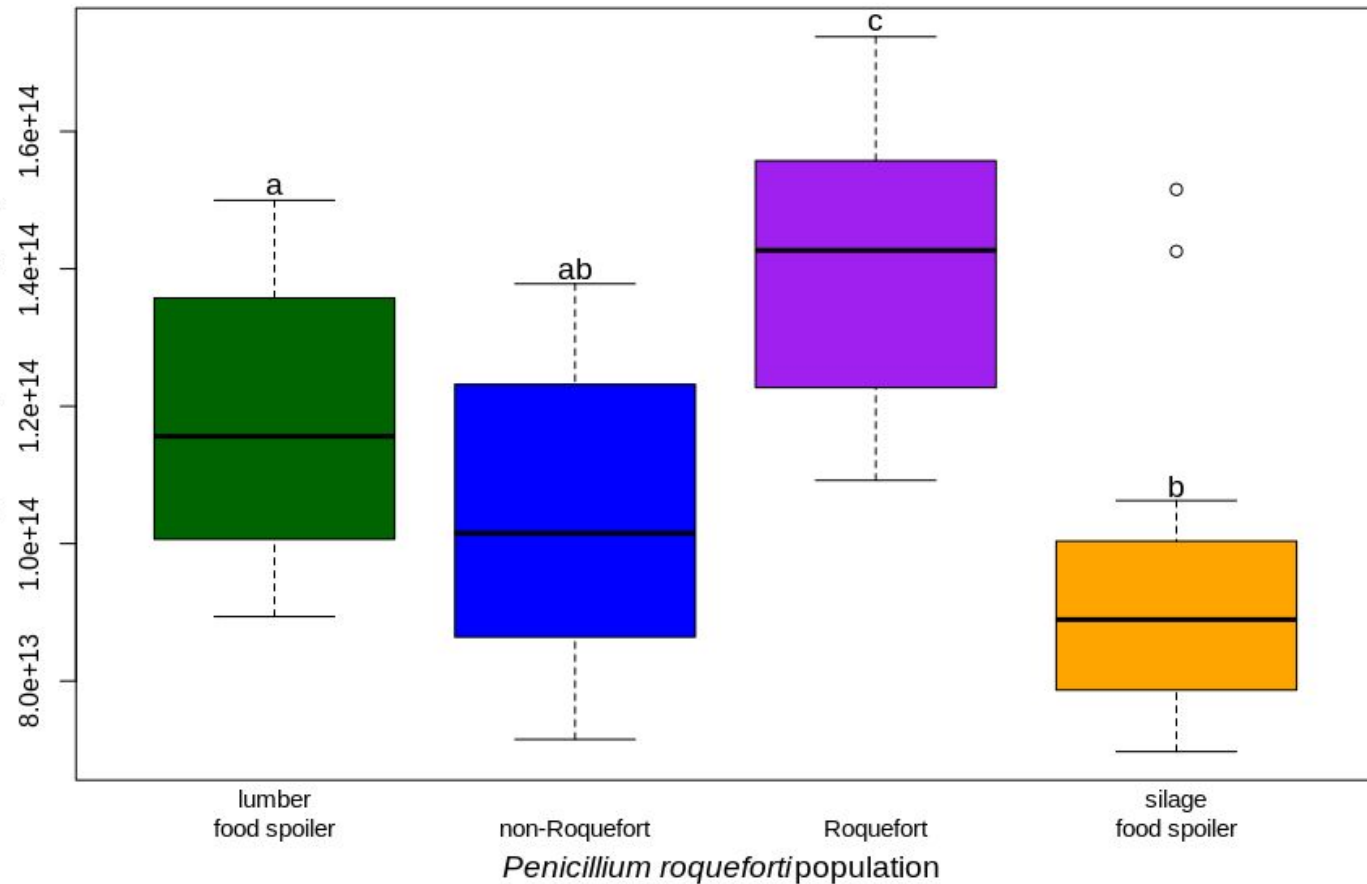


Figure 4: Sums of 3,864 non-targeted aqueous signal peak areas, weighted by their mass-to-charge ratios (“m/z”), obtained in positive ionization mode in 90-day cheeses made with strains from the four *Penicillium roqueforti* populations (lumber/food spoiler in green, non-Roquefort cheese in blue, Roquefort cheese in purple and silage/food spoiler in orange).

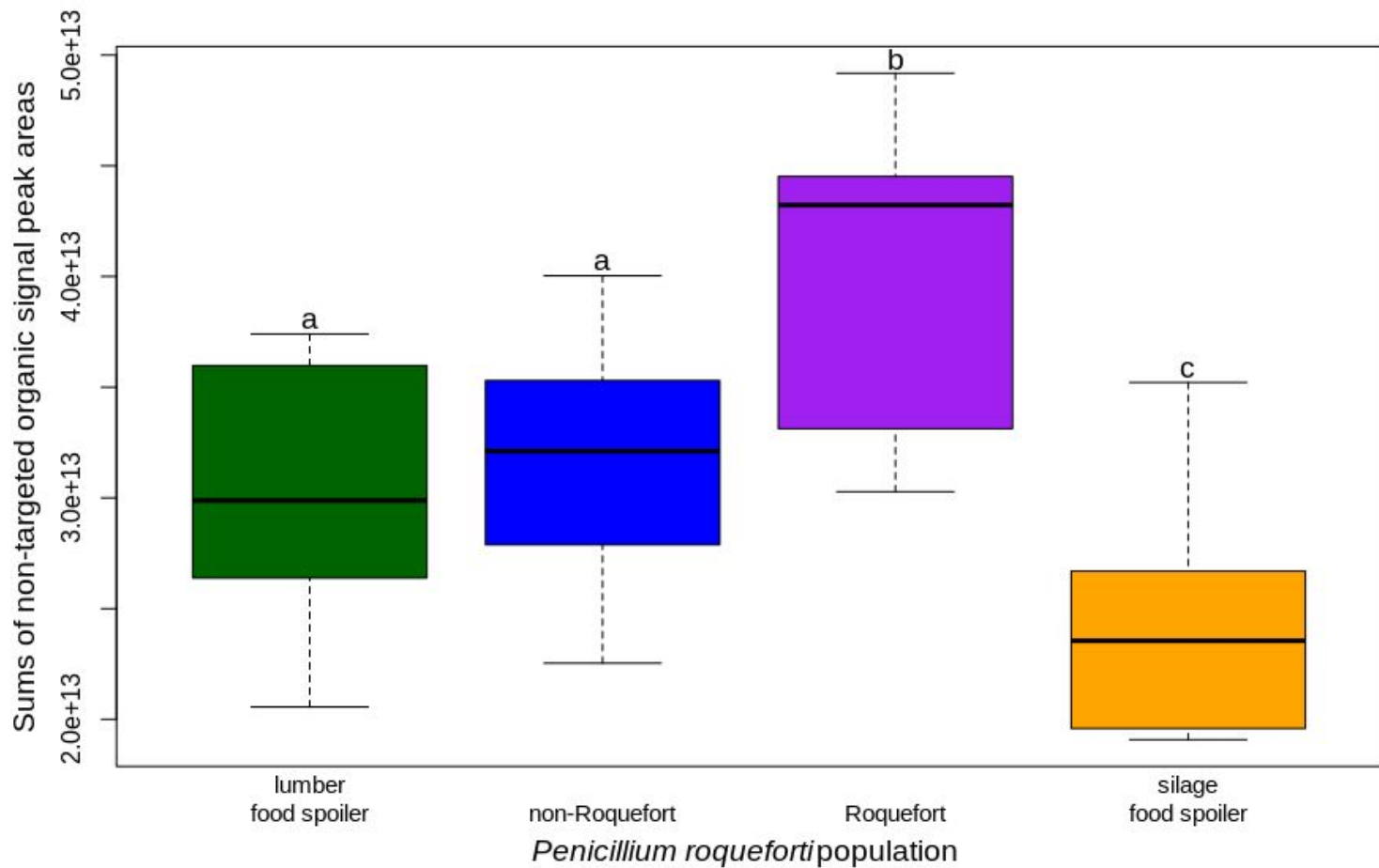


Figure 5: Sums of 3,094 non-targeted organic signal peak areas, weighted by their mass-to-charge ratios (“m/z”), obtained in negative ionization mode in 90-day cheeses made with strains from the four *Penicillium roqueforti* populations (lumber/food spoiler in green, non-Roquefort cheese in blue, Roquefort cheese in purple and silage/food spoiler in orange).

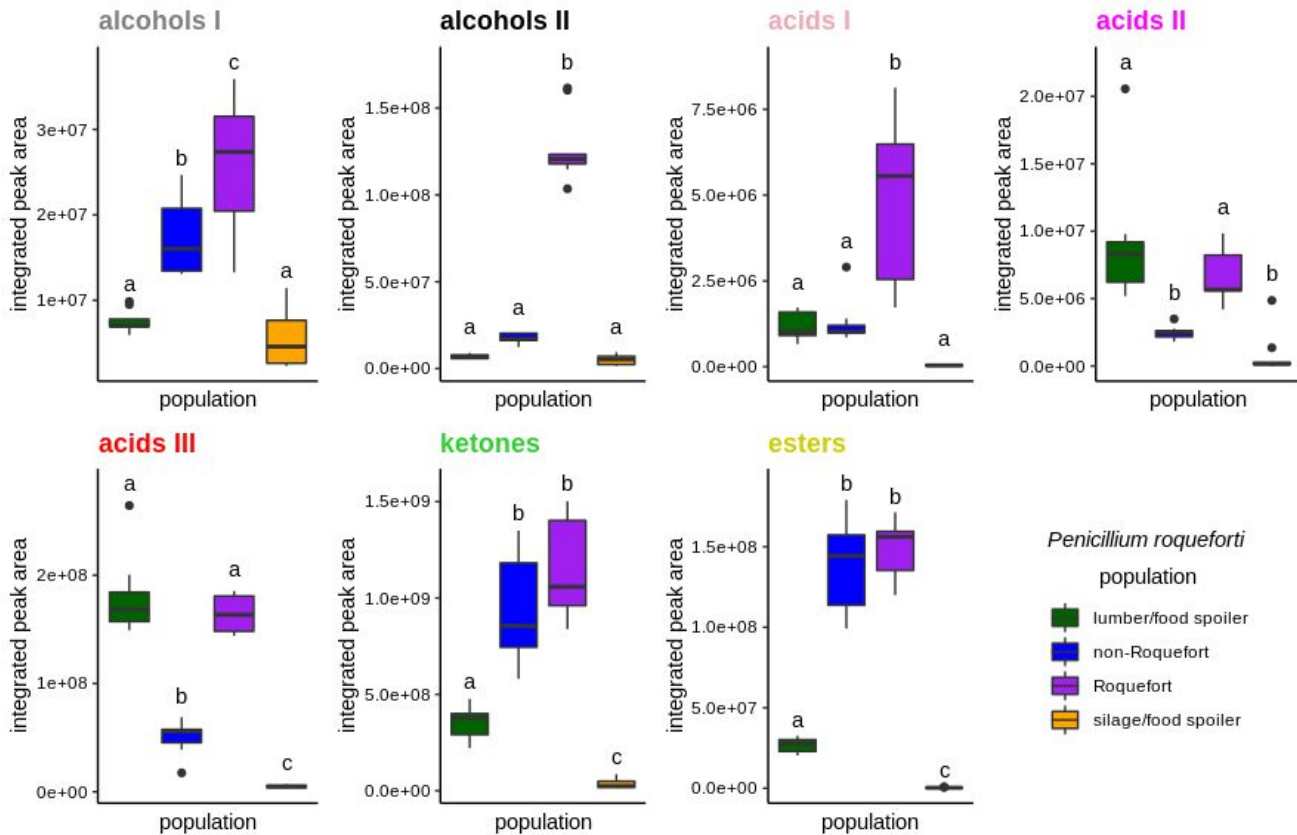


Figure 6: Volatile compound production (integrated peak areas from chromatograms in arbitrary units) in 90-day cheeses inoculated with strains from the four *Penicillium roqueforti* populations (non-Roquefort cheese in blue, Roquefort cheese in purple, silage/food spoiler in orange and lumber/food spoiler in green). The areas for each family of compounds are the sum of the integrated areas of the compounds belonging to the family concerned. Alcohols I and II are derived from proteolysis and lipolysis, respectively. Acids I, II and III are derived from proteolysis, glycolysis and lipolysis, respectively (Supplementary Table 3). The color of the titles indicates the affiliation of the compounds to their families, as in Figure S7.

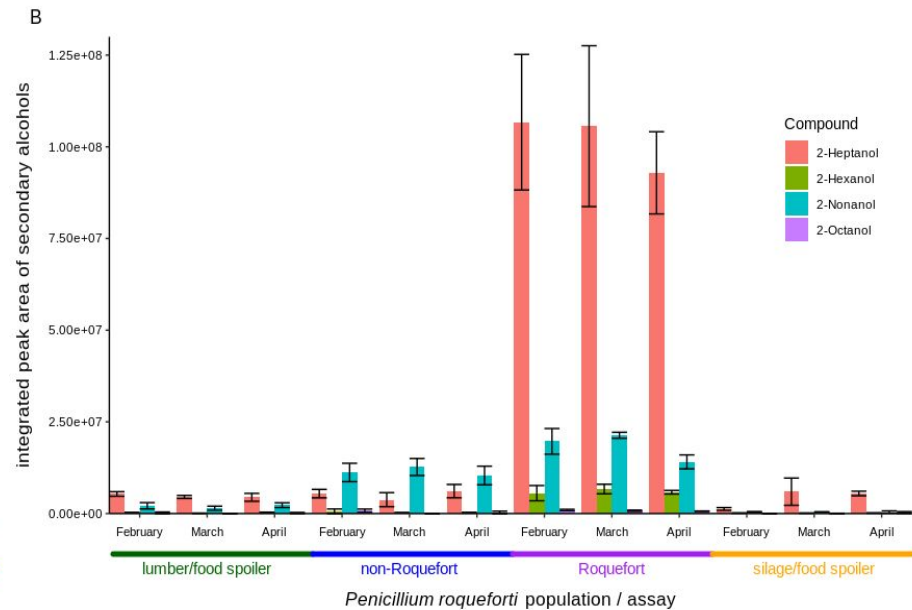
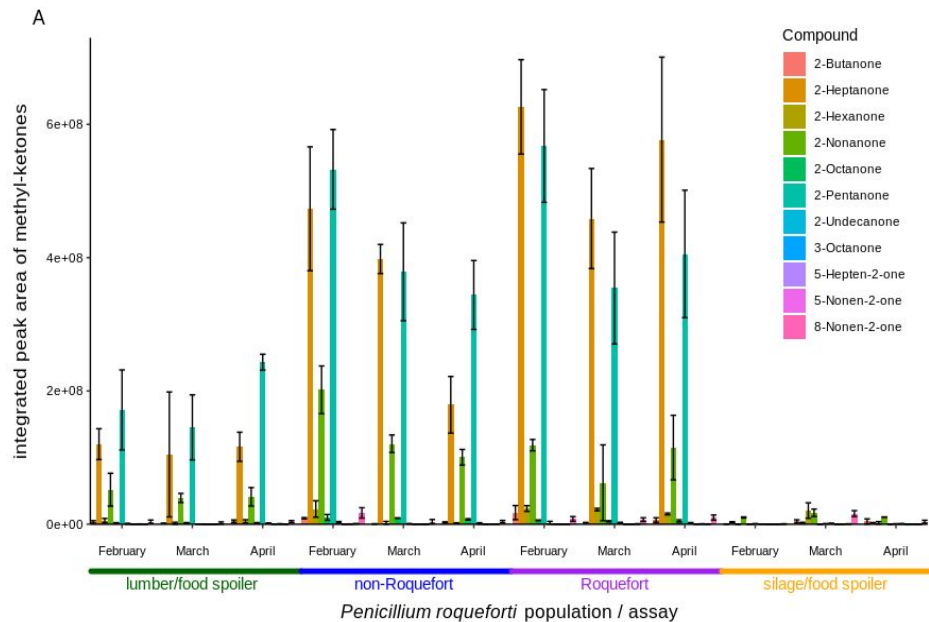


Figure 7: Integrated surface area (from chromatograms in arbitrary units) of methyl ketones (A) and secondary alcohols (B) for each assay (February, March, April) for the three strains of each *Penicillium roqueforti* population (lumber/food spoiler in green, non-Roquefort cheese in blue, Roquefort cheese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