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

Authors:

Thibault CARON[1,4], Mélanie LE PIVER[4], Anne-Claire PÉRON[3], Pascale LIEBEN[3], René LAVIGNE[2], Sammy BRUNEL[4], Daniel ROUEYRE[4], Michel PLACE[4], Pascal BONNARME[3], Tatiana GIRAUD[1]*, Antoine BRANCA[1]*, Sophie LANDAUD[3]*, Christophe CHASSARD[2]*

1: Ecologie Systematique Evolution, Université Paris Saclay, CNRS, AgroParisTech, 91400 Orsay, France

2: Université Clermont Auvergne, INRAE, Vetagro Sup, UMRF, 20 Côte de Reyne, 15000 Aurillac, France

3: Université Paris-Saclay, INRAE, AgroParisTech, UMR SayFood, 78850 Thiverval-Grignon, France

4: Laboratoire Interprofessionnel de Production – SAS L.I.P., 34 rue de Salers, 15 000 Aurillac, France

*These authors jointly supervised the study

Corresponding author: Antoine Branca antoine.branca@u-psud.fr

Running title: *Penicillium roqueforti* population impact on cheeses

Keywords: Roquefort cheese, fungi, *Penicillium*, domestication, volatile compounds

**Abstract**: The study of food microorganism domestication can bring important insights on adaptation mechanisms and have industrial applications. The *Penicillium roqueforti* mold is divided into four main populations, with two populations domesticated for blue-cheese making and two populations thriving in other environments. While most blue cheeses worldwide are made with the same *P. roqueforti* clonal lineage, the emblematic Roquefort cheeses are inoculated with a specific population. To study the differences among *P. roqueforti* populations in the context of domestication for cheesemaking, we compared blue cheeses made with the four fungal populations following Roquefort-type production specifications. We found that the *P. roqueforti* populations had a minor impact on the cheese bacterial diversity and none on the main microorganism abundance. The cheese *P. roqueforti* populations produced cheeses with higher percentages of blue area and with different sets and higher quantities of desired volatile compounds. The Roquefort *P. roqueforti* population in particular produced higher quantities of positive aromatic compounds in cheeses, which was related due to its most efficient proteolysis and lipolysis, and also produced cheeses with lower water activity, thus restricting spoiler microorganisms. Our results show the strong influence of *P. roqueforti* populations on several important aspects of cheese safety, appearance and flavour. The typical appearance and flavours of blue cheeses are therefore the result of human selection on *P. roqueforti*, thus constituting domestication, and the two cheese populations have acquired specificities. This has important implications for our understanding of adaptation and domestication processes as well as for improving cheese production.

**Importance**: Several fungi have been domesticated for food fermentation, with selection for traits beneficial for food production. The mold used for making blue cheeses, *Penicillium roqueforti*, is subdivided into four genetically different populations, two being found in cheese, one being specific of the Roquefort protected designation of origin, and two in other environments. The cheese *P. roqueforti* populations produced bluer cheeses with higher quantities of desired volatile compounds. The Roquefort *P. roqueforti* population in particular produced higher quantities of positive aromatic compounds in cheeses, in relation to its most efficient proteolysis and lipolysis, and also produced cheeses with lower water activity, thus restricting spoiler microorganisms. Our results support that the blue cheese typical aspect and flavors are the result of a selection by humans and show the strong influence of *P. roqueforti* populations for several important aspects of cheese safety, aspect and flavor, paving the way for improving cheese production.

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 a strong and recent selection on traits that are often known and of interest for humans (1.Larson 2014). In addition, studying domestication often has important applied consequences for the improvement of cultivated organisms. Domesticated fungi have however been little studied so far compared to crops, despite representing excellent models in this field (2.Gladieux 2014; 3.Giraud 2017). Most fungi can be cultivated in Petri dishes, stored alive for decades in freezers and propagated asexually, which facilitate experiments. Their metabolisms are used to produce various compounds of interest such as fuels, enzymes and antibiotics (4.Bigelis 2001). Their oldest and most frequent use 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* for soy sauce and sake fermentation (5.Dupont 2017) and these models have provided important insights into the mechanisms of adaptation and domestication (6.Almeida 2014; 7.Baker 2015; 8.Gallone 2016; 9.Gibbons 2012; 10.Gonçalves 2016; 11.Libkind 2011; 12.Sicard and Legras 2011).

The *Penicillium* genus contains more than 300 species, several of them being used by humans; for example *P. rubens* led to the discovery of penicillin and *P. nalgioviense* and *P. salamii* are used for the production of dry-cured meat (13.Fleming 1929; 14.Ludemann 2010, 15.Perrone 2015). For centuries, *Penicillium roqueforti* has been used for the maturation of all the numerous varieties of blue cheeses worldwide (16,17.Labbe and Serres, 2004, 2009; 18.Vabre 2015), the fungus being responsible for the cheese blue veined aspect through the production of melanized spores in cheese cavities, where oxygen is available (19.Moreau 1980). *Penicillium roqueforti* can be found in other environments than cheeses, thriving in

spoiled food and silage (20.Pitt 2009; 21.Ropars 2012). Genomic and experimental approaches have recently elucidated several important aspects of *P. roqueforti* domestication (22.Cheeseman 2014; 23,24,25,26Ropars 2015, 2016 a and b, 2017; 27.Gillot 2015; 28.Gillot 2017; 29.Dumas 2020). Four main populations have been identified, two being used for cheesemaking, and the two other populations thriving in silage, lumber or spoiled food (30.Ropars 2014; 27.Gillot 2015; 29.Dumas 2020). Populations of *P. roqueforti* used to make blue cheeses display characteristic features of domesticated organisms, with genetic and phenotypic differences compared to non-cheese populations, and in particular for traits of interest for cheese production (29.Ropars 2014; 27.Gillot 2015; 29.Dumas 2020). While both cheese populations harbour lower genetic diversity than the two other populations, the two cheese populations differ from each other, both genetically and phenotypically, and resulted from independent domestication events (29.Dumas 2020). One of the cheese populations, called the non-Roquefort population, is a single clonal lineage, used to produce most types of blue cheeses worldwide; the second cheese population, called the Roquefort population, is genetically more diverse and contains all the strains used to produce blue cheeses from the Roquefort protected designation of origin (PDO) (29.Dumas 2020). Based on *in vitro* tests, the non-Roquefort population was found to display faster tributyrin degradation (*i.e.* a certain type of lipolysis) and higher salt tolerance, faster *in vitro* growth on cheese medium and better exclusion of competitors, compared to the Roquefort population (30.Ropars 2014; 23.Ropars 2015; 29.Dumas 2020). Horizontally-transferred genes only present in the non-Roquefort population are involved in the production of an antifungal peptide and in lactose catabolism (30.Ropars 2014; 23.Ropars 2015; 22.Cheeseman 2014). Positive selection has been detected in genes with predicted functions involved in flavor compound production in each of the cheese populations (29.Dumas 2020). The specific features of the Roquefort population may result from the PDO, requiring the use of local strains and 90 days of

maturation, preventing the use of the worldwide clonal lineage more suited to modern industrial production modes (29.Dumas 2020).

The four *P. roqueforti* populations thus likely harbour multiple specific traits that can generate cheeses with different physicochemical properties and flavors. *Penicillium roqueforti* is used as a secondary starter for flavor production, mainly through proteolysis (*i.e.* casein degradation) and lipolysis during ripening (19.Moreau 1980). The main characteristic feature of blue cheeses, and in particular Roquefort PDO cheeses, is their intense and spicy flavors (31.Kinsella & Hwang 1976; 32.Rothe 1982). The specific volatile and metabolic compounds responsible for these flavors are mainly generated by lipolysis in blue cheeses (33.Cerning 1987; 34.Collins 2003a), which intensity however varies among *P. roqueforti* strains (35.Larsen & Jensen 1999; 29.Dumas 2020). The fatty acids released by lipolysis are the precursors of aldehydes, alcohols, acids, lactones and methyl-ketones, the latter providing the moldy aromas typical of blue cheeses (34.Collins 2003a). Regarding proteolysis, *P. roqueforti* degrades ca. 50% of casein, with however varying intensity across strains (33.Cerning 1987; 36.Larsen 1998; 29.Dumas 2020). The resulting peptides contribute to flavors and their degradation into amino acids further influences cheese aroma and the growth of other microorganisms (37.Williams 2004; 38.McSweeney & Sousa 2000). *Penicillium roqueforti* also contributes to lactate degradation, which is necessary for deacidification, and also promotes the development of less acid-tolerant microorganisms (39.McSweeney & Fox 2017). Through these effects, as well as through the production of secondary metabolites with anti-microbial properties, *P. roqueforti* could also impact cheese microbial composition (40.Kopp 1979; 41.Vallone 2014). Another factor potentially affected by *P. roqueforti* populations restricting the occurrence of spoiler microorganisms is the lack of free water, i.e., a low water activity (Aw), which is highly controlled for Roquefort cheese

sales and is affected by the degree of proteolysis (42.Ardö 2017). The four *P. roqueforti* populations may thus also impact cheese features indirectly, by different effects on beneficial or undesired contaminants.

So far, differences among *P. roqueforti* populations have only been studied *in vitro* or in very rudimentary cheese models. Here, we aimed at investigating the differences among cheeses produced by the four *P. roqueforti* populations following conditions close to modern cheese production. Better understanding the specificities of *P. roqueforti* populations for cheesemaking, e.g., in terms of ripening dynamics and specific flavors, could allow important applications and developments, in addition to increasing knowledge on domestication and adaptation processes. We produced blue cheeses in conditions very close to those in industrial Roquefort PDO production, in particular with local “Lacaune” breed ewe milk, and with strains from the four *P. roqueforti* populations, and we compared several important cheese features among populations: i) physicochemical features, related to texture and nutritional quality, ii) cheese microbiota composition and abundance, which can impact several cheese features, iii) the proportion of blue area in cheese slices, which is important for the blue-veined aspect and depends on *P. roqueforti* growth and sporulation in cheese cavities, and iv) metabolic and volatile compound identities and quantities, which influence flavor and aromas. We tested the existence of differences among cheeses produced by the four *P. roqueforti* populations (Roquefort cheese, non-Roquefort cheese, silage and lumber/food spoiler populations) for these different features. We also tested whether there were differences between cheeses made by cheese versus non-cheese populations and between the cheese Roquefort and non-Roquefort populations.

## Results

**Design and cheesemaking.** We made cheeses using three different strains for each of the four *P. roqueforti* populations (Figure 1). We divided the production into three assays, each including one strain from each of the four populations (Figure 1). For each strain within each assay, we made three production replicates, with two cheeses per strain in each replicate. Because the assays were made sequentially from February to April, the effect of the seasonal change in milk composition was confounded with the strain effect, hereafter referred to as the "assay effect". The three replicates within each assay were also done at different times and with different batches of raw milk.

**Influence of *P. roqueforti* populations on the cheese bacterial diversity but not on the abundance of the main microorganisms.** In order to test whether *P. roqueforti* populations had an influence on the cheese microbiota composition, we estimated the concentrations of key microbial communities with cell counts on various specific culture media one the one hand (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 targeting bacteria in cheeses at several maturation stages based on 16S sequencing on the other hand. The identity and abundance of the studied microorganisms (Supplementary figure 1A and 1B) were similar to those in four commercial Roquefort cheeses (43.Devoyod 1968; personal information from C. Callon) and close blue cheeses (44.Diezhandino). Based on microbial counts, we found no significant effect of the *P. roqueforti* population on the abundance of any of the counted microorganisms, including molds (i.e., mainly *P. roqueforti)*, at any maturation stage (Supplementary Table 1A).

Based on the metabarcoding approach targeting bacteria, we found a majority of sequences belonging to the *Lactococcus* and *Leuconostoc spp.* starters, ensuring acidification and the formation of cavities in cheeses, respectively. The remaining sequences corresponded to twelve bacterial genera frequently found in raw milk cheeses, such as *Lactobacillus*, *Staphylococcus* and *Arthrobacter*. The large predominance of starters however prevented to have sufficient data on other bacteria to assess differences in the abundance of particular bacteria among cheeses made with the four *P. roqueforti* populations (Supplementary Table 1B). We estimated three OTU (operational taxonomic unit) diversity parameters based on bacterial barcode sequence abundances, measuring OTU richness and/or evenness. Using the Bray-Curtis dissimilarity measure, we did not find that communities were more similar between cheeses made with any given *P. roqueforti* population than among *P. roqueforti* populations. However, we found a significant effect of the *P. roqueforti* population, in addition to a stage effect, on the Shannon and Simpson diversity indexes. Cheeses made with the cheese *P. roqueforti* populations tended to show higher bacterial OTU diversity, and in particular at 9 days of maturation and in the Roquefort population (Supplementary figure 1C and 1D), although the post-hoc analyses did not have enough power to detect any significant pairwise differences (Supplementary Table 1B). The differences in the cheese bacterial diversity, although minor, suggest that the differences among cheeses made with the four *P. roqueforti* populations may be also due, in addition to a direct effect of *P. roqueforti* populations, to an indirect effect through the induction of more or less diverse bacterial community.

**Higher proportion of blue area in cheeses produced by cheese *P. roqueforti* populations.** We estimated the percentage of cheese area covered by blue on fresh inner cheese slices, which depended on the formation of cavities in cheeses, the growth of *P. roqueforti* in

cheeses and its sporulation efficiency. We found significantly higher percentages of blue area in cheeses made by cheese than by non-cheese populations (Figure 2; Figure 3; Supplementary Table 1C). We also found a significant decrease of blue area from 20 to 180-day maturation, except for the Roquefort population, which maintained a rather steady blue area proportion (Figure 2; Supplementary Table 1C).

**Most efficient proteolysis and lipolysis by the Roquefort *P. roqueforti* population.** Because the *P. roqueforti* strains used for cheesemaking are known to have high proteolytic and lipolytic activities, which are key functionalities for cheese ripening, we tested whether the four populations displayed different proteolysis and lipolysis efficiencies. Using both targeted and non-targeted chromatographic analyses, we found that the Roquefort *P. roqueforti* population displayed the most efficient proteolysis. We performed the targeted analysis using standards for 23 main amino acids (Supplementary Table 2A). We found that eight amino acids significantly discriminated among the cheeses made by the different *P. roqueforti* populations (Supplementary Table 1D), 15 between the cheese and non-cheese populations and 14 between the Roquefort and non-Roquefort populations (Supplementary figure 2A). The cheeses made with cheese populations, and in particular with the Roquefort population, contained a higher total concentration of amino acids (Supplementary Tables 1D and 2B).

For analyzing proteolysis activity, we further performed a non-targeted analysis (fingerprint approach) on the whole chromatograms (8,364 signals) allowing much more powerful discrimination among metabolites. Each metabolite generates a signal specific to its mass-to-charge (m/z) at a given retention time. We found more aqueous signals, indicating more efficient proteolysis, in cheeses inoculated with the Roquefort population than with other

populations, followed by lumber and non-Roquefort populations being non significantly different from each other, the silage population being the least efficient (Figure 4; Supplementary Table 1E).

We also found that the Roquefort population had a more efficient lipolysis than the other populations. We tested whether the *P. roqueforti* population influenced the quantities of free fatty acids and residual glycerides as a proxy for lipolysis efficiency in 90-day cheeses, with again targeted and non-targeted chromatographic analyses in positive and negative ionization modes, more specifically targeting glycerides and free fatty acids, respectively. In the targeted analysis, we identified seven free fatty acids and 20 triglycerides and found three free fatty acids that were significantly more concentrated in cheeses made with Roquefort than non-Roquefort populations (Supplementary Table 1F). In the non-targeted analysis, we obtained 3,094 signals and observed higher amounts of organic signals specific to free fatty acids, indicating more efficient lipolysis, in cheeses made using the Roquefort population, followed by lumber and non-Roquefort populations very similar to each other, the silage population being the least efficient (Figure 5; Supplementary Table 1G). Regarding the residual glycerides, we obtained 8,472 signals and detected no significant differences (Supplementary figure 3; Supplementary Table 1H).

Regarding the main physico-chemical properties, we found as expected a maturation stage effect for 11 out of 16 parameters (Supplementary Table 1I). We found significantly higher non-protein nitrogenous content in cheeses inoculated with cheese *P. roqueforti* populations, suggesting more efficient proteolysis by cheese populations (Supplementary figure 4). We found a significant difference in cheese water activity among the cheeses made with the four *P. roqueforti* populations (Supplementary figure 4), with significantly lower water activity in

the Roquefort population than in the non-Roquefort and silage populations (Supplementary Table 1I).

**Strong influence of the *P. roqueforti* population on volatile compound production.** In order to test if *P. roqueforti* populations had an influence on cheese aroma and flavor, we measured the relative abundance of the most relevant volatile compounds in 90-day cheeses. After GC-MS data processing, we focused on the 40 main volatile compounds considered as markers of the aromatic quality of blue cheeses (32.Rothe 1982): 11 acids, 12 ketones, 10 esters, six alcohols and one aldehyde (Supplementary Table 3). We found a strong influence of *P. roqueforti* populations on the amount of all these aromatic compound families in cheeses (Supplementary Table 1J; Figures 6 and 7). As a matter of fact, the cheeses showed huge differences in their odors: while the cheeses made with the cheese *P. roqueforti* populations smelled as good as typical ripened blue cheeses, cheeses made with non-cheese *P. roqueforti* populations had unpleasant odors, similar to wet swab (Supplementary figure 5; personal observation).

We found higher quantities of acids, methyl-ketones and secondary alcohols resulting from proteolysis and lipolysis, and contributing to the typical flavor of blue cheese, in cheeses produced by cheese than non-cheese populations, and in particular in the Roquefort population. Among the 40 analysed compounds, four were by-products of proteolysis (3-methyl-butanal, 3-methyl-butanol and isopropyl-alcohol, i.e. alcohols I and 3-methyl-butanoic acid i.e. acids I; Supplementary Table 3). We found alcohols I in significantly higher abundances in cheeses made with cheese than non-cheese *P. roqueforti* populations, especially with the Roquefort population (Supplementary Table 1J). We also found higher amounts of acids I in cheeses made with the Roquefort population than in other cheeses. Two

acids, by-products of glycolysis (i.e. acids II), were found at higher levels in cheeses made with Roquefort and lumber/food spoiler *P. roqueforti* populations than in other cheeses (Supplementary Tables 1J and 3). The 35 other aromatic compounds (i.e. acids III, ketones, alcohols II and esters families) were almost all direct or indirect by-products of lipolysis (Supplementary Table 3). We found higher abundances of acids III in cheeses made with Roquefort and lumber/food spoiler populations compared to the non-Roquefort population, with the silage population showing the lowest value. We found higher amounts of esters and methyl-ketones (especially 2-pentanone and 2-heptanone) in cheeses made with cheese *P. roqueforti* populations (Supplementary Table 1J), with the Roquefort population showing the highest methyl-ketone quantities and the silage population containing almost no methyl-ketones (Figure 7A). We also found a much higher level of alcohols II, especially 2-heptanol, in cheeses made with the Roquefort population than in other cheeses (Supplementary Table 1J; Figure 7B).

## Discussion

**Cheese *P. roqueforti* populations have been domesticated for producing better cheeses.** By measuring multiple features of blue cheeses made under conditions similar as in the typical Roquefort industrial production, we found a strong influence of the differentiated *P. roqueforti* populations on cheese quality, with the cheese populations appearing adapted to cheesemaking both in terms of aspect and aromatic quality. We found that the cheese *P. roqueforti* populations produced higher percentages of blue area on cheese slices, which is an important visual aspect of blue cheeses. We also found more efficient proteolysis and lipolysis in cheeses made with the Roquefort population than with the other *P. roqueforti* populations, which resulted in the production of higher quantities of desired volatile compounds, in particular alcohols and associated acids. We also found lower cheese water activity in cheeses made with strains from the Roquefort population, which is also likely due to its more efficient proteolysis (42.Ardö 2017). As we could not find any significant difference in microorganism identity and abundance among cheeses made by the four *P. roqueforti* populations, but only some minor differences in species diversity, the differences among cheeses were likely mostly a direct effect of *P. roqueforti* population specificities, although there may also be some minor indirect effects through the induction of more diverse bacterial communities by cheese *P. roqueforti* populations. Our findings overall strongly support the view that cheese *P. roqueforti* populations have been selected by humans for improving cheese safety, aspect and aroma. Previous studies had found differences among *P. roqueforti* populations, in terms of growth, lipolysis and proteolysis, however using synthetic media (29.Dumas 2020; 23.Ropars 2015). Here, using experimental cheeses made following commercial cheese production conditions, we revealed important specificities of the cheese *P. roqueforti* populations, and of each of the Roquefort and non-Roquefort populations. These findings are important in the context of domestication, for understanding rapid

adaptation and diversification, and future studies using quantitative trait mapping could identify the genomic changes responsible for the population specificities. Progenies among different populations can indeed be obtained in *P. roqueforti* (23.Ropars 2015), which could also allow strain improvement by recombination among the different populations. Our results are therefore also important for improving blue cheese production.

**Little difference in the microbiota induced by the four *P. roqueforti* populations but lower water availability induced by cheese populations, restricting spoiler microorganism occurrence.** Based on microbiological counts, we found no significant differences in abundance for any of the floras monitored among cheeses made from the four populations of *P. roqueforti*. In particular, we found no significant difference in mold abundance on Petri dishes. However, microbiological counts are known to provide poor estimates of fungal biomass, especially for mycelium growth (45.Schnurer 1993).

The metabarcoding approach suggested that the different *P. roqueforti* populations induced more or less diverse bacterial communities, with the cheese population showing the highest associated diversity, and in particular the Roquefort population. The large predominance of bacterial starters however prevented having sufficient data to assess sub-dominant bacterial relative abundance differences based on the metabarcoding approach. We also found a significant difference in water activity among cheeses made with the four *P. roqueforti* populations, the minimum being observed for the Roquefort population. This may also be a result of selection by humans, as a low water activity restricts the occurrence of spoiler microorganisms, and is therefore highly controlled for Roquefort cheese sales, and in particular for export.

**Cheese *P. roqueforti* populations produce bluer cheeses.** We found significantly higher percentages of blue areas in slices of cheeses made with cheese than with non-cheese *P. roqueforti* populations, which can be due to greater *P. roqueforti* growth in cheese and/or higher sporulation efficiency in cavities. The percentage of blue areas in cheese slices also depends on the formation of cavities in cheese, as *P. roqueforti* can only sporulate in cavities where oxygen is available. The cavities are mainly generated by the gas-producing bacteria *Leuconostoc mesenteroides*, whose abundance was not different among the cheeses made by the different *P. roqueforti* populations, showing that there was a direct effect of *P. roqueforti* populations on the blueness of cheese slices. The significantly higher percentages of blue areas in slices of cheeses made with the cheese than non-cheese *P. roqueforti* populations is therefore most likely the result of better cheese and cavity colonization and sporulation, probably resulting from a selection for visual aspect. The percentage of blue area decreased at the end of the maturation time, perhaps due to fungal mortality. Only the cheeses made with the Roquefort strains kept their high percentage of blue area until the end of the 90 days of maturation, which may again result from a selection in the pre-industrial times when Roquefort cheeses had to be stored several months at cave temperature before sell; even nowadays, the minimum maturation time for Roquefort PDO remain 90 days, i.e. longer than the other blue cheeses. These findings contrast with a previous one showing that the non-Roquefort population colonized the cavities of cheese models better than the other populations (29.Dumas 2020); this discrepancy may be due to different measures (total percentage of blue area versus percentage of blue areas within cavities), to the type of milk (ewe versus goat) or to the mode of cheese making (rudimentary models versus commercial-like cheeses), that were different between the two studies. However, these findings are consistent with the presence of horizontally-transferred genes in cheese populations with predicted functions in fungal development, including sporulation and hyphal growth

(29.Dumas 2020).

**The Roquefort *P. roqueforti* population has more efficient proteolysis and lipolysis.** Based on chemical analyses and powerful chromatographic discrimination methods, we found the highest abundance of amino acids and small peptides, i.e., residual products of proteolysis, in cheeses made with the Roquefort *P. roqueforti* population, showing that it has the highest proteolytic capacities, which is an important process in cheesemaking. Proteolysis indeed contributes to the development of cheese texture, flavors and aromas (42.Ardö 2017; 46.Andersen 2010; 47.McSweeney 1997; 48.Roudot-Algaron 1996; 49.Ardö 2002). Previous measures of proteolytic activities in synthetic media could detect significant differences among *P. roqueforti* populations but not between the two cheese populations (29.Dumas 2020). Here, we found that the experimental cheeses made with the Roquefort population had a higher content of proteolysis residual products, thus showing more advanced ripening.

We also found a more efficient lipolysis in the cheeses made with the Roquefort *P. roqueforti* population. Previous measures in synthetic media found in contrast that the non-Roquefort population had the most efficient lipolysis (29.Dumas 2020), showing that measures in real cheeses are needed to reliably assess metabolic activities. Lipolytic activity is known to impact texture and the production of volatile compounds affecting cheese pungency (50.Alonso 1987a; 51,52.González De Llano 1990, 1992; 53.Martín and Coton 2016; 54.Thierry 2017; 55.Woo & Lindsay 1984). The more efficient proteolysis and lipolysis in the Roquefort *P. roqueforti* population should have strong impacts on cheese texture and flavor, and likely results from selection to obtain better cheeses, i.e. from a domestication process, as has been reported previously in other fungi (6.Almeida 2014; 7.Baker 2015;

8.Gallone 2016; 9.Gibbons 2012; 10.Gonçalves 2016; 11.Libkind 2011; 12.Sicard and Legras 2011). The Roquefort cheeses are in fact commonly considered as the blue cheeses with the strongest aromas and flavours; the less efficient lipolysis and lipolysis 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 important differences among cheeses made with the different *P. roqueforti* populations in terms of the volatile compounds resulting from lipolysis and, to a lesser extent, also from proteolysis. Among the aromatic compounds detected in our cheeses, only four (3-methyl-butanal, 3-methyl-butanol, isopropyl-alcohol and 3-methyl-butanoic acid) were by-products of the proteolysis of caseins (38.McSweeney 2000) and their concentrations were significantly higher for the Roquefort *P. roqueforti* population, in agreement with its higher proteolysis efficiency and their amino acid precursor concentrations (i.e. valine, leucine and isoleucine). These compounds produce fruity (banana), cheesy and alcoholic notes, which has likely been an important selection criterion in the Roquefort *P. roqueforti* population. Regarding the products of the metabolic pathways leading from amino-acids to alcohol (Ehrlich pathway with reduction of aldehyde) or to acid (oxidation of aldehyde; 56.Ganesan 2017), the higher concentration of the alcohols versus acids observed in all populations is consistent with the general micro-aerobic conditions in the blue cheese cavities.

The majority of the aromatic compounds identified were directly or indirectly by-products of lipolysis, which is consistent with the known key role of lipolysis in the generation of typical blue cheeses aroma (33.Cerning 1987; 34.Collins 2003). The aromatic compounds resulting from lipolysis corresponded to four chemical families (acids, methyl-ketones, secondary alcohols and esters), the methyl-ketones being the most diverse and abundant for cheese *P. roqueforti* populations, especially the Roquefort one, with 2-pentanone and 2-heptanone

having the highest level, the latter one displaying the characteristic “blue cheese” sensory descriptor (51,52.De Llano 1990, 1992; 57.Moio 2000; 58.Anderson & Day 1966). Methyl-ketones with odd carbon numbers are mainly produced by *P. roqueforti* via beta-oxidation of fatty acids, whereas methyl-ketones with even carbon numbers can be produced by beta-oxidation but also by autoxidation of fatty acids (59.Spinnler 2011). These compounds are produced by the decarboxylation of hexanoic acid and octanoic acid, respectively, which are also the most abundant acids found in our cheeses. This reaction is considered as a way of detoxification because methyl-ketones are less toxic than acids (60.Kinderlerer 1993; 59.Spinnler 2011). Interestingly, this pathway seems to be more active in the cheese *P. roqueforti* populations, as the level of methyl-ketones was four-fold and 10 fold lower in the cheeses made with lumber and silage populations, respectively, than cheeses made with the cheese populations. As methyl-ketone concentrations were not directly associated with the concentrations of their precursors (acids), the highest concentrations being found in lumber and Roquefort populations, the biosynthesis pathway producing methyl-ketones must be more efficient in cheese populations, especially in the non-Roquefort population. The cheese *P. roqueforti* populations have likely been selected for their higher ability of acid detoxification, thus producing aromatic compounds with very positive impact on flavour (59.Spinnler 2011).

The concentrations of secondary alcohols (resulting from the reduction of methyl-ketones) were also higher in cheeses produced by cheese *P. roqueforti* populations, and especially for the Roquefort population, being 7-fold and 20-fold higher than for non-Roquefort and silage/lumber populations, 2-heptanol being the major alcoholic compound. The reduction of 2-heptanone to 2-heptanol is specific to anaerobic conditions and much stronger in the Roquefort population, while the aerobic conditions were similar for all the populations. The Roquefort *P. roqueforti* population may therefore have also been selected for this

functionality, as secondary alcohols provide “fruity notes” improving the aromatic quality (59.Spinnler 2011). The reduction of methyl-ketone to alcohol may be performed by an alcohol dehydrogenase, such as in the reduction of aldehyde to alcohol via the Ehrlich pathway. Alcohol dehydrogenase genes may thus have been targets of selection in the Roquefort *P. roqueforti* population, although they were not detected as evolving under positive selection in a previous study (29.Dumas 2020).

We also found higher levels of esters in cheeses made with cheese *P. roqueforti* populations; esters are mainly produced by the esterification of ethanol esterification by acids resulting from beta-oxidation. Ethanol can be produced by *Leuconostoc* starters and ester synthesis is also described as a detoxification mechanism (61.Mason 2000). These results further indicate that the cheese *P. roqueforti* populations, and especially the Roquefort one, have been selected for better ability to detoxify acids, leading to a large variety of less toxic aromatic compounds with great aromatic properties.

Overall, we thus found more appealing aromas in cheeses made with the cheese *P. roqueforti* populations, and especially the Roquefort population, which likely results from selection by humans. The cheeses made by silage and lumber populations displayed mild and unpleasant smell whereas cheeses made with cheese populations smelled as typical blue cheeses, the Roquefort population leading to the cheeses with the strongest smell. This may be partly related to the previously detected horizontal gene transfers in cheese populations involving genes with predicted functions in lipolysis or amino-acid catabolism and the positive selection on genes involved in aroma production (29.Dumas 2020). We compared *P. roqueforti* populations based on real cheeses, which is a major step forward in comparison with the previous studies only based on experimental models or synthetic media (28.Gillot 2017; 29.Dumas 2020). We used raw ewe milk as in the Roquefort PDO production, which also impacts cheese aromas; it would be interesting in future studies to assess whether using

ewe versus cow milk or raw versus pasteurized milk leads to similar specificities of the Roquefort versus non-Roquefort *P. roqueforti* populations, as there may have been a selection during domestication leading to an adaptation of the Roquefort population for the catabolism of raw ewe milk.

**Conclusion.** We showed that the *P. roqueforti* population had a strong impact on cheese quality, aspect and aromas. The populations used for cheesemaking led to bluer cheeses, with better aromas, which likely results from domestication, i.e. selection on multiple fungal traits by humans of the strains making the best cheeses. French cheese producers have inoculated cheeses with *P. roqueforti* spores from moldy rye bread since the end of the 19$^{th}$ century (16,17.Labbe and Serres, 2004, 2009; 18.Vabre 2015), which allowed them to re-inoculate the strains producing the best cheeses and thus to apply strong selection. The two cheese populations exhibited multiple specificities, 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* (8.Gallone 2016; 60.Legras 2018). Previous studies on *P. roqueforti* detected in the cheese populations recurrent changes in amino acids and horizontal gene transfers, both allowing rapid adaptation (29.Dumas 2020; 23.Ropars 2015). Our findings allow a better understanding of domestication in *P. roqueforti* and open the way for strain improvement by targeting relevant traits. A protocol inducing sexual selection has been developed in *P. roqueforti* (30.Ropars 2014), which will allow to perform crosses between the two cheese populations, harboring each very little genetic diversity (29.Dumas 2020), to generate variability and identify performant strains.

**Data accessibility**

Metabarcoding nucleic sequences are available at https://www.ebi.ac.uk/ena/data/search?query=ERS4329370, study PRJEB36799, samples ERS4329370 to ERS4329465. Metabolomic and volatilomic data are available at https://www.ebi.ac.uk/metabolights/, study MTBLS1509.

**Material and Methods**

The cheesemaking protocol corresponded to the typical mode used by the main Roquefort producers and complied with the Roquefort protected designation of origin (PDO) specifications, except that the ripening process occurred in artificial cellars in Aurillac INRA facilities and with strains from different *P. roqueforti* populations (Figure 1). We estimated the concentration of different microorganism communities on the initial raw milk and on various cheese maturation stages. We performed a metabarcoding analysis on the experimental cheeses at 9 and 20 days of maturation, by sequencing the 16S DNA fragment using Illumina Miseq technology and analysing sequences using Find Rapidly OTUs with Galaxy Solution (FROGS), v3.0 (63.Escudie 2018). For each OTU, taxonomic assignment was determined with Silva-132 (https://www.arb-silva.de/) and 16S rDNA RefSeq databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi). We estimated the area percentage of cheese covered by blue on fresh inner cheese slices by analysing pictures of fresh slices using imageJ and by counting the number of dark pixels. We performed standard nutritional quality controls of cheeses by measuring dry, fat over dry matter content, moisture of the defatted cheese, total, soluble and non-protein nitrogen contents, chloride and salt content, water activity and pH at various maturation stages according to reference methods. We measured glucose, lactose, lactate, acetate and butyrate in 9 and 20-day cheeses by high performance liquid chromatography (HPLC). We tested whether the four populations displayed different

proteolytic and lipolytic activities following two procedures of extraction (water or organic solvents) and UHPLC-MS analysis. We analysed the quantities of free fatty acids and residual glycerides in 90-day cheeses by coupling a global extraction (accelerated solvent extraction with hexane-isobutanol) with a UHPLC-MS analysis in positive (triglycerides) and negative (fatty acids) ionization modes. We investigated the identity and abundance of volatile compounds involved in flavor and aromas 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 a quadrupole mass spectrometer Agilent 5977B (Santa Clara, United States). Statistical analyses were performed with the R software (http://www.r-project.org/). Further details on the material and methods are given in Supplementary methods.

**Acknowledgments:**

We thank Béatrice DESSERRE, Céline DELBES and Cécile CALLON for advice and technical assistance in microbiology, Sébastien THEIL for the technical support of metabarcoding analyses, Patricia LE THUAUT, Manon SURIN and Brigitte POLLET for the technical support of metabolomic analyses, Sara PARISOT for milk delivery and quality, Pierre CONCHON for the technical support of image analysis, LIAL-MC for the various reference measurements in physico-chemistry, Christophe LACROIX and Alfonso DIE for the determination of short-chain fatty acids in fermentation supernatants.

This study has been funded by the LIP SAS, ANRT (association nationale recherche technologie), by the ERC Genomefun 309403 Stg and Blue Proof of Concept grants, the Fondation Louis D grant (French Academy of Sciences) and the ANR-19-CE20-0002-02 Fungadapt ANR grant.

Thibault Caron, Mélanie Le Piver, Sammy Brunel, Daniel Roueyre and Michel Place have

been employed by the funder SAS LIP which produces starters for fermented food products during the course of the study and therefore declare a competitive financial interest. None of them, except TC, played a role in the design decision, data analysis and interpretation, or in the decision to submit the work for publication. All other authors declare no conflict of interest.

## References

1. Larson G, Fuller DQ. The Evolution of Animal Domestication. Annual Review of Ecology, Evolution, and Systematics. 2014;45:115–136. https://doi.org/10.1146/annurev-ecolsys-110512-135813. doi:10.1146/annurev-ecolsys-110512-135813

2. Gladieux P, Ropars J, Badouin H, Branca A, Aguileta G, De Vienne DM, Rodríguez De La Vega RC, Branco S, Giraud T. Fungal evolutionary genomics provides insight into the mechanisms of adaptive divergence in eukaryotes. Molecular Ecology. 2014;23(4):753–773. https://doi.org/10.1111/mec.12631. doi:10.1111/mec.12631

3. Giraud T, Koskella B, Laine A-L. Introduction: microbial local adaptation: insights from natural populations, genomics and experimental evolution. Molecular Ecology. 2017;26(7):1703–1710. https://doi.org/10.1111/mec.14091. doi:10.1111/mec.14091

4. Bigelis R. Fungal Fermentation: Industrial. Encyclopedia of Life Sciences. 2001:1–8. https://doi.org/10.1038/npg.els.0000357. doi:10.1038/npg.els.0000357

5. Dupont J, Dequin S, Giraud T, Le Tacon F, Masit S, Ropars J, Richard F, Selosse M-A. Fungi as a Source of Food. Microbiology Spectrum. 2017;5(3):1-22. doi:10.1128/microbiolspec

6. Almeida M, Hébert A, Abraham A-L, Rasmussen S, Monnet C, Pons N, Delbès-Paus C, Loux V, Batto J-M, Leonard P, et al. Construction of a dairy microbial genome catalog opens new perspectives for the metagenomic analysis of dairy fermented products. BMC Genomics. 2014;15(1):1101. https://doi.org/10.1186/1471-2164-15-1101. doi:10.1186/1471-2164-15-1101

7. Baker EP, Wang B, Bellora N, Peris D, Hulfachor AB, Koshalek JA, Adams M, Libkind D, Hittinger CT. The Genome Sequence of Saccharomyces eubayanus and the Domestication of Lager-Brewing Yeasts. Molecular biology and evolution. 2015;32(11):2818–2831. https://doi.org/10.1093/molbev/msv168. doi:10.1093/molbev/msv168

8. Gallone B, Steensels J, Prahl T, Soriaga L, Saels V, Herrera-Malaver B, Merlevede A, Roncoroni M, Voordeckers K, Miraglia L, et al. Domestication and Divergence of Saccharomyces cerevisiae Beer Yeasts. Cell. 2016;166(6):1397–1410. http://dx.doi.org/10.1016/j.cell.2016.08.020. doi:10.1016/j.cell.2016.08.020

9. Gibbons JG, Salichos L, Slot JC, Rinker DC, McGary KL, King JG, Klich MA, Tabb DL, McDonald WH, Rokas A. The evolutionary imprint of domestication on genome variation and function of the filamentous fungus Aspergillus oryzae. Current Biology. 2012;22(15):1403–1409. https://doi.org/10.1016/j.cub.2012.05.033. doi:10.1016/j.cub.2012.05.033

10. Gonçalves M, Pontes A, Almeida P, Barbosa R, Serra M, Libkind D, Hutzler M, Gonçalves P, Sampaio JP. Distinct Domestication Trajectories in Top-Fermenting Beer Yeasts and Wine Yeasts. Current Biology. 2016;26(20):2750–2761. https://doi.org/10.1016/j.cub.2016.08.040. doi:10.1016/j.cub.2016.08.040

11. Libkind D, Hittinger CT, Valeŕio E, Gonçalves C, Dover J, Johnston M, Gonçalves P,

Sampaio JP. Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(35):14539–14544. https://doi.org/10.1073/pnas.1105430108. doi:10.1073/pnas.1105430108

12. Sicard D, Legras JL. Bread, beer and wine: Yeast domestication in the Saccharomyces sensu stricto complex. Comptes Rendus - Biologies. 2011;334(3):229–236. http://dx.doi.org/10.1016/j.crvi.2010.12.016. doi:10.1016/j.crvi.2010.12.016

13. Fleming A. On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to their Use in the Isolation of B. influenzæ. British Journal of Experimental Pathology. 1929;10(3):226–236. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2048009/. doi:10.1093/clinids/2.1.129

14. Ludemann V, Greco M, Rodríguez MP, Basílico JC, Pardo AG. Conidial production by Penicillium nalgiovense for use as starter cultures in dry fermented sausages by solid state fermentation. LWT - Food Science and Technology. 2010;43(2):315–318. https://doi.org/10.1016/j.lwt.2009.07.011. doi:10.1016/j.lwt.2009.07.011

15. Perrone G, Samson RA, Frisvad JC, Susca A, Gunde-Cimerman N, Epifani F, Houbraken J. Penicillium salamii, a new species occurring during seasoning of dry-cured meat. International Journal of Food Microbiology. 2015;193:91–98. http://dx.doi.org/10.1016/j.ijfoodmicro.2014.10.023. doi:10.1016/j.ijfoodmicro.2014.10.023

16. Labbe M, Serres JP. Chroniques du Roquefort - De la préhistoire à l'aube industrielle. Grand Imprimeur, La Primaube, France; 2004.

17. Labbe M, Serres JP. Chroniques du Roquefort: des Hommes, des Entreprises, des Marques, Période Moderne. Graphy Imprimeur, editor. La Primaube, France; 2009.

18. Vabre S. Le sacre du Roquefort. Presses universitaires François Rabelais, Tours, France; 2015.

19. Moreau C. Le Penicillium roqueforti, morphologie, physiologie, intérêt en industrie fromagère, mycotoxines. Le Lait. 1980;60(595-596):254–271. https://doi.org/10.1051/lait:1980595-59615%0A. doi:10.1051/lait:1980595-59615

20. Pitt JI, Hocking AD. Fungi and Food Spoilage. Springer. Springer, Boston, MA; 2009. https://link.springer.com/book/10.1007%2F978-0-387-92207-2. doi:10.1007/978-0-387-92207-2

21. Ropars J, Cruaud C, Lacoste S, Dupont J. A taxonomic and ecological overview of cheese fungi. International Journal of Food Microbiology. 2012;155(3):199–210. http://dx.doi.org/10.1016/j.ijfoodmicro.2012.02.005. doi:10.1016/j.ijfoodmicro.2012.02.005

22. Cheeseman K, Ropars J, Renault P, Dupont J, Gouzy J, Branca A, Abraham A-L, Ceppi M, Conseiller E, Bensimon A, et al. Multiple recent horizontal transfers of a large genomic region in cheese making fungi. Nature Communications. 2014;5, 2879 (2014). https://doi.org/10.1038/ncomms3876. doi:10.1038/ncomms3876

23. Ropars J, Rodríguez De La Vega RC, López-Villavicencio M, Gouzy J, Sallet E, Dumas É, Lacoste S, Debuchy R, Dupont J, Branca A, et al. Adaptive horizontal gene transfers between multiple cheese-associated fungi. Current Biology. 2015;25(19):2562–2569. https://doi.org/10.1016/j.cub.2015.08.025. doi:10.1016/j.cub.2015.08.025

24. Ropars J, Rodríguez De La Vega RC, Gouzy J, Dupont J, Swennen D, Dumas É, Giraud T, Branca A. Diversity and Mechanisms of Genomic Adaptation in Penicillium. In: Aspergillus and Penicillium in the post-genomic era. de Vries RP, Benoit Gelber I, Rordam A, editors. HAL. Caister Academic Press; 2016. https://hal.archives-ouvertes.fr/hal-01302706/. doi:⟨hal-01302706⟩

25. Ropars J, Lo Y-C, Dumas É, Snirc A, Begerow D, Rollnik T, Lacoste S, Dupont J, Giraud T, López-Villavicencio M. Fertility depression among cheese-making Penicillium roqueforti strains suggests degeneration during domestication. Evolution. 2016;70(9):2099–2109. https://doi.org/10.1111/evo.13015. doi:10.1111/evo.13015

26. Ropars J, López-Villavicencio M, Snirc A, Lacoste S, Giraud T. Blue cheese-making has shaped the population genetic structure of the mould Penicillium roqueforti. PLoS ONE. 2017;12(3):e0171387. https://doi.org/10.1371/journal.pone.0171387. doi:10.1371/journal.pone.0171387

27. Gillot G, Jany J-L, Coton M, Le Floch G, Debaets S, Ropars J, López-Villavicencio M, Dupont J, Branca A, Giraud T, et al. (Fifty shades of blue :) Insights into Penicillium roqueforti morphological and genetic diversity. PLoS ONE. 2015;10(6):e0129849. https://doi.org/10.1371/journal.pone.0129849. doi:10.1371/journal.pone.0129849

28. Gillot G, Jany J-L, Poirier E, Maillard M, Debaets S, Thierry A, Coton E, Coton M. Functional diversity within the Penicillium roqueforti species. International Journal of Food Microbiology. 2017;241:141–150. http://dx.doi.org/10.1016/j.ijfoodmicro.2016.10.001. doi:10.1016/j.ijfoodmicro.2016.10.001

29. Dumas E, Feurtey A, Vega RCR de la, Prieur S Le, Snirc A, Coton M, Thierry A, Coton E, Piver M Le, Roueyre D, et al. Independent domestication events in the blue-cheese fungus Penicillium roqueforti. Molecular Ecology. 2020;(January):451773. https://doi.org/10.1111/mec.15359. doi:10.1101/451773

30. Ropars J, López-Villavicencio M, Dupont J, Snirc A, Gillot G, Coton M, Jany J-L, Coton E, Giraud T. Induction of sexual reproduction and genetic diversity in the cheese fungus Penicillium roqueforti. Evolutionary Applications. 2014;7(4):433–441. https://doi.org/10.1111/eva.12140. doi:10.1111/eva.12140

31. Kinsella JE, Hwang DH. Enzymes of Penicillium Roqueforti Involved in the Biosynthesis of Cheese Flavor. C R C Critical Reviews in Food Science and Nutrition. 1976;8(2):191–228. https://doi.org/10.1080/10408397609527222. doi:10.1080/10408397609527222

32. Rothe M, Engst W, Erhardt V. Studies on characterization of Blue cheese flavour. Die Nahrung. 1982;26(7/8):591–602. https://doi.org/10.1002/food.19820260704. doi:10.1071/RJ12042

33. Cerning J, Gripon JC, Lamberet G, Lenoir J. Les activités biochimiques des Penicillium

utilisés en fromagerie. Le Lait. 1987;67(1):3–39. https://doi.org/10.1051/lait:198711%0A. doi:10.1051/lait:198711

34. Collins YF, McSweeney PLH, Wilkinson MG. Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. International Dairy Journal. 2003;13(11):841–866. https://doi.org/10.1016/S0958-6946(03)00109-2. doi:10.1016/S0958-6946(03)00109-2

35. Larsen MD, Jensen K. The effects of environmental conditions on the lipolytic activity of strains of Penicillium roqueforti. International Journal of Food Microbiology. 1999;46(2):159–166. https://doi.org/10.1016/S0168-1605(98)00191-3. doi:10.1016/S0168-1605(98)00191-3

36. Larsen MD, Kristiansen KR, Hansen TK. Characterization of the proteolytic activity of starter cultures of Penicillium roqueforti for production of blue veined cheeses. International Journal of Food Microbiology. 1998;43(3):215–221. https://doi.org/10.1016/S0168-1605(98)00114-7. doi:10.1016/S0168-1605(98)00114-7

37. Williams AG, Beattie SH, Banks JM. Enzymes involved in flavour formation by bacteria isolated from the smear population of surface-ripened cheese. International Journal of Dairy Technology. 2004;57(1):7–13. https://doi.org/10.1111/j.1471-0307.2004.00115.x. doi:10.1111/j.1471-0307.2004.00115.x

38. McSweeney PLH, Sousa MJ. Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. Le Lait. 2000;80(3):293–324. https://doi.org/10.1051/lait:2000127%0A. doi:10.1051/lait:2000127

39. McSweeney PLH, Fox PF, Ciocia F. Metabolism of Residual Lactose and of Lactate and Citrate. In: Cheese: Chemistry, Physics and Microbiology. McSweeney PLH, Fox PF, Cotter PD, Everett DW, editors. Fourth Edi. Elsevier Ltd; 2017. p. 411–421. http://dx.doi.org/10.1016/B978-0-12-417012-4/00016-8. doi:10.1016/B978-0-12-417012-4.00016-8

40. Kopp B, Rehm HJ. Antimicrobial action of roquefortine. European Journal of Applied Microbiology and Biotechnology. 1979;6:397-401 (1979). https://doi.org/10.1007/BF00499170. doi:10.1007/BF00499170

41. Vallone L, Giardini A, Soncini G. Secondary metabolites from Penicillium roqueforti, a starter for the production of Gorgonzola cheese. Italian Journal of Food Safety. 2014;3(3):173–177 (2118). https://doi.org/10.4081/ijfs.2014.2118. doi:10.4081/ijfs.2014.2118

42. Ardö Y, McSweeney PLH, Magboul AAA, Upadhyay VK, Fox PF. Biochemistry of Cheese Ripening: Proteolysis. In: Cheese: Chemistry, Physics and Microbiology. McSweeney PLH, Fox PF, Cotter PD, Everett DW, editors. Fourth Edi. Elsevier Ltd; 2017. p. 445–482. http://dx.doi.org/10.1016/B978-0-12-417012-4/00018-1. doi:10.1016/B978-0-12-417012-4.00018-1

43. Devoyod JJ, Muller M. La flore microbienne du fromage de Roquefort III. Les streptocoques lactiques et les Leuconostoc Influence de différents micro-organismes de contamination. Le Lait. 1969;487:369–399. https://doi.org/10.1051/lait:196948715

44. Diezhandino I, Fernández D, González L, McSweeney PLH, Fresno JM. Microbiological, physico-chemical and proteolytic changes in a Spanish blue cheese during ripening (Valdeón cheese). Food Chemistry. 2015;168:134–141. http://dx.doi.org/10.1016/j.foodchem.2014.07.039. doi:10.1016/j.foodchem.2014.07.039

45. Schnurer J. Comparison of methods for estimating the biomass of three food-borne fungi with different growth patterns. Applied and Environmental Microbiology. 1993;59(2):552–555. doi:10.1128/aem.59.2.552-555.1993

46. Andersen LT, Ardö Y, Bredie WLP. Study of taste-active compounds in the water-soluble extract of mature Cheddar cheese. International Dairy Journal. 2010;20(8):528–536. http://dx.doi.org/10.1016/j.idairyj.2010.02.009. doi:10.1016/j.idairyj.2010.02.009

47. McSweeney PLH. The flavour of milk and dairy products: III. Cheese: Taste. International Journal of Dairy Technology. 1997;50(4):123–128. https://doi.org/10.1111/j.1471-0307.1997.tb01752.x. doi:10.1111/j.1471-0307.1997.tb01752.x

48. Roudot-Algaron F. Le goût des acides aminés, des peptides et des protéines: Exemple de peptides sapides dans les hydrolysats de caséines. Lait. 1996;76(4):313–348. https://doi.org/10.1051/lait:1996425%0A. doi:10.1051/lait:1996425

49. Ardö Y. Flavour formation by amino acid catabolism. Biotechnology Advances. 2006;24(2):238–242. https://doi.org/10.1016/j.biotechadv.2005.11.005. doi:10.1016/j.biotechadv.2005.11.005

50. Alonso L, Juarez M, Ramose M, Martin-Alvarez PJ. Overall composition, nitrogen fractions and fat characteristics of Cabrales cheese during ripening. Zeitschrift für Lebensmittel-Untersuchung und -Forschung. 1987;185:481–486 (1987). https://doi.org/10.1007/BF01042813. doi:10.1007/BF01042813

51. De Llano DG, Ramos M, Polo C, Sanz J, Martinez-Castro I. Evolution of the volatile components of an artisanal blue cheese during ripening. Journal of Dairy Science. 1990;73(7):1676–1683. https://doi.org/10.3168/jds.S0022-0302(90)78842-X. doi:10.3168/jds.S0022-0302(90)78842-X

52. González de Llano D, Ramos M, Rodriguez A, Montilla A, Juarez M. Microbiological and physicochemical characteristics of Gamonedo blue cheese during ripening. International Dairy Journal. 1992;2(2):121–135. https://doi.org/10.1016/0958-6946(92)90005-7. doi:10.1016/0958-6946(92)90005-7

53. Martín JF, Coton M. Blue Cheese: Microbiota and Fungal Metabolites. In: Fermented Foods in Health and Disease Prevention. Elsevier Inc.; 2016. p. 275–303. http://dx.doi.org/10.1016/B978-0-12-802309-9.00012-1. doi:10.1016/B978-0-12-802309-9.00012-1

54. Thierry A, Collins YF, Abeijón Mukdsi MC, McSweeney PLH, Wilkinson MG, Spinnler H-E. Lipolysis and Metabolism of Fatty Acids in Cheese. In: Cheese: Chemistry, Physics and Microbiology. McSweeney PLH, Fox PF, Cotter PD, Everett DW, editors. Fourth Edi. Elsevier Ltd; 2017. p. 423–444. http://dx.doi.org/10.1016/B978-0-12-417012-4/00017-X.

doi:10.1016/B978-0-12-417012-4.00017-X

55. Woo AH, Lindsay RC. Concentrations of Major Free Fatty Acids and Flavor Development in Italian Cheese Varieties. Journal of Dairy Science. 1984;67(5):960–968. http://dx.doi.org/10.3168/jds.S0022-0302(84)81394-6. doi:10.3168/jds.S0022-0302(84)81394-6

56. Ganesan B, Weimer BC. Amino Acid Catabolism and Its Relationship to Cheese Flavor Outcomes. In: Cheese: Chemistry, Physics and Microbiology. Vol. 1. McSweeney PLH, Fox PF, Cotter PD, Everett DW, editors. Fourth Edi. Elsevier Ltd; 2017. p. 483–516. http://dx.doi.org/10.1016/B978-0-12-417012-4/00019-3. doi:10.1016/B978-0-12-417012-4.00019-3

57. Moio L, Piombino P, Addeo F. Odour-impact compounds of Gorgonzola cheese. Journal of Dairy Research. 2000;67(2):273–285. https://doi.org/10.1017/S0022029900004106. doi:10.1017/S0022029900004106

58. Spinnler H-E. Rôle des lipides dans la perception olfactive des produits laitiers. Sciences des Aliments. 2011;30:103–120.

59. Anderson DF, Day EA. Quantitation, Evaluation, and Effect of Certain Microorganisms on Flavor Components of Blue Cheese. Journal of Agricultural and Food Chemistry. 1966;14(3):241–245. https://doi.org/10.1021/jf60145a012. doi:10.1021/jf60145a012

60. Kinderlerer JL. Fungal strategies for detoxification of medium chain fatty acids. International Biodeterioration and Biodegradation. 1993;32(1–3):213–224. https://doi.org/10.1016/0964-8305(93)90053-5. doi:10.1016/0964-8305(93)90053-5

61. Mason AB, Dufour JP. Alcohol acetyltransferases and the significance of ester synthesis in yeast. Yeast. 2000;16(14):1287–1298. https://doi.org/10.1002/1097-0061(200010)16:14%3C1287::AID-YEA613%3E3.0.CO;2-I. doi:10.1002/1097-0061(200010)16:14<1287::AID-YEA613>3.0.CO;2-I

62. Legras JL, Galeote V, Bigey F, Camarasa C, Marsit S, Nidelet T, Sanchez I, Couloux A, Guy J, Franco-Duarte R, et al. Adaptation of s. Cerevisiae to fermented food environments reveals remarkable genome plasticity and the footprints of domestication. Molecular Biology and Evolution. 2018;35(7):1712–1727. https://doi.org/10.1093/molbev/msy066. doi:10.1093/molbev/msy066

63. Escudié F, Auer L, Bernard M, Mariadassou M, Cauquil L, Vidal K, Maman S, Hernandez-Raquet G, Combes S, Pascal G. FROGS: Find, Rapidly, OTUs with Galaxy Solution. Bioinformatics. 2018;34(8):1287–1294. https://doi.org/10.1093/bioinformatics/btx791. doi:10.1093/bioinformatics/btx791

64. Gomri G. Buffers in the range of pH 6.5 to 9.6. Proceedings of Society for Experimental Biology and Medicine. 1946;6(1):33–34. https://doi.org/10.3181/00379727-62-15361. doi:10.3181/00379727-62-15361

65. Nelson FE. The effect of the new standard milk agar on the plate count of dairy products. Journal of Bacteriology. 1940;39(3):263–272.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC374570/

66. Terzaghi BE, Sandine WE. Improved medium for lactic streptococci and their bacteriophages. Applied Microbiology. 1975;29(6):807–813. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=187084&tool=pmcentrez&rendertype=abstract. doi:10.1.1.88.2825

67. Mayeux J V, Sandine WE, Elliker PR. A selective medium for detecting Leuconostoc organism in mixed-strain starter cultures. Journal of Dairy Science. 1962;45:655–656.

68. Mossel DAA, Kleynen Semmeling AMC, Vincentie HM, Beerens H, Catsaras M. Oxytetracycline Glucose Yeast Extract Agar for Selective Enumeration of Moulds and Yeasts in Foods and Clinical Material. Journal of Applied Bacteriology. 1970;33(3):454–457. https://doi.org/10.1111/j.1365-2672.1970.tb02220.x. doi:10.1111/j.1365-2672.1970.tb02220.x

69. Denis C, Gueguen M, Henry E, Levert D. New media for the numeration of cheese surface bacteria. Le Lait. 2001;81(3):365–379. https://doi.org/10.1051/lait:2001138%0A. doi:10.1051/lait:2001138

70. Mossel DAA, Eelderink I, Koopmans M, Van Rossem F. Optimisation of a MacConkey-type medium for the enumeration of Enterobacteriaceae. Laboratory Practices. 1978;27:1049–1050.

71. Duval P, Chatelard-Chauvin C, Gayard C, Rifa E, Bouchard P, Hulin S, Picque D, Montel M-C. Microbial dynamics in industrial blue veined cheeses in different packaging. International Dairy Journal. 2016;56:198–207. https://doi.org/10.1016/j.idairyj.2016.01.024. doi:10.1016/j.idairyj.2016.01.024

72. Liu Z, Lozupone C, Hamady M, Bushman FD, Knight R. Short pyrosequencing reads suffice for accurate microbial community analysis. Nucleic Acids Research. 2007;35(18):e120. https://doi.org/10.1093/nar/gkm541. doi:10.1093/nar/gkm541

73. Andersson AF, Lindberg M, Jakobsson H, Bäckhed F, Nyrén P, Engstrand L. Comparative analysis of human gut microbiota by barcoded pyrosequencing. PLoS ONE. 2008;3(7):e2836. doi:10.1371/journal.pone.0022109

74. Lazuka A, Auer L, Bozonnet S, Morgavi DP, O'Donohue M, Hernandez-Raquet G. Efficient anaerobic transformation of raw wheat straw by a robust cow rumen-derived microbial consortium. Bioresource Technology. 2015;196:241–249. https://doi.org/10.1016/j.biortech.2015.07.084. doi:10.1016/j.biortech.2015.07.084

75. Rognes T, Flouri T, Nichols B, Quince C, Mahé F. VSEARCH: a versatile open source tool for metagenomics. PeerJ. 2016;4:e2584. https://doi.org/10.7717/peerj.2584. doi:10.7717/peerj.2584

76. Mahé F, Rognes T, Quince C, de Vargas C, Dunthorn M. Swarm: robust and fast clustering method for amplicon-based studies. PeerJ. 2014;2:e593. https://doi.org/10.7717/peerj.593. doi:10.7717/peerj.593

77. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011;27(16):2194–2200. https://doi.org/10.1093/bioinformatics/btr381. doi:10.1093/bioinformatics/btr381

78. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nature Methods. 2012;9(7):671–675. http://dx.doi.org/10.1038/nmeth.2089. doi:10.1038/nmeth.2089

79. Cheese and processed cheese: determination of total solids content. International standards 4a. International Dairy Federation, Brussels, Belgium. 1982. https://www.iso.org/standard/35249.html. ISO 5534:2004 [IDF 4:2004]

80. Cheese and processed cheese: determination of chloride content potentiometric titration method. International standards 4a. International Dairy Federation, Brussels, Belgium. 1988. https://www.iso.org/standard/43922.html. ISO 5943:2006 [IDF 88:2006]

81. Determination of water activity NF ISO 21807. International Standardisation Organisation. 2004. https://www.iso.org/standard/34728.html. ISO 21807:2004

82. Pham VT, Lacroix C, Braegger CP, Chassard C. Early colonization of functional groups of microbes in the infant gut. Environmental microbiology. 2016;18(7):2246–2258. https://doi.org/10.1111/1462-2920.13316. doi:10.1111/1462-2920.13316

83. Le Boucher C, Courant F, Jeanson S, Chereau S, Maillard M, Royer AL, Thierry A, Dervilly-Pinel G, Le Bizec B, Lortal S. First mass spectrometry metabolic fingerprinting of bacterial metabolism in a model cheese. Food Chemistry. 2013;141(2):1032–1040. http://dx.doi.org/10.1016/j.foodchem.2013.03.094. doi:10.1016/j.foodchem.2013.03.094

84. Hébert A, Forquin-Gomez MP, Roux A, Aubert J, Junot C, Heilier JF, Landaud S, Bonnarmeb P, Beckerich JM. New insights into sulfur metabolism in yeasts as revealed by studies of Yarrowia lipolytica. Applied and Environmental Microbiology. 2013;79(4):1200–1211. http://dx.doi.org/10.1128/AEM.03259-12. doi:10.1128/AEM.03259-12

85. Gatto L, Lilley KS. Msnbase-an R/Bioconductor package for isobaric tagged mass spectrometry data visualization, processing and quantitation. Bioinformatics. 2012;28(2):288–289. https://doi.org/10.1093/bioinformatics/btr645. doi:10.1093/bioinformatics/btr645

86. Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G. XCMS: Processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. Analytical Chemistry. 2006;78(3):779–787. https://doi.org/10.1021/ac051437y. doi:10.1021/ac051437y

87. Kuhl C, Tautenhahn R, Böttcher C, Larson TR, Neumann S. CAMERA: An integrated strategy for compound spectra extraction and annotation of liquid chromatography/mass spectrometry data sets. Analytical Chemistry. 2012;84(1):283–289. https://doi.org/10.1021/ac202450g. doi:10.1021/ac202450g

88. Bates D, Mächloer M, Bolker BM, Walker SC. Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software. 2015;67(1):1–48.

https://www.jstatsoft.org/article/view/v067i01/0. doi:10.18637/jss.v067.i01

89. Fox J, Weisberg S. An {R} Companion to Applied Regression. Third. Oaks T, editor. Sage; 2019. https://socialsciences.mcmaster.ca/jfox/Books/Companion/. ISBN: 9781544336473.

90. van den Boogaart KG, Tolosana-Delgado R, Bren M. compositions: Compositional Data Analysis. Computers & Geosciences. 2018;34(4):320–338. (R package version 1.40-2.). https://doi.org/10.1016/j.cageo.2006.11.017. doi:10.1016/j.cageo.2006.11.017.

91. Bjørn-Helge M, Wehrens R, Hovde Liland K. pls: Partial Least Squares and Principal Component Regression. 2018. R package version 2.7-0. https://cran.r-project.org/package=pls

92. Searle SR, Speed FM, Milliken GA. Population marginal means in the linear model: An alternative to least squares means. American Statistician. 1980;34(4):216–221. http://dx.doi.org/10.1080/00031305.1980.10483031. doi:10.1080/00031305.1980.10483031

93. Oksanen J, Blanchet GF, Firendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, et al. vegan: Community Ecology Package. 2019. https://cran.r-project.org/package=vegan

94. Villanueva RAM, Chen ZJ, Wickham H. ggplot2: Elegant Graphics for Data Analysis Using the Grammar of Graphics. 2nd Edition. Springer-Verlag New York; 2016. https://doi.org/10.1080/15366367.2019.1565254. doi:10.1080/15366367.2019.1565254

95. Wilke CO. cowplot: Streamlined Plot Theme and Plot Annotations for "ggplot2". 2019. https://cran.r-project.org/web/packages/cowplot/index.html

96. Auguie B. gridExtra: Miscellaneous Functions for "Grid" Graphics. 2017. https://cran.r-project.org/web/packages/gridExtra/index.html

97. Wickham H. The split-apply-combine strategy for data analysis. Journal of Statistical Software. 2011;40(1):1–29. https://www.jstatsoft.org/article/view/v040i01. doi:10.18637/jss.v040.i01

98. Urbanek S, Horner J. Cairo: R Graphics Device using Cairo Graphics Library for Creating High-Quality Bitmap (PNG, JPEG, TIFF), Vector (PDF, SVG, PostScript) and Display (X11 and Win32) Output. 2019. https://cran.r-project.org/web/packages/Cairo/index.html

99. Snow G. TeachingDemos: Demonstrations for Teaching and Learning. 2016. https://cran.r-project.org/web/packages/TeachingDemos/index.html

## Figure legends

**Figure 1:** Experimental cheese making. (A) Experimental design for cheesemaking, using three different strains of each of the four *Penicillium roqueforti* populations (non-Roquefort in blue, Roquefort in purple, silage / food spoiler in orange, lumber / food spoiler in green). Each assay (February, March, April) includes one strain from each of the four populations with three production replicates at different times, with different batches of raw 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 maturation days.

**Figure 2:** Mean percentage of blue area per cheese slice at 20, 90 and 180 maturation days generated by the four *Penicillium roqueforti* populations (non-Roquefort in blue, Roquefort 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 proportion of blue area per cheese slice at 180 maturation days among the four *Penicillium roqueforti* populations (non-Roquefort in blue, Roquefort in purple, silage/food spoiler in orange and lumber/food spoiler in green). Contrast and brightness have been standardized and the edges cropped on the cheese slices.

**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 by inoculating the four *Penicillium roqueforti* populations (lumber/food spoiler in green, non-Roquefort in blue, Roquefort 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 by inoculating the four *Penicillium roqueforti* populations (lumber/food spoiler in green, non-Roquefort in blue, Roquefort in purple and silage/food spoiler in orange).

**Figure 6**: Volatile compound family production (integrated peak areas from chromatograms in arbitrary units) in 90-day cheeses inoculated with the four *Penicillium roqueforti* populations (non-Roquefort in blue, Roquefort in purple, silage/food spoiler in orange and lumber/food spoiler in green). The areas of each family are the sum of the integrated areas of associated compounds. 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 colour of the titles indicates the affiliation of the compounds to their families as in Supplementary figure 5.

**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 in blue, Roquefort in purple, silage/food spoiler in orange). Error bars represent standard deviations across cheese replicates.

**Figure legends of supplementary material**

**Figure S1A**: Abundance (in log colony-forming unit/g) of the eight types of microorganisms monitored along the different stages of cheese maturation (i.e. crude raw milk, 9, 20, 90 and 180 days), for each of the four *Penicillium roqueforti* populations inoculated in the cheeses

(non-Roquefort in blue, Roquefort in purple, silage/food spoiler in orange and lumber/food spoiler in green). Error bars represent standard deviations across assays.

**Figure S1B**: Relative abundance of the six main bacterial operational taxonomic units in cheeses made with the four *Penicillium roqueforti* populations (non-Roquefort in blue, Roquefort in purple, silage/food spoiler in orange and lumber/food spoiler in green) in each assay (February in light grey, March in grey and April in dark grey) in 9-day (45° striated) and 20-day (135° striated) cheeses.

**Figure S1C**: Mean Shannon index (bacterial genus diversity) of the operational taxonomic units detected by metabarcoding in 9-day cheeses (left) and 20-day cheeses (right) made with the four *Penicillium roqueforti* populations (lumber/food spoiler in green, non-Roquefort in blue, Roquefort in purple and silage/food spoiler in orange)

**Figure S1D**: Opposite of mean Simpson index (1-Simpson index; bacterial genus diversity) of the operational taxonomic units detected by metabarcoding in 9-day cheeses (left) and 20-day cheeses (right) made with the four *Penicillium roqueforti* populations (lumber/food spoiler in green, non-Roquefort in blue, Roquefort in purple and silage/food spoiler in orange)

**Figure S2A**: Discrimination between 90-day cheeses made by inoculating cheese (blue) and non-cheese (green) *Penicillium roqueforti* populations (left), or Roquefort (purple) and non-Roquefort (blue) *P. roqueforti* populations (right), based on the quantity of 23 identified amino acids using an orthogonal signal-corrected partial least squared (PLS) discriminant analysis. Vertical and horizontal axes represent PLS1 and PLS 2 scores and grey arrows

represent the relative contribution of loadings of signals significantly discriminating the group considered from a t-test on jackknife resampling.

**Figure S2B**: Quantities of molecular classes detected in cheeses: mean of integrated peak area from chromatograms in arbitrary units (bars, left axis) and cumulative percentage (line with dots, right axis) of aqueous extracts across all 90-day cheeses.

**Figure S3**: Sums of 8,472 non-targeted organic signal peak areas, weighted by their mass-to-charge ratios ("m/z"), obtained in positive ionization mode in 90-day cheeses made with the four *Penicillium roqueforti* populations (lumber/food spoiler in green, non-Roquefort in blue, Roquefort in purple and silage/food spoiler in orange).

**Figure S4**: Non-protein nitrogen at 20, 90 and 180 maturation days (left) and water activity at 90 and 180 maturation days (right) between cheeses made with different *Penicillium roqueforti* populations (non-Roquefort in blue, Roquefort in purple, silage/food spoiler in orange and lumber/food spoiler in green). Error bars indicate 95% confidence intervals.

**Figure S5**: Discrimination among 90-day cheeses inoculated with the four *Penicillium roqueforti* populations (non-Roquefort in blue, Roquefort in purple, silage/food spoiler in yellow and lumber/food spoiler in green) based on the quantities of 41 volatile compounds using an orthogonal signal-corrected partial least squared (PLS) discriminant analysis. Vertical and horizontal axes represent PLS1 and PLS2 variances and arrows represent the relative contribution of compound odor loadings significantly discriminating the group considered (according to www.thegoodscentscompany.com) from a t-test on jackknife resampling. The odour colours indicate the families in Figure 6 which the associated

compounds belong to.

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

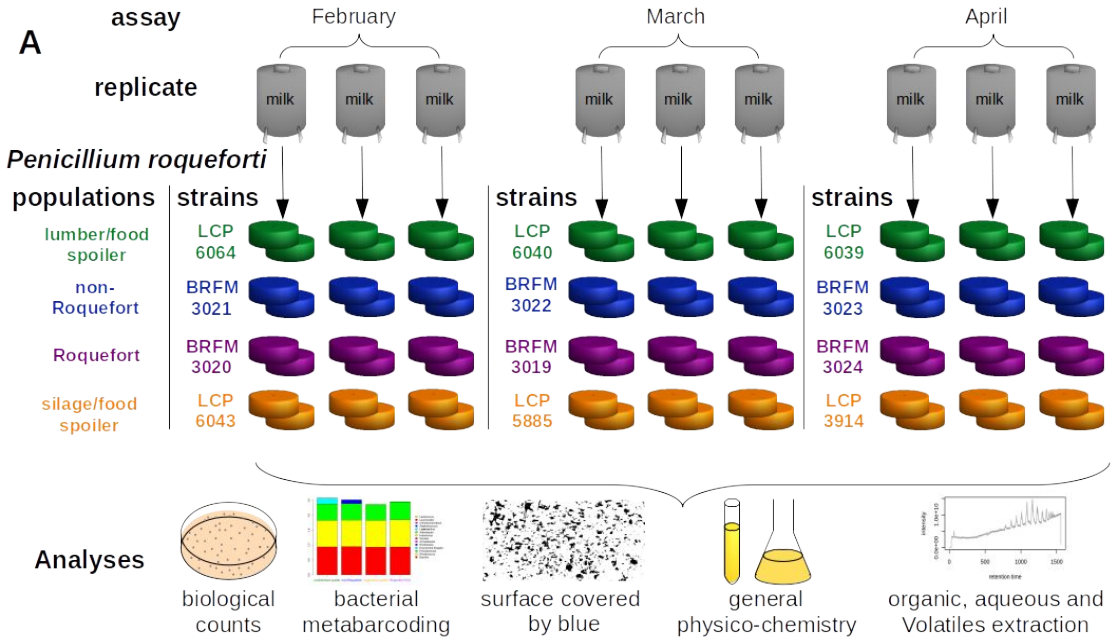

Figure 1: Experimental cheesemaking (A) Experimental design for cheesemaking, using three different strains of each of the four *Penicillium roqueforti* populations (non-Roquefort in blue, Roquefort in purple, silage / food spoiler in orange, lumber / food spoiler in green). Each assay (February, March, April) includes one strain from each of the four populations with three production replicates at different times, with different batches of raw 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 maturation days.

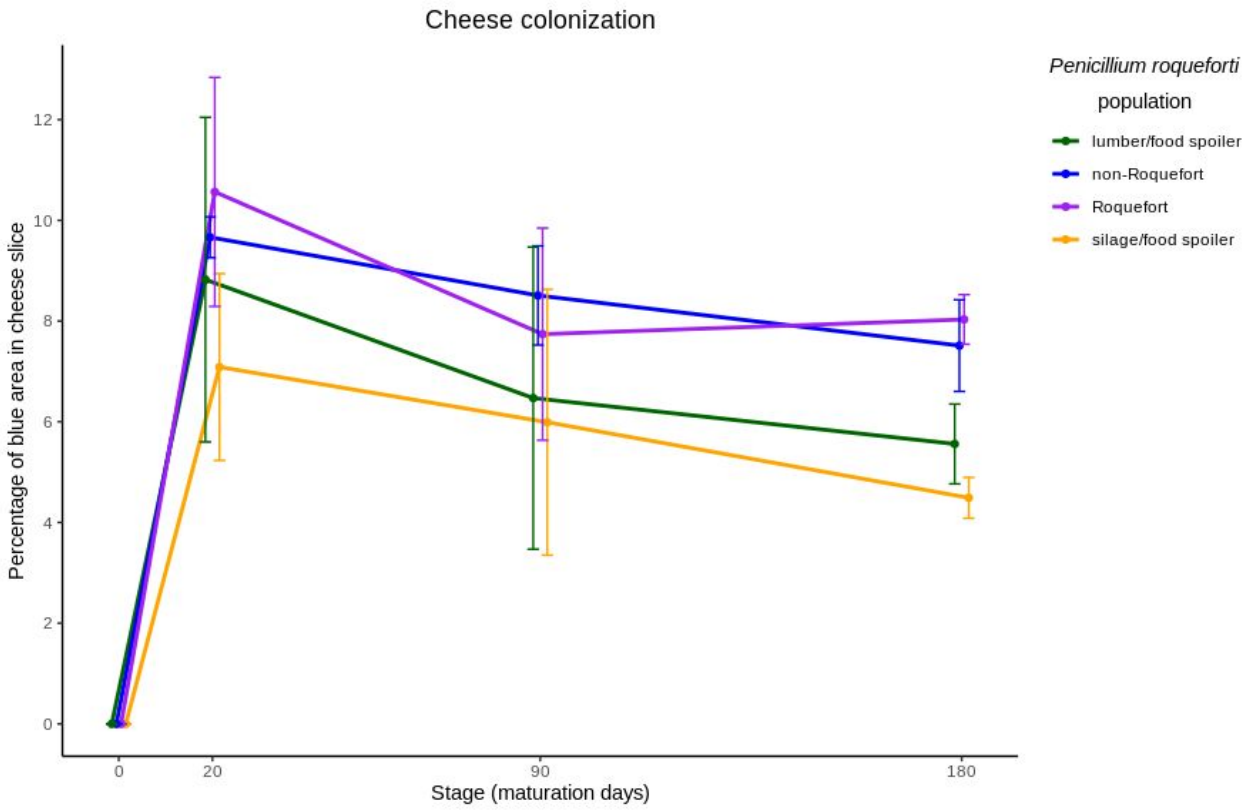


Figure 2: Mean percentage of blue area per cheese slice at 20, 90 and 180 maturation days generated by the four *Penicillium roqueforti* populations (non-Roquefort in blue, Roquefort in purple, silage/food spoiler in orange and lumber/food spoiler in green). Error bars indicate 95% confidence intervals.

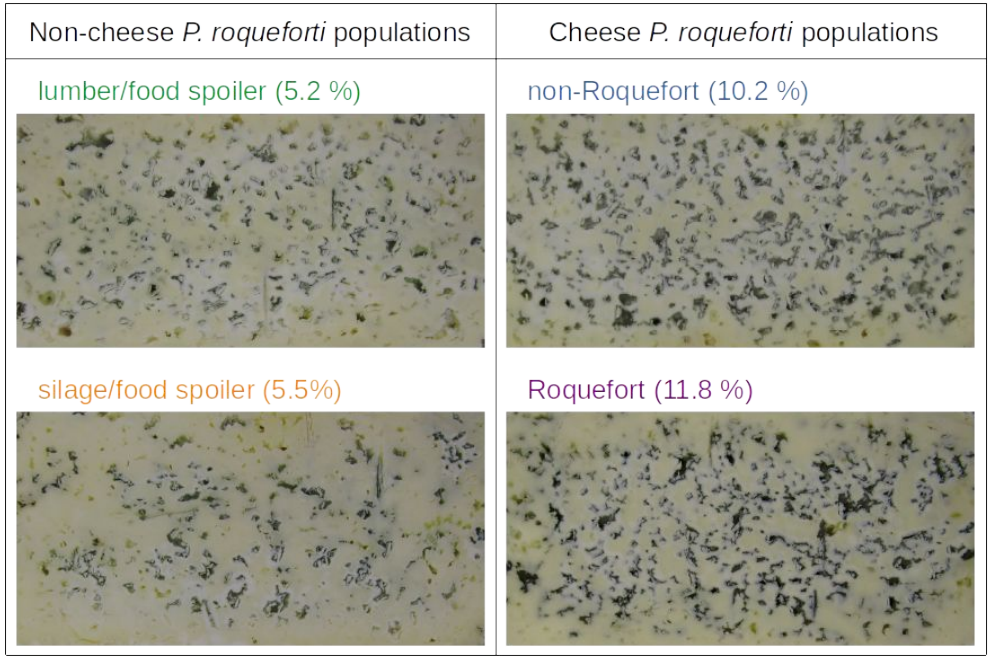


Figure 3: Illustration of the differences in the mean percentage of blue area per cheese slice at 180 maturation days among the four *Penicillium roqueforti* populations (non-Roquefort in blue, Roquefort in purple, silage/food spoiler in orange and lumber/food spoiler in green). Contrast and brightness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

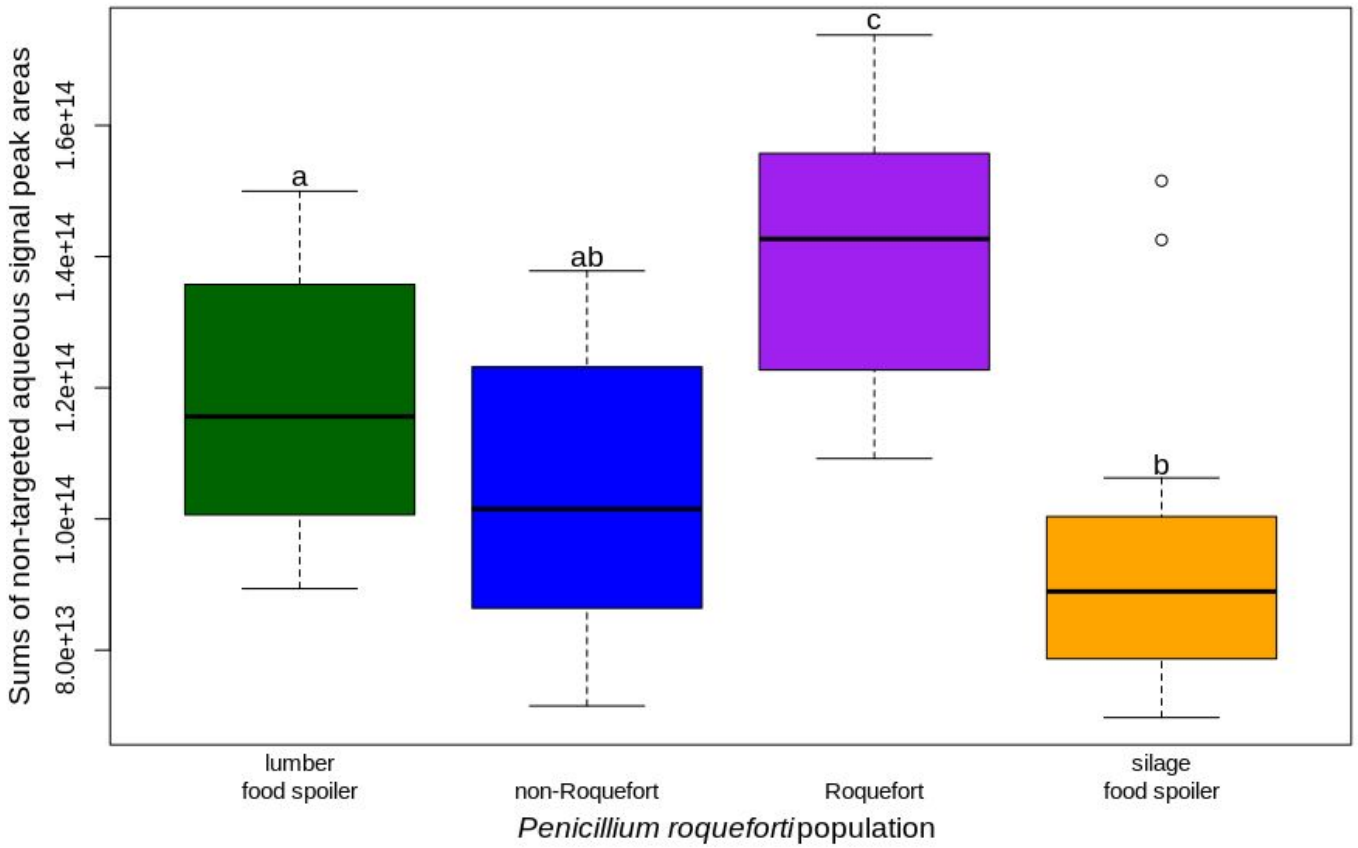


Figure 4: Sums of 3,864 non-targeted aqueous signal peak areas, weighted by their mass-to-charge ratios ("m/z"), obtained in positive ionization mode measured in 90-day cheeses made by inoculating the four *Penicillium roqueforti* populations (lumber/food spoiler in green, non-Roquefort in blue, Roquefort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

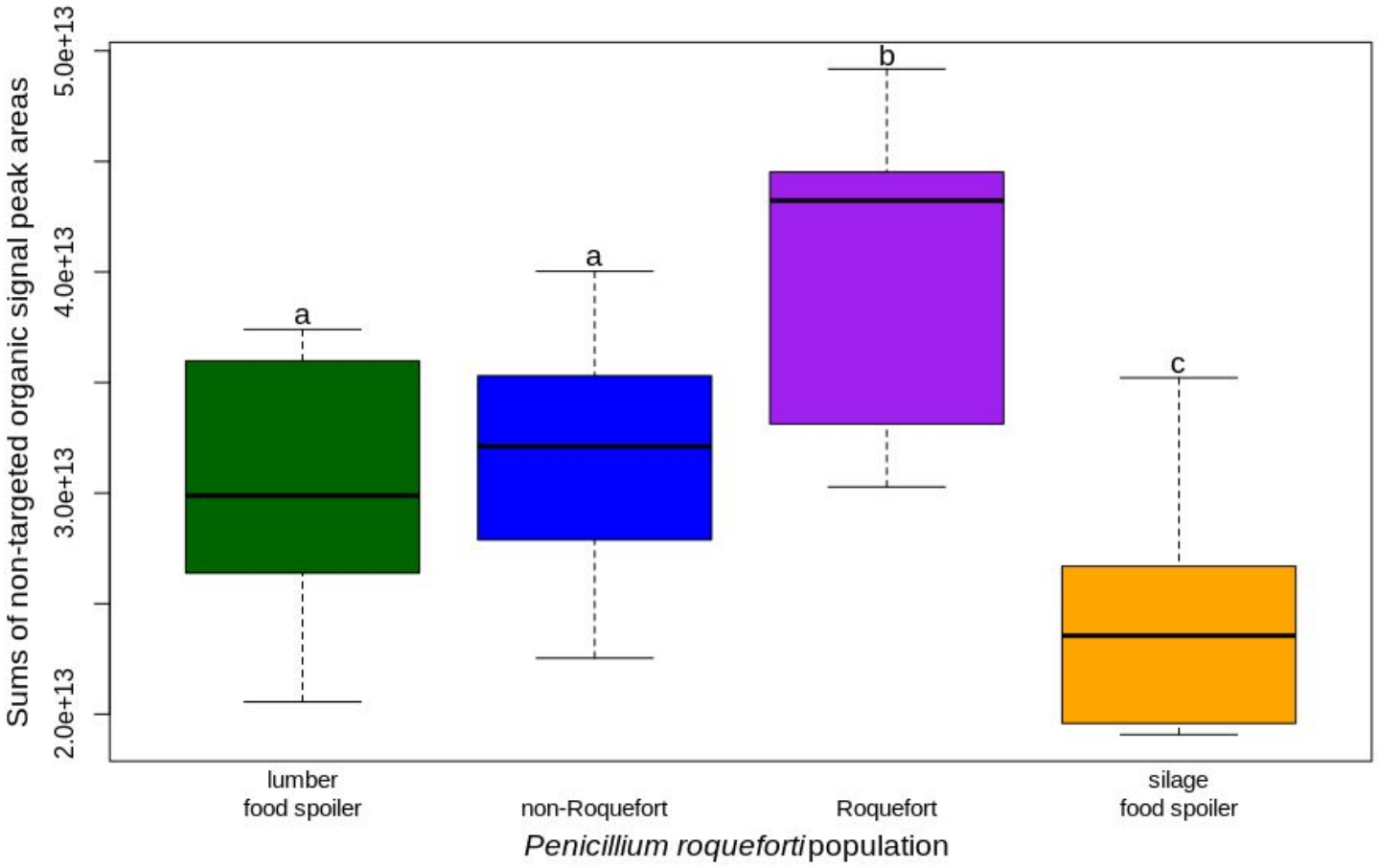


Figure 5: Sums of 3,094 non-targeted organic signal peak areas, weighted by their mass-to-charge ratios ("m/z"), obtained in negative ionization mode in 90-day cheeses made by inoculating the four *Penicillium roqueforti* populations (lumber/food spoiler in green, non-Roquefort in blue, Roquefort in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

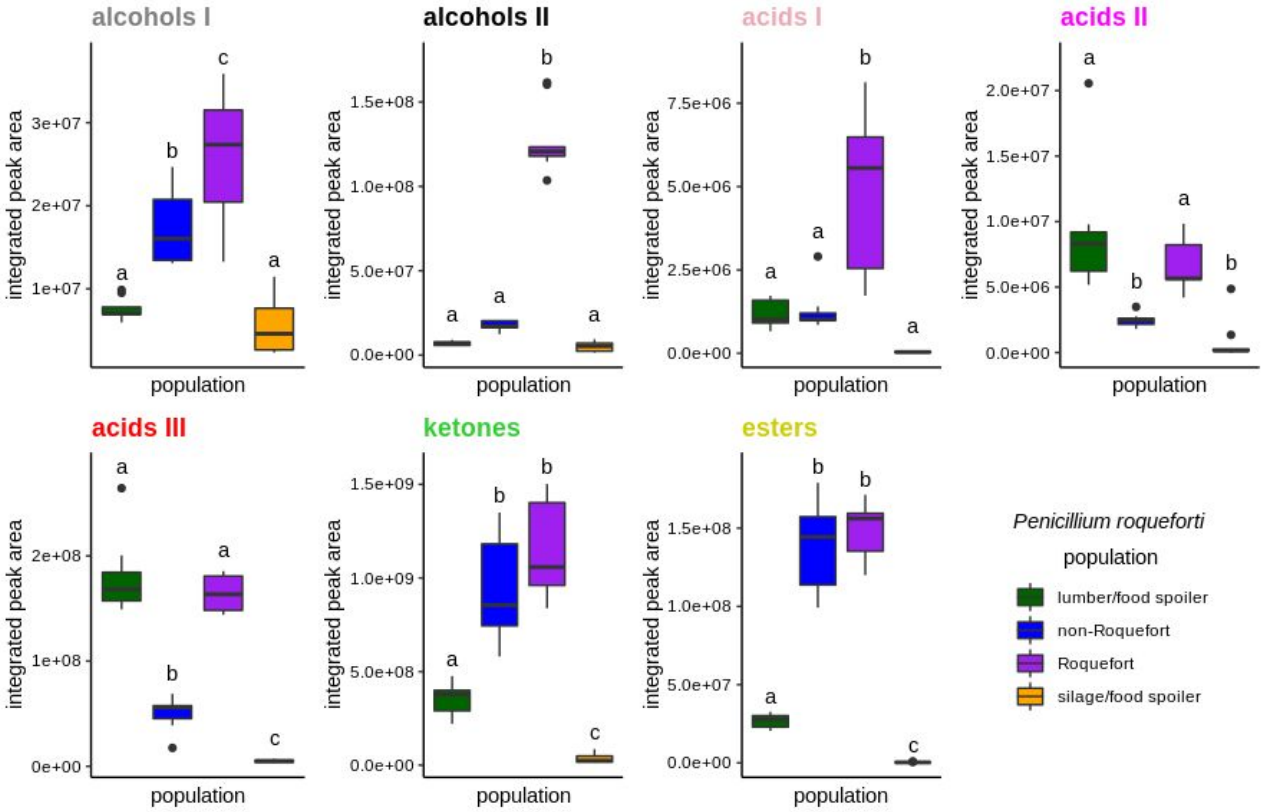


Figure 6: Volatile compound family production (integrated peak areas from chromatograms in arbitrary units) in 90-day cheeses inoculated with the four *Penicillium roqueforti* populations (non-Roquefort in blue, Roquefort in purple, silage/food spoiler in orange and lumber/food spoiler in green). The areas of each family are the sum of the integrated areas of associated compounds. Alcohols I and II are derived from proteolysis and lipolysis, respectively. Acids I, II and III are derived from proteolysis, glycolysis and lipolysis, respectively (see details in Supp. "compouds_precursors"). The colour of the titles indicates the affiliation of the compounds to their families as in Supplementary figure 5.

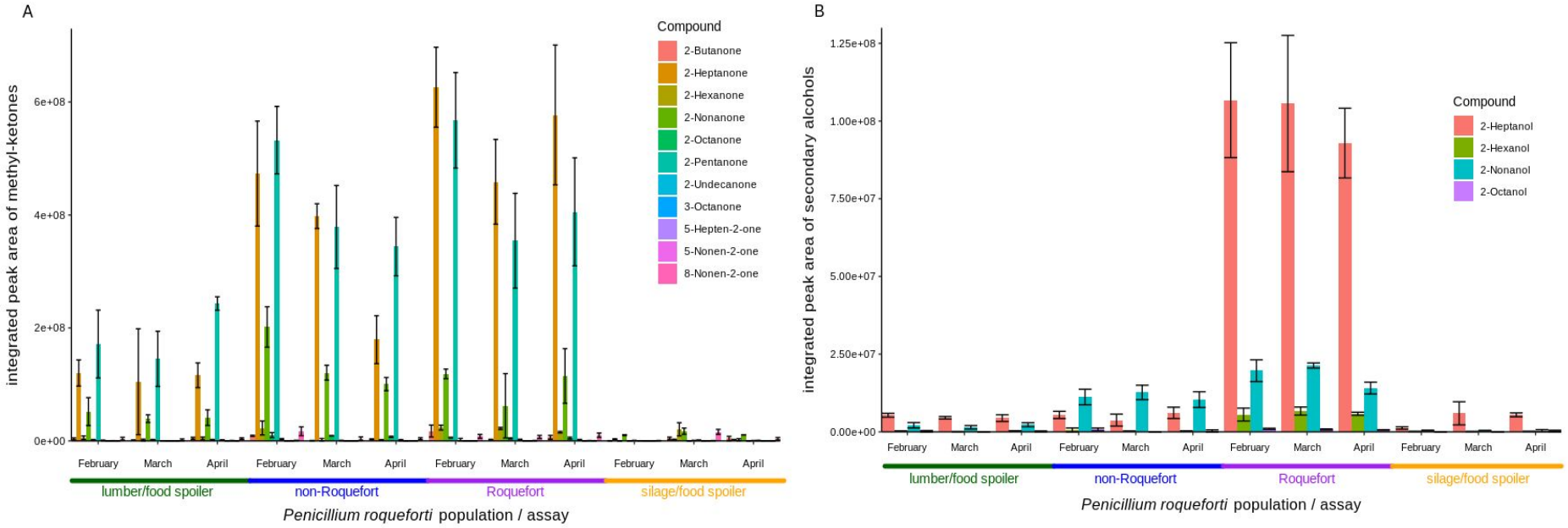


Figure 7: Integrated peak area (from chromatograms in arbitrary units) of methyl-ketones (A) and secondary alcohols (B) for each assay (February, March, April) for the three strains of each *Penicillium roqueforti* population (lumber/food spoiler in green, non-Roquefort in blue, Roquefort in purple,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