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

Running title: Impact of mold population impact on blue cheeses

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Abstract

Studies of food microorganism domestication can provide important insight into adaptation mechanisms and lead to commercial applications. The *Penicillium roqueforti* fungus consists of four genetically differentiated populations, two of which have been domesticated for blue cheese-making, the other two thriving in other environments. Most blue cheeses are made with strains from a single *P. roqueforti* population, whereas Roquefort cheeses are inoculated with strains from a second population. We made blue cheeses in accordance with the production specifications for Roquefort-type cheeses, inoculating each cheese with a single *P. roqueforti* strain and using three strains from each of the four populations. The strain population-of-origin had a minor impact on bacterial diversity and none on the main microorganism abundance. The strains from cheese populations produced cheeses with higher percentages of blue area and larger amounts of desired volatile compounds. In particular, the Roquefort strains produced larger amounts of appealing aromatic compounds, in part due to their greater efficiency of proteolysis and lipolysis. The typical appearance and flavors of blue cheeses thus result from human selection on *P. roqueforti*, and the two cheese populations have acquired specific features. This has important implications for our understanding of adaptation and domestication, and for cheese improvement.

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

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

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

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

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

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

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

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

Materials and Methods

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

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

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

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

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

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

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

Results

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

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

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

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

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

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

More efficient proteolysis and lipolysis by the Roquefort *P. roqueforti* population. The *P. roqueforti* strains used for cheesemaking are known to have high proteolytic and lipolytic activities, which play a key role in cheese ripening. We therefore investigated the proteolysis and lipolysis efficiencies of the four populations. Both targeted and non-targeted chromatographic analyses showed that proteolysis efficiency was highest in the Roquefort *P. roqueforti* population. We performed the targeted analysis with standards for the principal 23 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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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.

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.

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.

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.

Figure legends for the Supplementary Material

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

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

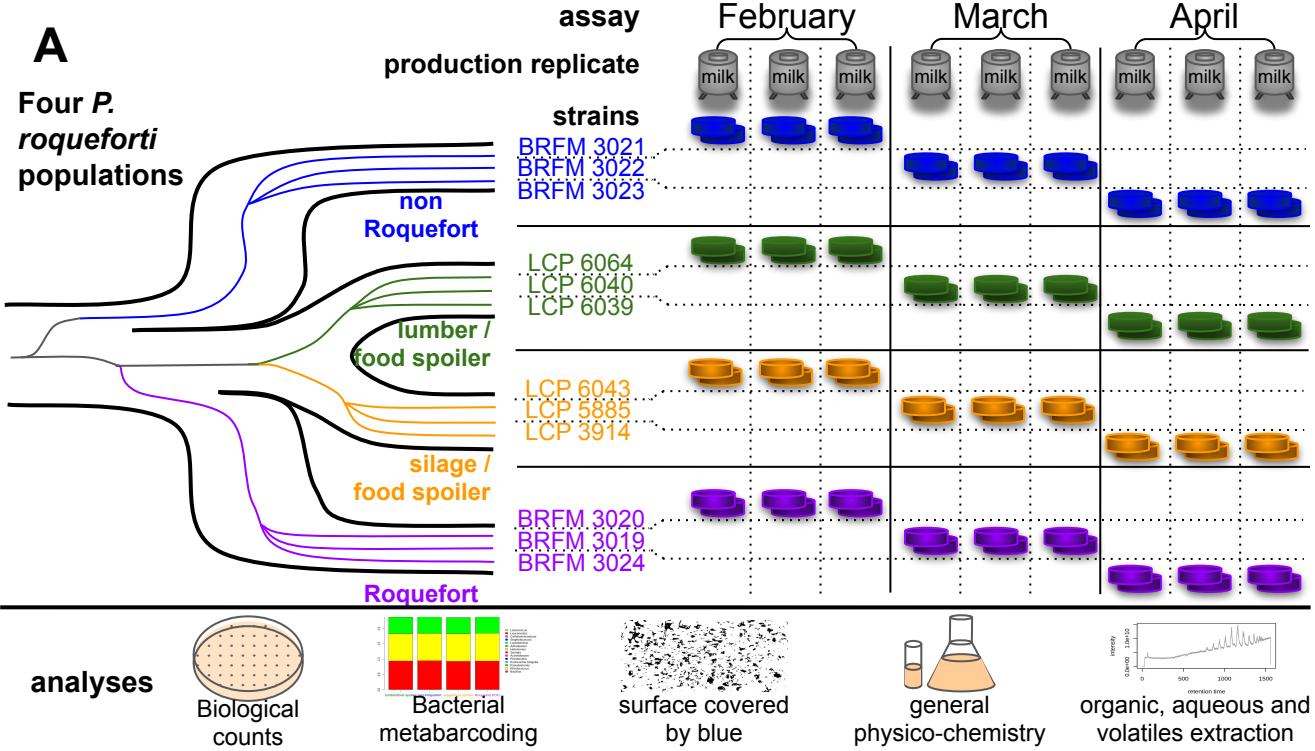
Figure S2: Mean bacterial genus diversity. A: Shannon index, B: Inverse of Simpson index= 1 - Simpson index) for the operational taxonomic units detected by metabarcoding in 9-day cheeses (left) and 20-day cheeses (right) made with strains from the four *Penicillium roqueforti* populations (lumber/food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

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

Figure S4: Differences in amino acid content between cheeses according to the population-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) 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).



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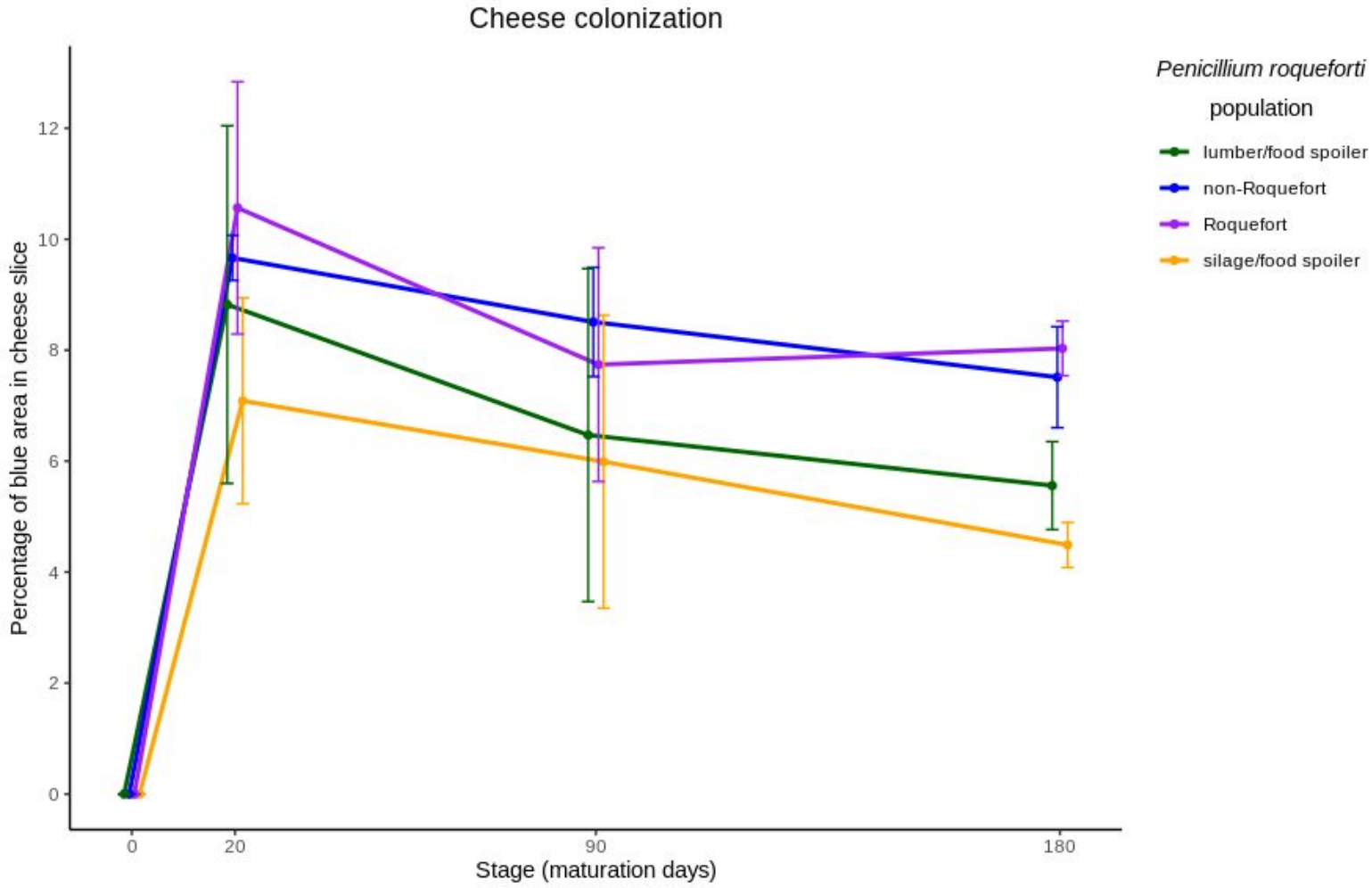

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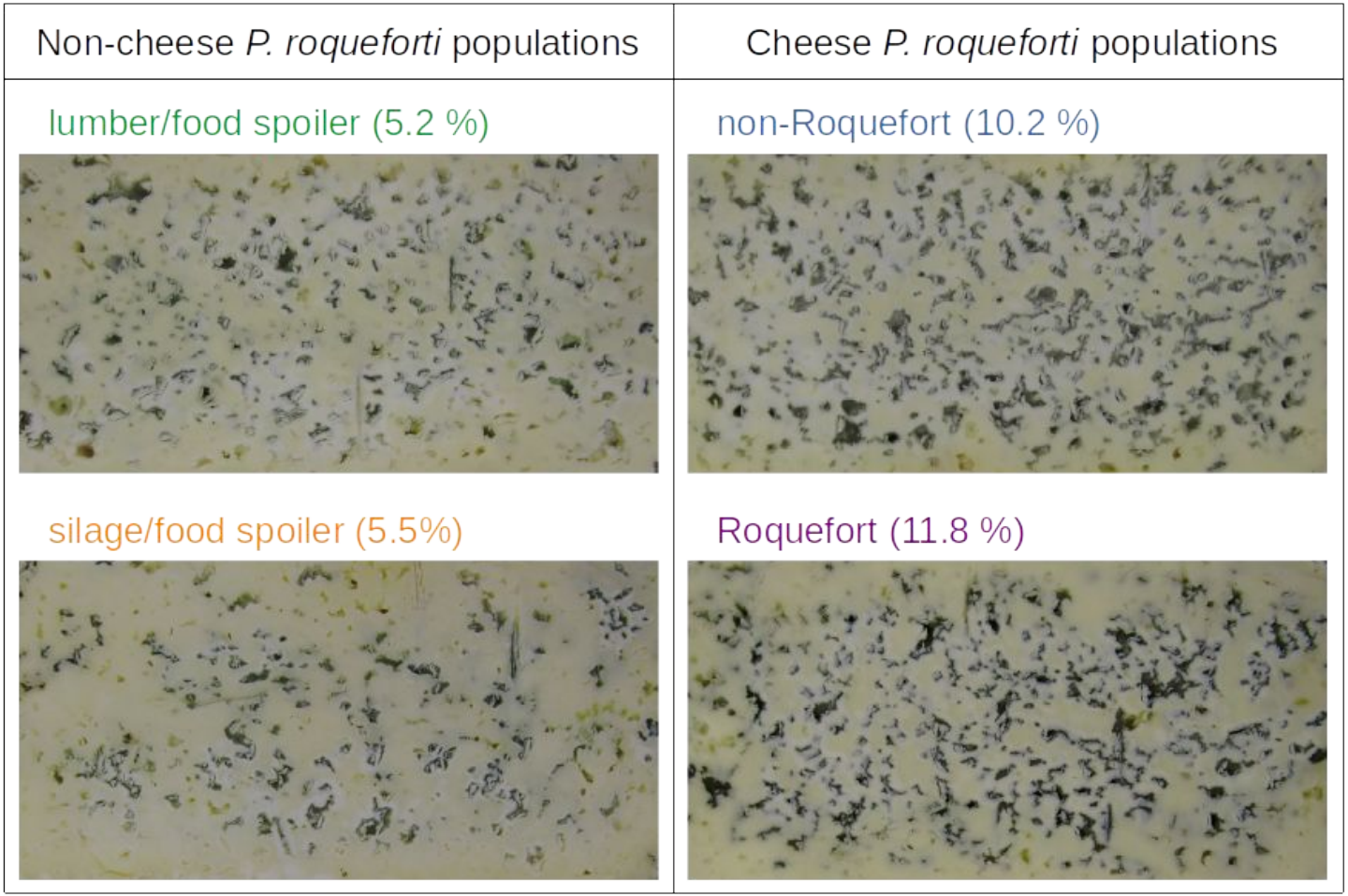

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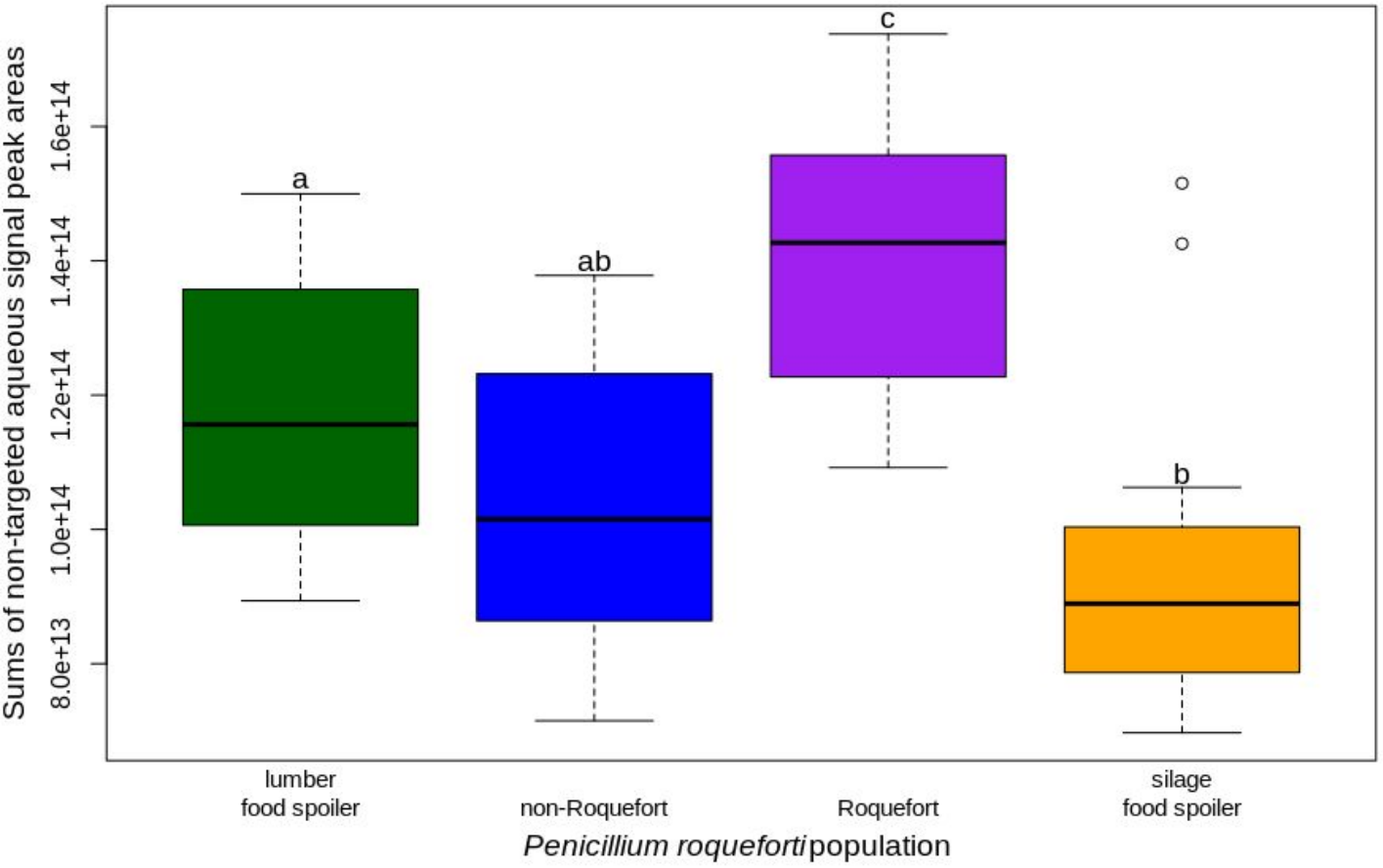

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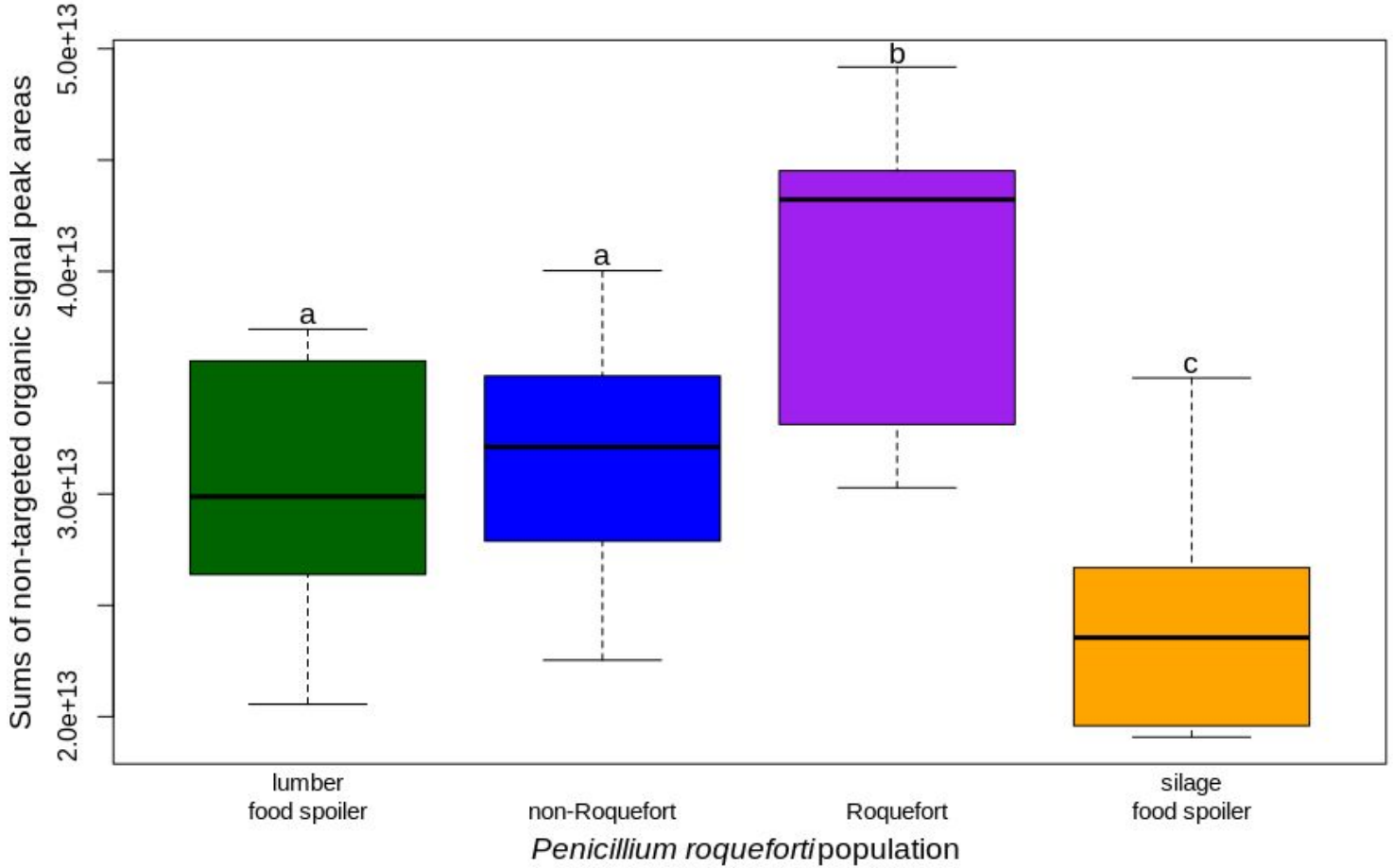

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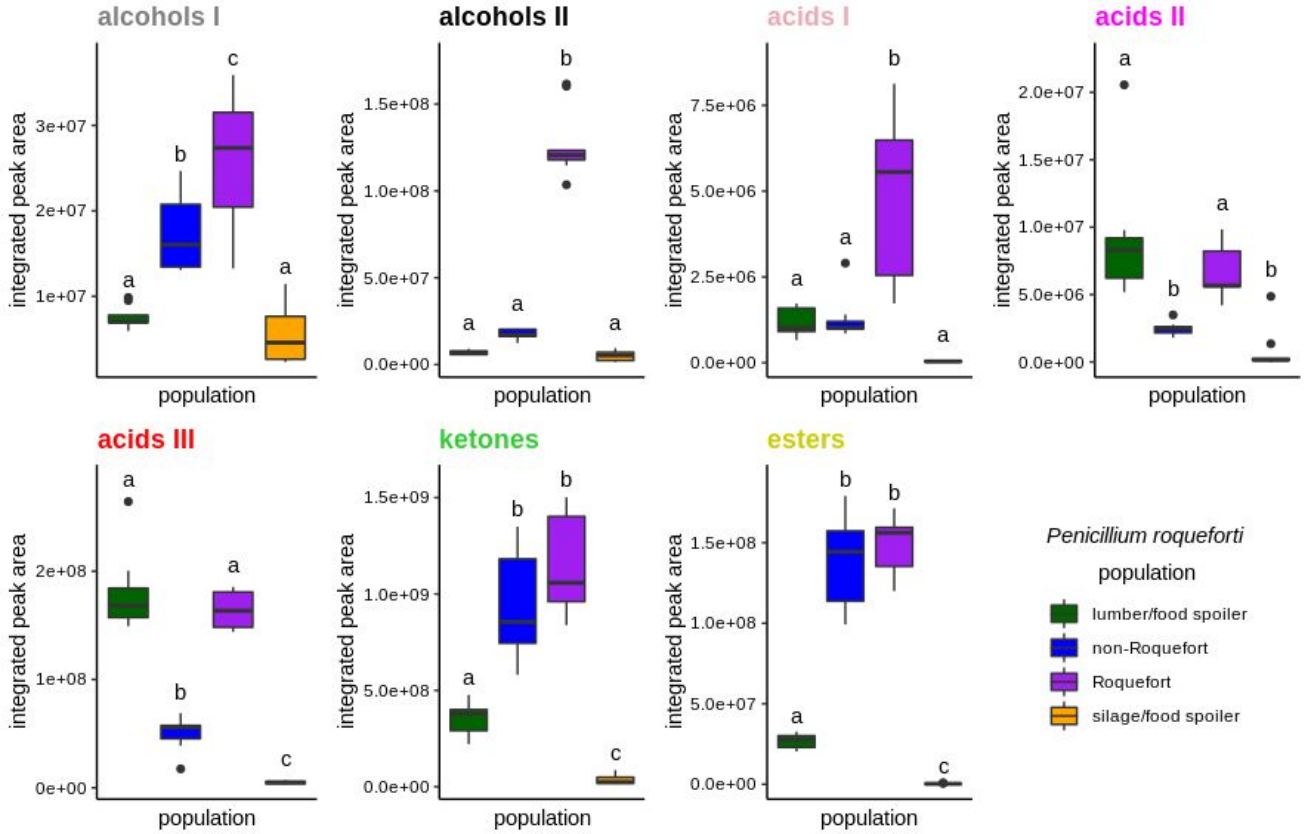

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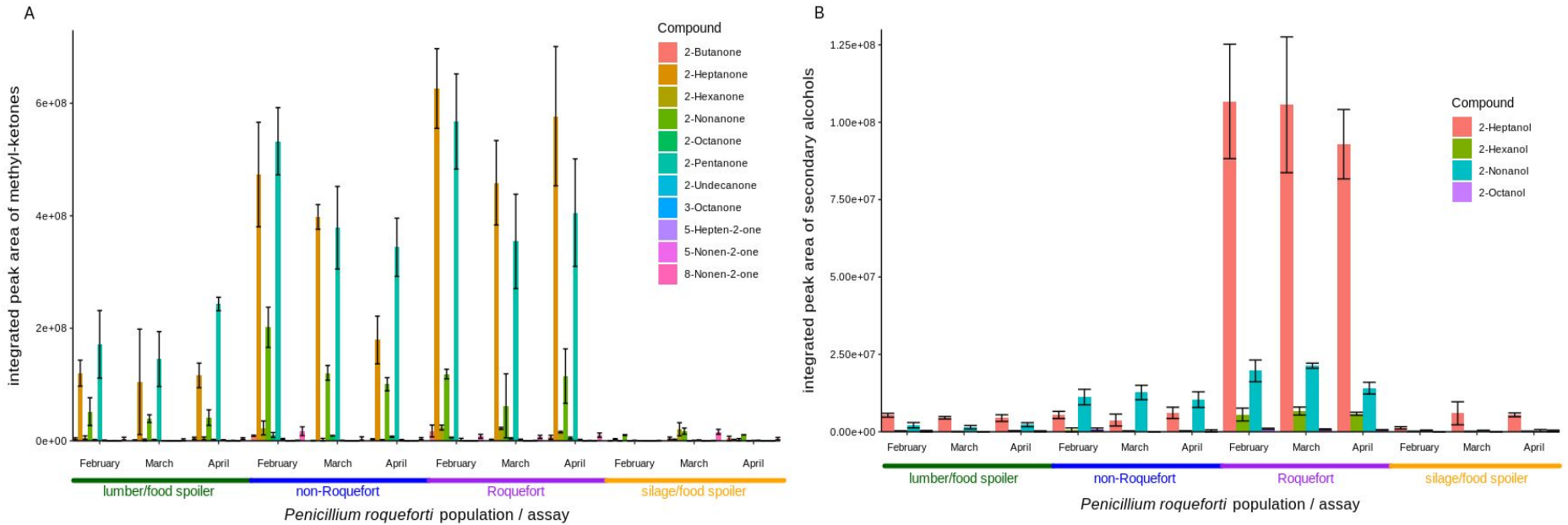

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