Domestication of different varieties in the cheese-making fungus *Geotrichum candidum*

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Abstract

Domestication is an excellent model for studying adaptation processes, constituting recent adaptation and diversification, as well as degeneration of unused functions. *Geotrichum candidum* is a fungus used for cheese-making and is also found in other environments such as soil and plants. By analyzing whole genome data from 98 strains, we found that all strains isolated from cheese formed a monophyletic clade. Within the cheese clade, we identified three differentiated populations and we detected footprints of recombination and hybridization. The genetic diversity in the cheese clade was the lowest but remained high compared to other domesticated fungi, indicating milder bottlenecks. Commercial starter strains were scattered across the cheese clade, not constituting a single clonal lineage. The cheese clade was phenotypically differentiated from other populations, with a slower growth on all media even cheese, a prominent production of attractive cheese flavors and a lower proteolytic activity. Furthermore, cheese populations displayed contrasted phenotypes, with one cheese population producing a typical blue cheese flavor and

another one displaying denser and fluffier colonies, excluding more efficiently cheese spoiler fungi. Cheese populations lost two beta lactamase-like genes, involved in xenobiotic clearance, and displayed transposable element expansion, likely due to relaxed selection. Our findings suggest the existence of genuine domestication in *G. candidum*, which led to diversification into three varieties with contrasted phenotypes. Some of the traits acquired by cheese strains indicate convergence with other, distantly related fungi used for cheese maturation.

Introduction

Understanding how populations adapt to their environment is a key question in evolutionary biology. Domestication is an excellent model for studying adaptation processes, as it involves recent adaptation events under strong selection on known traits and rapid diversification. Numerous studies have documented the specific traits acquired in domesticated animals (dog, horse, pig, cow) (Diamond, 2002; Frantz et al., 2015; Petersen et al., 2013; Warmuth et al., 2011) and plants (cabbage, corn, wheat) (Hufford et al., 2012; Mabry et al., 2021; Peng et al., 2011), as well as their genetic differentiation from wild populations and their adaptive genomic changes. For example, domesticated animals (dog, horse and cattle) have been selected for coat color, size, rapidity and docility (Liu et al., 2022; Plassais et al., 2022; Qanbari et al., 2014). On the other hand, functions essential in wild environments but unused in anthropic environments have often degenerated due to relaxed selection, for example, reductions in defense mechanisms in plants (Cornille et al., 2014; Hufford et al., 2012). Domestication also often leads to strong reduction in genetic diversity due to bottlenecks in animals (e.g. dog) (Marsden et al., 2016) and annual plants (e.g. rice) (Zhu et al., 2007).

Humans have domesticated several fungi for the fermentation of foods (e.g. beer, bread, wine, dry-cured meat and cheese), to produce secondary metabolites used in pharmaceutics (e.g. penicillin), or for their nutritional and gustatory values (e.g. button and shiitake mushrooms) (Steensels et al., 2021). Fungi are excellent models for studying evolution and adaptation in eukaryotes, given their many experimental assets (Gladieux et al., 2014): fungi have relatively small genomes, many are easy to culture in laboratory conditions, and spores can survive long periods in the freezer.

However, despite their economic and industrial importance, and their utility as biological models for studying adaptive divergence, the fungi used by humans have yet been little studied. An exception is the budding yeast *Saccharomyces cerevisiae* used in the production of beer, wine and bread (Fay and Benavides, 2005; Gallone et al., 2016; Legras et al., 2018; Libkind et al., 2011; Liti et al., 2009; Peter et al., 2018), and to a lesser extent the filamentous fungus *Aspergillus oryzae* used to ferment soy and rice products in Asia (Galagan et al., 2005; Gibbons et al., 2012; Machida et al., 2005) and the *Penicillium* species used for cheese ripening, e.g. *P. camemberti* for soft cheeses, (Ropars et al., 2020) and *P. roqueforti* for blue cheeses (Cheeseman et al., 2014; Dumas et al., 2020; Ropars et al., 2015). Phenotypic traits beneficial for food production have been acquired in domesticated fungi compared to wild populations; domestication having led to *P. camemberti* occurred in several steps, with the successive differentiation of several lineages with decreasing diversity and increasing beneficial traits for cheese maturation, from the wild *P. fuscoglaucum*, to *P. biforme* and then the two clonal *P. camemberti* varieties, *caseifulvum* and *camemberti* (Ropars et al., 2020). Domesticated populations of fermented food microorganisms can for example better assimilate the carbon sources present in the anthropic environment, *e.g.* lactose for *Penicillium* cheese fungi (Ropars et al., 2015) and maltose for *S. cerevisiae* sourdough strains (Bigey et al., 2021). Furthermore, volatile organic compounds crucial for cheese flavor are more appealing in cheese populations of *P. roqueforti* compared to wild populations (Caron et al., 2021).

The genomic processes involved in adaptation to human-made environments in domesticated fungi include gene family expansion for specific metabolism pathways, gene gain, hybridization, introgression and horizontal gene transfer (Almeida et al., 2014; Barros Lopes et al., 2002; Borneman et al., 2016; Cheeseman et al., 2014; Gallone et al., 2016; Libkind et al., 2011; Machida et al., 2005; Naumova et al., 2005; Novo et al., 2009; Ropars et al., 2015). Domesticated fungi have also lost parts of genes no longer useful in the food environment; for example a cheese *P. roqueforti* population and *P. camemberti* var. *caseifulvum* are no longer able to produce some of their toxins due to deletions in the corresponding genes (Gillot et al., 2017; Ropars et al., 2020). Such losses are likely due to relaxed selection in terms of competition ability in cheese, where desired fungi are often inoculated in large quantities compared to possible competitors. Bottlenecks and degeneration have also been

documented in domesticated fungi, with reduced fertility and genetic diversity in the cheese fungi *P. roqueforti* and *P. camemberti* (Dumas et al., 2020; Ropars et al., 2020).

Geotrichum candidum (teleomorph *Galactomyces candidus*) is a ubiquitous dimorphic fungus (i.e., able to grow as a yeast or a mycelial form), commonly used for cheese-making, but also thriving in other environments such as soil, plants and fruits. *Geotrichum candidum* is naturally present in raw milk and is also often added as a starter culture for the production of semi-hard, mold-ripened, smeared soft cheeses, fresh goat and ewe cheeses. Analyses based on genetic markers have revealed genetic differentiation between cheese and wild strains (Alper et al., 2013; Jacques et al., 2017; Perkins et al., 2020; Tinsley et al., 2022). Phenotypic diversity within *G. candidum* has been reported in terms of carbon and nitrogen assimilation, lipolysis and proteolysis (Boutrou and Gueguen, 2005; Perkins et al., 2020). However, it has not been tested whether *G. candidum* cheese populations have evolved specific traits that could be beneficial for cheese-making.

By analyzing the genomes of 98 strains isolated from different kinds of cheeses and other environments, we confirmed the genetic differentiation between cheese and wild strains. Within the cheese clade, we identified three differentiated cheese populations, including one with all goat cheese strains, as well as footprints of recombination and hybridization. The genetic diversity in the cheese clade was the lowest but remained high compared to other domesticated fungi indicating milder bottlenecks. Industrial strains were scattered within the cheese clade, some corresponding to hybrid strains. We found phenotypic differentiation between cheese and wild populations: cheese strains grew more slowly and had weaker proteolysis than wild strains on all tested media, even cheese, and some cheese populations had a denser growth. Cheese populations produced lower quantities of volatiles providing solvent type notes than wild populations, thereby producing in highest proportions the volatiles corresponding to attractive cheese flavors. One of the cheese populations was the most efficient at excluding common food spoilers; volatile compounds were able to inhibit alone the growth of some challengers in all *G. candidum* populations. We identified genomic regions of higher differentiation than the rest of the genome between wild and cheese populations that could be responsible for these differences. We also reveal the loss, in cheese populations, of

two of the three tandem beta lactamase-like genes present in other populations and involved in xenobiotic clearance; such a loss is likely due to relaxed selection in the cheese environment as cheese-making fungi are often inoculated in high quantities compared to possible competitors. Some transposable element families expanded in cheese populations, also likely due to relaxed selection. Altogether our findings suggest the existence of genuine domestication in *G. candidum,* resulting in both the genetic and phenotypic differentiation of cheese strains from their wild counterparts, and their convergence with other domesticated cheese fungi. We also found diversification within the cheese clade, with three genetic clusters with contrasted traits and levels of diversity.

Results and Discussion

Genetic differentiation between wild and cheese strains of *Geotrichum candidum*

We collected and sequenced the genomes of 88 *G. candidum* strains with Illumina technology and included in our analyses ten available genomes. Our dataset included 61 strains isolated from different kinds of cheeses (semi-hard, mold-ripened, smeared soft and fresh goat cheeses), 16 industrial strains used for cheese-making, seven strains from dairy products, four strains from other food substrates (e.g., sausage or vegetables) and 10 wild strains (e.g. from soil or plant) (Table S1). We identified 699,755 SNPs across the 98 strains by mapping against the CLIB 918 reference genome (cheese strain).

The maximum likelihood tree, principal component analysis (PCA) and neighbor-net (SplitsTree) analyses all identified the same three clades (Figure 1A), with one containing mostly wild strains (corresponding to the GeoC group identified previously based on genetic markers) (Perkins et al., 2020), one composed of strains of varying origins (i.e. dairy products and other environments, corresponding to the group previously named GeoB) and one containing mostly cheese and dairy strains (previously named GeoA). The larger sampling and the genome sequencing of the

present study further revealed genetic subdivision in the cheese clade, with three clearly differentiated populations and several admixed strains (Figure 1B).

We performed an admixture analysis by inferring *K* populations, *K* ranging from two to ten. At *K*=3, the cheese, mixed-origin and wild clades were separated (Figure S1). At *K*=5, the cheese clade was divided into three genetic clusters, corresponding to monophyletic groups in the maximum likelihood tree and well-separated genetic clusters in the PCA and the neighbor-net (Figure 1 B, D).

Some cheese strains could not be assigned to any genetic cluster with the admixture analysis and were placed on the PCA between the three well-delimited cheese genetic clusters (Figure 1C-D), suggesting that they resulted from admixture events. To test this hypothesis, we investigated whether these strains had mosaic genomes, with different genomic regions assigned to distinct clusters. We calculated pairwise identity between unassigned strains and the other strains, computing mean identity to the different genetic clusters along scaffolds using sliding windows. For all unassigned strains in the cheese clade, we observed shifts in identity values along scaffolds, confirming that these strains are the results of admixture between clusters (Figure S2). In contrast, the three unassigned strains outside of the cheese clade did not show changes in similarity level to the different clusters along their genome; these strains may belong to yet additional genetic clusters that could not be distinguished by the analyses because too few strains belonged to these clusters in the sampling (Figure 1B).

The second order rate of change in the likelihood (ΔK) peaked at *K*=6. However the additional population distinguished at *K*=6, compared to *K*=5, only encompassed two strains that were not that differentiated from others in the splistree (MUCL 14462 and CBS 9194; FIGURE 1B.b.). Therefore, we chose to set the number of populations to five, the *K* value at which the structure was the strongest and the clearest, with three cheese populations, several admixed cheese strains, a population of mixed origins and a wild population. We found no particular cheese type distribution among the three cheese populations, except that all strains from goat cheeses clustered in the Cheese_1 population. The wild clade was the most differentiated population from all other *G. candidum* populations with F_{ST} values above 0.70 (Table S2). Its differentiation level was similar to that found between the domesticated *P. camemberti* mold and its wild closest relative species, *P. fuscoglaucum* (F_{ST} = 0.83; d_{xy} = 6E-03), and much higher than the differentiation between the cheese Roquefort

population and the lumber/food spoiler population in *P. roqueforti* (F_{ST} = 0.27). The percentage of private SNPs in the five populations was also high (Table S3). F3 tests based on the number of shared sites (Table S4) supported the differentiation between these populations.

High nucleotide diversity within cheese populations and footprints of recombination

Each of the three cheese populations of *G. candidum* had reduced nucleotide diversities compared to wild and mixed-origin populations (Table S5), by at least a factor of two, suggesting that the cheese populations underwent bottlenecks. The Cheese_2 population showed the lowest genetic diversity, by a factor of four compared to the two other cheese populations (Table S5), being of the same order of magnitude as in the Roquefort *P. roqueforti* population (Dumas et al., 2020).

The Cheese_1 population had a nucleotide diversity similar to that in the domesticated cheese species *P. biforme,* whereas the Cheese_3 population was genetically as diverse as *P. fuscoglaucum,* the closest wild relative of the clonal lineage *P. camemberti.*

Geotrichum candidum is a heterothallic fungus, meaning that sexual reproduction can only occur between two haploid cells carrying different mating types. Two mating types have been described in *G. candidum* (Morel et al., 2015): MATA, encoded by a HMG box gene homolog to the MATA2 *Kluyveromyces lactis* allele, present in CLIB 918 (sequence id: HF558448.1), and MATB, encoded by an alphabox gene homolog to the MATα1 *S. cerevisiae* allele, present in the strain CBS 615.84 (sequence id: HF558449.1). In the Cheese_2 population, we found a significant departure from the 1:1 mating-type ratio expected under regular sexual reproduction, all the 12 strains carrying the MATB allele, suggesting that this population is at least partly clonal (Table S6). The absence of linkage disequilibrium decay with physical distance between two SNPs (Figure S3), together with the absence of reticulation in the neighbor-net (Figure 1), are consistent with a lack of recombination in the Cheese_2 population. However, pairwise homology index (PHI) tests, testing with permutations the null hypothesis of no recombination by looking at the genealogical association among adjacent sites, were significant in all the *G. candidum* populations (Table S7); this indicates that recombination did occur at least in a recent past in the Cheese_2

population. In contrast to the Cheese_2 population, we found both mating-type alleles in balanced proportions in both Cheese_1 and Cheese_3 populations (Table S6) and we observed sharp decays in linkage disequilibrium (LD) with genomic distance, although LD levels remained higher than in the mixed-origin and wild populations (Figure S3). We observed reticulations in the neighbor-net within and, to a lesser extent, between populations; only the Cheese_2 population lacked reticulations (Figure 1A).

As previously mentioned, the 16 commercial starter strains in our *G. candidum* dataset were scattered in the maximum likelihood tree (in yellow, Figure 1B.a.) and we detected above footprints of recombination in the Cheese_1 and Cheese_3 populations (Figure 1A, Figure S3); the situation in *G. candidum* is thus very different from the use of a single clonal lineage in commercial cheeses as in both *P. camemberti* and *P. roqueforti*. The bottlenecks in *G. candidum* have been milder, similar to those reported in *P. biforme* and the Roquefort *P. roqueforti* population (Dumas et al., 2020; Ropars et al., 2020). We nevertheless detected a few groups of clonemates, by the lack of branches in the trees and the presence of fewer than 90,000 SNPs between strains (Figure 1; Table S1, clonal group column). Since strains within these clonal lineages were isolated from different cheeses, it indicates that some lineages may be clonally cultivated for cheese-making; some of the commercial starter strains indeed clustered within these clonal groups ("$" symbol on Figure 1B). Within the cheese admixed strains, 19 out of 23 were part of clonal lineages, suggesting that hybrid lineages may have been selected for beneficial traits for cheese-making, as in other domesticated fungi (e.g. *S. pastorianus*; Gallone et al., 2018).

Copy number variation: loss of two tandem beta lactamase-like genes in the cheese populations and repeat expansion

Expansions of gene families involved in specific metabolism pathways, of transposable elements and loss of genes no longer required in the new environment can be involved in adaptation to new environments. For example, variations in gene copy number were associated with the adaptation of *S. cerevisiae* to beer making, with duplications of genes involved in maltose and maltotriose metabolism

specifically in domesticated beer strains (Gallone et al., 2016; Giannakou et al., 2020; Gonçalves et al., 2016). We therefore analyzed gene copy-number variation (CNV) to detect structural variants specific to either wild or cheese populations, using 500bp sliding windows and two reference genomes, belonging to the Cheese_3 and the wild populations, respectively (Table S8). Using the Cheese_3 reference, we found 61 CNV regions (mean length of 1515bp and 45 non-genic CNVs), encompassing in total 16 genes, half having predicted functions, none being obviously related to cheese adaptation (e.g. methylglyoxal reductase and tRNAs, Table S8). Using the wild genome reference, we found 132 CNV regions (mean length of 1664bp and 105 non genic CNVs), encompassing 29 genes (seven with unknown functions).

One of these regions, 20kb long, included only two genes, both matching the Pfam hidden markov model for beta-lactamases; these two genes (g5112 and g5113) were absent from all cheese populations, and were present in most wild strains (except one that had partially the region) and in four strains belonging to the mixed-origin population (Figure 2). The nucleotide identity between the two beta lactamase-like genes was 93%. A third beta lactamase-like gene (g5111) was found immediately next to this CNV region in all *G. candidum* strains, and displayed a nucleotide identity of 87% with the two other beta lactamase-like genes within the CNV. Surrounding these different genes, we found several Tc1/*mariner*, a LINE/Tad1 and other DNA transposons, that may have contributed to the beta-lactamase-like gene deletion (Figure 2). Fungal beta lactamase-like genes are known to contribute to hydrolysis of microbial and plant xenobiotics, and thus may be important in the wild environment to compete with other microorganisms (Gao et al., 2017). The cheese populations may have lost these two copies of the beta-lactamase genes due to relaxed selection; indeed, these functions may not be useful in the cheese environment if *G. candidum* is inoculated in high quantity compared to potential competitors.

De novo detection of repeats using the wild strain LMA-244 yielded a library containing 176 types of repeated elements (including 24 types of DNA transposons and 15 of retroelements and 3 rolling-circles). We identified 19 types of repeated elements present in at least one other *G. candidum* genome with five times more copies than in the LMA-244 wild strain (this threshold was set based on the fat tail of

the distribution; Figure S4). Among these 19 types of repeated elements, several DNA transposons of the Tc1/*mariner* repeat family showed a cheese-clade specific expansion (Table S9, Figure 3). Several unidentified repeat types also showed expansions in the cheese clade, alongside a milder expansion in the mixed origin clade. These transposable element expansions in the cheese clade could be due to relaxed selection because of bottlenecks and lack of recombination in the cheese clade.

Genomic footprints of adaptation: genomic islands of differentiation in cheese populations and genes under positive selection

We looked for the genomic regions with a greater differentiation or a lower genetic diversity than the genomic background when comparing each of the three cheese populations to the wild population, to detect footprints of divergent selection and recent selective sweeps, respectively. We scanned the whole genome using 7500bp non-overlapping windows and explored the windows with the 1% highest d_{xy} (high differentiation) or 5% lowest π (low diversity) values. Regions of high-differentiation appeared as outliers in the distribution of d_{xy} values, representing a small peak of high d_{xy} values (Figure S5), and were often located in non-genic or in low gene-density regions (Figure S6). We however detected 69 genes in the 64 windows of 1% highest d_{xy} values across the three populations, including 26 genes with predicted functions. Two of them encoded proteases, ,one of them being an ADAM metalloprotease which was the only enriched function in the high-differentiation regions when compared to the rest of the genome (Table S10). Proteases are important in cheese-making as the breakdown of milk caseins greatly contributes to cheese texture and decreases water activity by degrading proteins into molecules with free carboxyl and amino groups (McSweeney, 2004). *Geotrichum candidum* was shown to be prevalent during the amino-acid catabolism ripening step of Pelardon fresh cheese (Penland et al., 2021), indicating that *G. candidum* plays an important role in proteolysis in cheese-making.

In the 198 windows representing the pooled set of the 5% lowest π values in the three cheese populations, we detected 497 genes, 323 of which had predicted

functions. For this analysis, we excluded the windows for which the wild population also had a low diversity in order to exclusively isolate regions potentially impacted by recent selective sweeps. Among the 323 annotated genes, five were predicted to encode proteases or lipases. Although these functions were not enriched compared to the rest of the genome (Table S10), this could still represent footprints of selection on some of these individual genes. Lipases are key enzymes for cheese flavor as they enable the breakdown of milk fats through fatty acid production (Collins et al., 2003), and are thus crucial for cheese-making.

We searched for genes evolving under positive selection in terms of high rates of non-synonymous substitutions by performing four runs of McDonald and Kreitman (MK) tests (Table S11), i.e., comparing the mixed-origin population to each cheese population and to the cheese clade as a whole. We detected 25 different orthologous genes as evolving under positive selection in at least one cheese population (9 for Cheese_1, 18 for Cheese_2, two in Cheese_3 and one in all three cheese populations at once; TABLE S11). Among them, a metalloendopeptidase evolved under positive selection in all three cheese population, and it may play a role in casein degradation through cell lysis, as previously suggested in *G. candidum* and described in *D. hansenii* (Dugat-Bony et al., 2015; KUMURA et al., 2002). A Glucan 1,3-beta-glucosidase was also detected under positive selection in the Cheese_2 population; this enzyme could be involved in fungal inhibition through fungal cell degradation (Adams, 2004). The other genes under positive selection had either no predicted function or putative function that could not be related to cheese adaptation (Table S11).

Missing evidence of relaxed selection in cheese populations

Domesticated organisms often display degeneration in some traits compared to their sister wild populations, due to bottlenecks and unused functions (e.g. Fages et al., 2019). However, we did not detect any accumulation of nonsense or missense mutations in the cheese populations compared to silent mutations (Felsenstein, 1974; Table S12). This was the case even in the clonal Cheese_2 population, while degeneration can be expected to be particularly strong in clonally replicated populations as recombination allows more efficient selection. The absence of sexual reproduction in the Cheese_2 population may be too recent to observe degeneration

(Carpentier et al., 2022). We also investigated the presence of premature stop codons due to nonsense mutations in all genes with predicted functions in both CLIB 918 and LMA-244 reference genomes. Thirty-five genes contained a premature stop codon when mapped to the CLIB 918 reference (cheese_3 population) and 28 when mapped to the LMA-244 reference (wild population), with some fixed in at least one population (18 and 11 fixed stop-inducing SNPs when mapped to CLIB 918 and LMA-244, respectively). None of the genes with premature stop codons had predicted functions that could be detected as specific either to the wild or cheese environments (Figure S7).

Phenotypic differentiation between cheese and wild populations

Denser mycelial growth and/or faster proteolysis in cheese populations of *Geotrichum candidum*

Strains selected by humans are expected to display specific traits beneficial for cheese-making, such as faster growth in cheese at cave temperature or colonies of attractive aspect or color. For example, the *P. camemberti* strains used for soft cheese production were selected for their white and fluffy aspect, to make cheeses more attractive to consumers compared to the blue-grey crust produced by the *P. camemberti* ancestor (Pitt et al., 1986). In contrast, the ability to grow in harsh conditions may have been lost in cheese strains due to relaxed selection, as often reported for unused traits in human-made environments in domesticated organisms (Gallone et al., 2018; Price, 2002; Ropars et al., 2015).

We therefore measured colony radial growth of 31 strains from the five *G. candidum* populations on different agar media (cheese, rich and poor media) at different temperatures. The ANOVA showed significant effects on growth of temperature and population, but not of their interaction (Table S13, Figure S8). Wild populations grew faster than cheese populations on all media at all temperatures, with a more pronounced difference at 25°C. This may result from trade-offs with other traits, such as a fluffier mycelium, i.e. more vertical growth at the expense of less radial growth.

To test whether cheese populations had a denser mycelium or had become whiter and/or fluffier, we compared the opacity of populations on cheese agar at cave temperature (10°C), which integrates the brightness and fluffiness of a colony. The

Cheese_1 and Cheese_3 populations were not more opaque than wild populations (Figure 4B). The Cheese_2 population had a significantly higher opacity than all other *G. candidum* populations, except the mixed-origin population (post-hoc Tukey test in Table S13). This represents a convergence with *P. camemberti* var. *camemberti*, with independent evolution of similar phenotypes in two distantly related cheese fungi.

Lipolysis and proteolysis are crucial biochemical processes during cheese ripening, that influences flavor and texture of the final product; lipolysis and proteolysis contribute to energy and nutrient uptake, and they affect the production of volatile compounds, which are key flavor factors in cheeses (McSweeney, 2004). We therefore tested whether cheese populations of *G. candidum* performed faster lipolysis and proteolysis than wild populations. All populations of *G. candidum* had similar lipolysis rates. The wild and mixed-origin populations had degraded a significantly higher amount of proteins than the cheese populations and we did not detect any proteolysis in the Cheese_2 population in our experiment (Figure S9; Table S14).

No adaptation to high salt concentration or milk origin in cheese populations

Cheese is a salty medium, with the percentage of salt varying from 0.5g/100g for Emmental to 3g/100g for Roquefort. Salt is added on the surface of cheeses to prevent the growth of contaminants, and cheese populations of *G. candidum* may thus have adapted to high salt concentrations. Cheeses display a wide range of salt concentrations so we tested four cheese mediums: unsalted media, 1% salt as St Nectaire and cream cheeses, 2% as Camembert and goat cheeses and 4% as Roquefort blue cheeses. Wilcoxon tests were used instead of Tukey tests because residues deviated from normality. Wild populations grew faster than cheese populations in all salt concentrations tested, as on YPD and minimal media (Figure S10A ;Table S13).

Because all strains sampled from goat cheeses belonged to the Cheese_1 population, we tested whether this population was able to grow faster on goat cheese medium (1% salt) compared to other populations. There was no significant interaction between population and media on radial growth effects, meaning that

there was no specific adaptation to any particular kind of milk by the different populations (Figure S10B).

Contrasting volatile compound production between wild and cheese populations

Cheese ripening fungi, including *G. candidum*, contribute to cheese flavor through the production of volatile compounds (McSweeney, 2004). Flavor is a crucial criterion for cheese consumers and the cheese populations may have been selected for desirable and specific volatile compounds. We grew 14 *G. candidum* strains on a sterilized Camembert curd for 21 days at 10°C, *i.e.,* the ripening conditions of a Camembert. On average across compounds, the wild population produced five times higher quantities of volatiles than cheese populations. In order to compare the relative proportions of the different compounds, which is also an important aspect for flavor, we standardized the values by dividing all compound quantities by the total quantity of volatiles per sample. The PCA indicated a differentiation between wild and cheese strains in terms of volatile relative proportions (Figure 4D). The wild population thus produced combinations of volatile compounds different from cheese populations, with a high proportion of ethyl esters and ethyl acetates (Figure S10C), known to be key compounds in fermented beverages such as wine and beer. However, the impact of ethyl acetate on flavor is rather negative because it brings solvent type notes. In cheese, these esters are never predominant (Liu et al., 2004; Urbach, 1997). Ethyl esters are involved in anaerobic metabolism and may be important for survival in the wild. By contrast, cheese strains produced many alcohols, ketones, aldehydes and sulfur compounds (Figure S10C), known for producing attractive flavors such as buttery, cheesy, fermented and aldehydic notes (Curioni and Bosset, 2002). These volatile compounds, attractive in cheese, were present in similar absolute quantities in wild strains but were in minor proportions compared to other volatile compounds (Table S13), suggesting that cheese populations evolved a lower production of undesirable and unused volatiles. The overall balance between different volatile compounds is as important as volatile absolute quantities for flavor perception (Liu et al., 2004). The dimethyl sulfone, a compound previously reported to be produced during the catabolism of L-methionine

in *G. candidum*, was actually specifically produced by the cheese populations (Bonnarme et al., 2001; Penland et al., 2021).

Cheese populations inhibit more the growth of food spoilers than wild populations

Cheese is a protein- and fat-rich medium, where many microorganisms, including desired microbes, but also spoilers, can thrive and thus compete for nutrients; for example, iron is limiting in cheese (Mayo et al., 2021; Monnet et al., 2015, 2012). Cheese *G. candidum* populations may have been selected for excluding competitors by inhibiting their growth (Boutrou and Gueguen, 2005). This fungus is known to inhibit fungal and bacterial food spoilers, such as *Aspergillus* species and *Listeria monocytogenes,* but these inhibitory activities have only been investigated in cheese *G. candidum* strains so far (Dieuleveux et al., 1998; Nielsen et al., 1998; Omeike et al., 2021). We therefore tested whether cheese populations displayed better growth inhibition abilities than the wild population, using common fungal food spoilers as competitors: *Debaryomyces hansenii, Penicillium biforme, P. roqueforti* and *Scopulariopsis asperula*. We also tested whether growth inhibition of challengers occurred via secreted and/or volatile compounds.

Inhibition by a mycelium lawn - In the first experiment, we grew challengers in a central spot for 24h, alone or after spreading out *G. candidum* to let it grow as a lawn; growth inhibition could occur in this setting by secreted molecules in the medium, volatile compounds and/or a physical barrier to reach nutrients and grow. The growth of *D. hansenii* was completely inhibited by all populations of *G. candidum*. *Penicillium roqueforti* was strongly inhibited by *G. candidum*, in particular by the Cheese_2 population that completely prevented *P. roqueforti* growth (Figure 5; Table S13). The growth of *Scopulariopsis asperula* and *P. biforme* was also inhibited by *G. candidum,* with a significant difference between competitor growth when spread alone or on a *G. candidum* lawn; the Cheese_2 population again inhibited better competitors than any other population (Figure 5; Table S13). The Cheese_2 population was the most opaque population on cheese and had a beta-glucanase gene under positive selection, suggesting that challenger inhibition would be due to either mycelium density as a physical barrier or degradation of competitor cell wall.

Inhibition by volatile compounds - In a second experiment, we used splitted Petri dishes (the two parts being separated by a plastic barrier) to test whether cheese populations inhibited competitors to a greater extent than the wild population when only volatile compounds can reach challengers. No significant growth difference was observed between the growth alone and at the side of *G. candidum* for neither *S. asperula* nor *P. roqueforti* (Figure 5B, Table S13). Only *P. biforme* showed a significant growth inhibition by *G. candidum* in this setup (Table S13)*;* such a growth inhibition by *G. candidum* from an isolated Petri dish compartment indicates that volatile compounds produced by *G. candidum* are able to impair the growth of some competitors.

The two sets of experiments enabled us to assess by which mechanism *G. candidum* can inhibit competitors: *P. biforme* growth was inhibited by *G. candidum* in the splitted Petri dishes, suggesting that volatile compounds are able to impair its growth. On the contrary, *P. roqueforti* and *S. asperula* were only inhibited by *G. candidum* when molecules could diffuse in the medium of the challenger and *G. candidum* mycelium form a physical barrier. The Cheese_2 population had a stronger inhibition ability than the other *G. candidum* populations only when molecules could diffuse in the medium and the mycelium could act as a barrier.

Conclusions

Analyzing the genome of 98 *G. candidum* strains isolated from different kinds of cheeses, other food substrates and other environments revealed three monophyletic clades, corresponding to strains isolated from cheese, mixed-origins (dairy and other environments) and the wild, respectively. Within the cheese clade, we found three distinct clusters alongside several admixed strains. In terms of genetic diversity, the mixed-origin clade contained the most diversity, followed by the wild clade and, lastly, the cheese populations. However, the nucleotide diversity within the cheese clade is still relatively high compared to other cheese fungi. Indeed, the Cheese_2 population, while being four times less diverse than the two other cheese populations, is as diverse as the Roquefort *P. roqueforti* cheese population. The low diversity, the presence of a single mating type, a high level of linkage disequilibrium and the absence of reticulation in the neighbor-net indicated a lack of recombination

in the Cheese_2 population, that may thus correspond to a clonally cultivated line for cheese-making. Additional clonal lineages may be cultivated for cheese-making, as we found clonemates in all cheese populations, even in the clusters of hybrids, and including some commercial starter strains. The Cheese_1 population was as diverse as the domesticated cheese species *P. biforme* and the Cheese_3 population as its wild relative *P. fuscoglaucum.* The genetic relationships between *G. candidum* populations and their contrasting levels of diversity suggest that domestication occurred in several steps, with an ancient domestication event separating the mixed-origin and the wild clades, then the cheese and the mixed-origin clades, and yet more recently the three cheese clusters. The domestication of *P. camemberti* similarly occurred in several steps, the last steps involving the selection of a white and fluffy clonal lineage (Ropars et al., 2020). Considering the genetic diversity, the situation in *G. candidum* is very different from that of *P. camemberti* and *P. roqueforti,* for which a single or a few clonal lineages are sold by spore producers for all kinds of cheeses (Ropars et al., 2020). The domestication of *G. candidum* involved milder bottlenecks compared to other domesticated cheese fungi, such as *P. camemberti, P. roqueforti* and *S. cerevisiae*, perhaps because it is more abundant spontaneously in raw milk. It may also be that the domestication of *G. candidum* is more recent or has not involved a selection as strong as in other cheese fungi.

We found evidence of phenotypic adaptation to cheese-making in *G. candidum* cheese populations compared to the wild population, in terms of proteolysis activity, mycelium growth density, volatile compounds and competitive abilities. Denser mycelial growth leading to a fluffy aspect has also been selected in *P. camemberti* var. *camemberti* (Ropars et al., 2020), thus representing a convergent phenotype between two distantly related cheese fungi. *Geotrichum candidum* is increasingly inoculated in milk in the place of *P. camemberti* for industrial soft cheese production, as it provides the fluffy desired aspect without the disadvantage of *P. camemberti* that browns the surface of Camembert cheeses at the end of the ripening process (Carreira et al., 2002). The volatile proportions produced by cheese strains corresponded to attractive flavor for cheese-making, in contrast to wild strains. *Geotrichum candidum* is able to efficiently inhibit the growth of common food spoilers, in particular *P. biforme* and *P. roqueforti,* and the clonal Cheese_2 population is the most efficient competitor. We also found genomic footprints of

adaptation to cheese, with the presence of genomic islands of differentiation in cheese populations and the loss of genes no longer required in the human-made environment, i.e. tandem beta lactamase-like genes.

Our findings have industrial implications, as they reveal high genetic diversity and subdivision in a fungus widely used in the cheese industry, and the existence of different varieties, *i.e.,* genetically and phenotypically different populations used for cheese-making, with specific and contrasted traits beneficial for cheese-making. The most fluffy and most competitive cheese population corresponded to a clonal lineage which may represent the most recent selection event. The occurrence of recombination between cheese strains is highly relevant for cheese producers as it opens possibilities for further improvement for the agrofood sector. It is crucial to maintain the larger genetic diversity in cheese *G. candidum* populations as genetic diversity is essential in domesticated organisms for variety improvement and diversification and to avoid degeneration (Harlan et al., 2012).

Material and Methods

Sampling

We isolated 53 strains from different kinds of cheeses (e.g. Camembert, Brie, Saint Nectaire, Ossau Iraty, comté, bleu de chèvre) from five European countries, Canada and the USA . Cheese crusts were left in the freezer for 24h to kill acarians. Then, we diluted a piece of each crust in sterile water and spread 50 uL of the suspension on a malt agar Petri dish. When colonies appeared on the Petri dish, typically after three days, we isolated the different morphotypes with a sterile toothpick and inoculated them on new Petri dishes. After seven days of growth, we performed monospore isolation by several dilution steps, in order to obtain separated colonies arising each from a single spore. We identified the species of these pure strains after DNA extraction by sequencing the 5’ end of the nuclear ribosomal large subunit (LSU rDNA) using the LROR/LR6 oligonucleotide primers (Vilgalys and Hester, 1990). We also gathered 24 strains from INRAE, isolated from cheeses but also other environments (e.g. sand, hay, rainforest) and 15 strains from a French spore

seller. For each strain, single spore cultures were generated to ensure the presence of a single genotype before DNA extraction.
The LMA-244 strain were inoculated on Yeast Extract Glucose (YEG) agar plates (10 g.L-1 of yeast extract (Fischer Scientific), 10 g.L-1 of D-glucose (EMD Chemicals) and 15 g.L-1 of Bacto agar (BD Diagnostics)) directly from 15% glycerol (v/v) stock cultures stored at -80°C. The plates were incubated in the dark for five days at 25°C.

DNA extraction, genome sequencing, assembly, annotation and mapping

We used the Nucleospin Soil Kit (Macherey-Nagel, Düren, Germany) to extract DNA from 88 *G. candidum* strains cultured for five days on malt agar. Sequencing was performed with Illumina HiSeq 3000 paired-end technology (Illumina Inc.), 2x150bp. For the eight LMA strains, sequencing was performed using the Illumina HiSeq paired-end technology.

All Illumina reads were trimmed and adapters cleaned with Trimmomatic v0.36 (Bolger et al., 2014). Leading or trailing low quality or N bases below a quality score of three were removed. For each read, only parts that had an average quality score higher than 20 on a four base window are kept. After these steps, only reads with a length of at least 36 bp were kept.

Cleaned Illumina reads were assembled with SPAdes v3.15.3 not using unpaired reads with “--careful” parameter.

For the LMA-244 strain, Genomic DNA was extracted using the Fungi/Yeast Genomic DNA Isolation Kit (Norgen Biotek Corp.) with the following modifications. Thirty milligrams of frozen grounded mycelium were thawed and homogenized in 500 µL of a 0.9% NaCl solution. The elution buffer was replaced by a Tris 10 mM buffer (pH 8). Following the extraction step, gDNA suspensions were purified and concentrated using Agencourt AMPure XP magnetic beads (Beckman-Coulter), according to the manufacturer’s protocol.
DNA concentration and purity were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, U.S.A.) and a Qubit Fluorometer 3.0 (Thermo Fisher Scientific Inc., Wilmington, U.S.A.).

The DNA library was prepared following the Pacific Biosciences 20 kb template preparation using BluePippin Size-Selection System protocol and the Pacific Biosciences Procedure & Checklist – Preparing Multiplexed Microbial Libraries Using SMRTbell Express Template Prep Kit 2.0 protocol. No DNA shearing was performed. The DNA damage repair, end repair and SMRT bell ligation steps were performed as described in the template preparation protocol with the SMRTbell Template Prep Kit 1.0 reagents and the SMRTbell Express Template Prep Kit 2.0 reagents (Pacific Biosciences, Menlo Park, CA, USA). The DNA library was size selected on a BluePippin system (Sage Science Inc., Beverly, MA, USA) using a cut-off range of 10 kb to 50 kb. The sequencing primer was annealed at a final concentration of 0.83 nM and the P6 v2 polymerase was bound at 0.50 nM while the sequencing primer was annealed with sequencing primer v4 at a final concentration of 1 nM and the Sequel 3.0 polymerase was bound at 0.5 nM.. The libraries were sequenced on a PacBio RS II instrument at a loading concentration (on-plate) of 160 pM using the MagBead OneCellPerWell loading protocol, DNA sequencing kit 4.0 v2, SMRT cells v3 and 4 hours movies.

Raw PacBio reads were corrected using Illumina reads already available and described in a previous article (Perkins et al., 2020), with the default parameters of the LoRDEC software and trimmed with Canu v1.6 (Koren et al., 2017; Salmela and Rivals, 2014). Corrected and trimmed PacBio reads were then assembled using Canu v1.6. Illumina polishing of the Canu assembly was performed using Pilon v1.22 (Walker et al., 2014). A final assembly step was then performed with the hybrid assembler SPAdes v3.11.1 using the trimmed PacBio reads, the Illumina reads and the Pilon corrected assembly as trusted contigs (Antipov et al., 2016; Prjibelski et al., 2020) . Additionally, the CLIB 918 assembly (Bioproject PRJEB5752) was used as a reference in the SPAdes script for the assembly of each *G. candidum* genome (Morel et al., 2015). Scaffolds were filtered using the khmer software with a length cut-off of 1,000 bp (Crusoe et al., 2015).

The LMA-244 PacBio assembly and reads have been deposited in GenBank: nbPROJECT. To annotate short read assemblies and the LMA-244 genome, gene prediction was performed using Augustus v3.4.0 (Stanke et al., 2008). The training annotation file “saccharomyces” was used, with parameters as follows: “--gff3=on”,

“--protein=on”, “--codingseq=on”, “--exonnames=on”, “--cds=on” and “--uniqueGeneId=true”. The output of Augustus and the CLIB918 gff was provided to Funannotate v1.8.9 (ref DOI:10.5281/zenodo.4054262) for functional annotation. InterProscan was used under Funannotate pipeline locally (Blum et al., 2021). Funannotate then searched in the Pfam database v34.0 and dbCAN database version 10.0 with Hmmer v3.3.2 (Eddy, 2011; Huang et al., 2018; Mistry et al., 2021), in database UniProt version 2021_03 and database MEROPS version 12.0 with diamond blastp v2.0.11 (Rawlings et al., 2018; The UniProt Consortium et al., 2021), eggNOG-mapper v2 on the database eggNOG 5.0 (Cantalapiedra et al., n.d.; Huerta-Cepas et al., 2019).
Cleaned reads were mapped on the reference genomes CLIB 918 and LMA-244 using Bowtie2 v2.4.2 (Langmead and Salzberg, 2012). Maximum fragment length was set to 1000 and the preset “very-sensitive-local” was used.
SAMtools v1.7 (Li et al., 2009) was used to filter out duplicate reads and reads with a mapping quality score above ten for SNP calling and above one for CNV analyses.

In total, we have a dataset of 98 genomes, 88 being sequenced, eight from the University of Laval (LMA strains: Bioproject PRJNA482576, PRJNA482605, PRJNA482610, PRJNA482613, PRJNA482616, PRJNA482619, PRJNA490507, PRJNA490528), one strain CLIB 918 from the Collection de Levures d'Intérêt Biotechnologique (Bioproject PRJEB5752), and one of the strain Phaff72-186 from the 1000 Fungal Genomes project (Bioproject PRJNA334358 NCBI).

SNP calling

Single nucleotide polymorphisms (SNPs) were called using GATK v4.1.2.0 HaplotypeCaller, which provides one gVCF per strain (option -ERC GVCF). GVCFs were combined using GATK CombineGVCFs, genotypes with GATK GenotypeGVCFs, SNPs were selected using GATK SelectVariants (option -select-type SNP). SNPs were filtered using GATK VariantFiltration and options QUAL < 30, DP < 10, QD < 2.0, FS > 60.0, MQ < 40.0, SOR > 3.0, QRankSum < -12.5, ReadPosRankSum < -8.0. All processes from cleaning to variant calling were performed with Snakemake v5.3.0 (script available at https://github.com/BastienBennetot/Article_Geotrichum_2022).

Phylogenetic analysis

We inferred phylogenetic relationships among the 98 isolates using the dataset of 699,755 SNPs in a maximum likelihood framework using IQ-Tree2 v2.1.1 (Minh et al., 2020). The tree has been midpoint rooted. The best-fit model chosen according to Bayesian information criterion (BIC) was TVMe+R2 . Branch supports are ultrafast bootstrap support (1000 bootstrap replicates, Minh et al., 2013).

Genetic structure

We used the dataset of 699,755 SNPs to infer population structure based on the mapping on the CLIB 918 reference genome. We used Splitstree v4.16.2 (Huson and Bryant, 2006) for the neighbor-net analysis. We used the R package *Ade4* (Bougeard and Dray, 2018; Chessel et al., 2004; Dray et al., 2007; Dray and Dufour, 2007; Thioulouse et al., 2018) for principal component analyses (PCA, centered and unscaled). We used NGSadmix v.33 (Jørsboe et al., 2017) from the ANGSD (Korneliussen et al., 2014) package (version 0.933-110-g6921bc6) to infer individual ancestry from genotype likelihoods based on realigned reads, by assuming a given number of populations. A Beagle file was first prepared from bam using ANGSD with the following parameters: "-uniqueOnly 1 -remove_bads 1 -only_proper_pairs 1 -GL 1 -doMajorMinor 1 -doMaf 1 -doGlf 2 -SNP_pval 1e-6". The Beagle file was used to run NGSadmix with 4 as the minimum number of informative individuals. Given the high number of strains genetically highly similar among cheese strains (that may represent clonal lineages), we randomly sampled one of the individuals for each group of clonemates identified on the ML tree as having fewer than 90,000 SNPs and filtered out the other strains (N=64 strains kept) to avoid biasing the analysis. The analysis was run for different *K* values, ranging from 2 to 10. A hundred independent runs were carried out for each number of clusters (*K*).

The nucleotide diversity π (Nei's Pi; Hudson et al., 1992; Nei and Li, 1979), the Watterson's θ (Watterson, 1975), the fixation index F_{ST} (Hudson et al., 1992) and the absolute divergence d_{XY} (Nei and Li, 1979) were calculated using the *popgenome* package in R (Pfeifer et al., 2014). Fixed, private and shared sites were counted using custom scripts available at https://github.com/BastienBennetot/fixed_shared_private_count, with bcftools version 1.11 (using htslib 1.13+ds). F3 tests were computed using the *admixr*

package v0.9.1. The pairwise homology index (PHI) test was performed using PhiPack v1.1 and CLIB 918 genome as reference.
Linkage disequilibrium was calculated using vcftools v0.1.17 with the --hap-r2 parameter and a minimum distance between SNPs of 15,000 bp. Values were averaged when SNPs had the same distance.
Pairwise identity between an admixed strain and each non-admixed strain was calculated using overlapping sliding windows of 30kb span and 5kb step. Admixed clusters are indicated in Table S1. The custom script is available on https://github.com/BastienBennetot/Article_Geotrichum_2022

Copy number variation and identification of premature stop codons in CDS

Copy number variation (CNV) was analyzed using Control-FREEC v11.6 with the following parameters: ploidy was set to 1, non-overlapping windows of 500bp, telomeric and centromeric regions were excluded, expected GC content was set between 0.25 and 0.55, minimum of consecutive windows to call a CNV set to 1. This analysis was performed using as references the CLIB 918 (cheese_3) and LMA-244 (wild) genome sequences. CNVs were classified in different groups when the median of copy number was different between populations. We defined three groups: regions for which copy number was different between wild and cheese populations, between mixed-origin and cheese populations and when at least one cheese population differed from another population. For each InterPro term present in these regions, we performed enrichment tests, i.e., a fisher exact test comparing the number of a particular InterPro term found in these regions and the whole genome (Table S8).

We used snpeff (Cingolani et al., 2012) to assess how each SNP affected the coding sequence of predicted proteins, in the vcf file containing all SNPs and all genomes of our dataset. We detected premature stop codons in the 7,150 CDS of the CLIB 918 genome and the 5,576 CDS of the LMA-244 genome using a custom script and bcftools v1.11.

Analyzing the repeat landscape

In order to *de novo* detect repeats within *G. candidum*, RepeatModeler (v2.0.2; Flynn et al., 2020), using the ncbi engine (-engine ncbi) and the option -LTRStruct, was run on the pacbio genome assembly of LMA244 generating a library of 176 repeats. To estimate the per strain copy number of each repeat, illumina reads were aligned using bwa mem (v0.7.17; Li, 2013) to the repeat library and the median coverage for each repeat was then normalized by the LMA244 genome wide median coverage.

Detecting positive selection

The assemblies LMA-317, LMA-77 and LMA-563 have been excluded for this analysis because of a N50 under ten kb. All the 437441 predicted protein sequences from the 66 genomes of all cheese clades and mixed-origin clade were searched against each other with BLASTP using diamond v0.9.36 and clustered into orthologous groups using Orthagog v1.0.3 (Ekseth et al., 2014). For these analyses, we only kept single-copy orthologs shared between two populations. We compared the mixed origin population to each cheese population and the cheese clade. Multiple nucleotide sequence alignments with predicted gene sequences were then constructed using MACSE v2.0.3 with default parameters (Ranwez et al., 2018). We performed an approximative MacDonald Kreitman tests using the R package PopGenome (Pfeifer et al., 2014). The approximation comes from the fact that only codons with a single SNP are examined. The assumption of this version of the test is that the probability that two SNPs will appear in the same codon is very low. To identify genes evolving under positive selection in *G. candidum* genomes, α, i.e. the representation of the proportion of substitutions driven by positive selection was used. Genes with an alpha under 0 were filtered out. Of these genes, only those with a Fisher's test p-value under 0.05 were kept.

Phenotypic characterization

Sampling and strain calibration

We used 36 *Geotrichum candidum* strains for laboratory experiments: seven from the Cheese_1 population, five from the Cheese_2 population, eleven from the Cheese_3 population, eight from the mixed-origin population and five from the wild

population (Table S1). This set encompassed 26 strains isolated from dairies, one from other food environments and nine isolated from environments other than food. Experiments were initiated with spore suspensions calibrated to 1.10^6 spores/mL with a hemocytometer.

Media preparation

All media were sterilized in an autoclave at 121°C for 20 minutes except those with cheese or milk for which the autoclave was run at 110°C for 15 minutes to avoid curdling. Each 94mm-diameter Petri dish was filled with 25mL of the appropriate medium. Cheese medium was prepared as follows for 800mL: 300g of unsalted cream cheese from La Doudou farm in Cheptainville, 16g agar, 8g NaCl dissolved in 200mL of deionized water. Deionized water was added to reach 800mL. pH was adjusted to 6.5 and drops of blue food dyes were added to enable fungal colony measures (white medium and white colonies are not distinguable). Yeast Peptone Dextrose (YPD) medium was prepared as follows for 1L: 10g Yeast extract, 10g Bacto Peptone, 10g glucose, 14g agar powder. Minimal medium was prepared as described in “Improved protocols for *Aspergillus* minimal medium: trace element and minimal medium salt stock solutions”, Terry W. Hill, Rhodes College, Etta Kafer, Simon Fraser University. Tributyrin agar was prepared as follows: Tributyrin medium 33 g/L, neutral Tributyrin 10 g/L, Bacto Agar 15 g/L. Ingredients were bought at Nutri-Bact company, Québec, Canada. Caseinate agar was prepared according to Frazier and Rupp, modified as follows: Calcium caseinate medium 37.2 g/L, Bacto Agar 15 g/L. Ingredients were bought at Nutri-Bact company, Québec, Canada. For yogurt media we used three different types of raw milk, i.e. sheep, goat and cow milks, coming from d’Armenon farm near Les Molières (Esonne, France), Noue farm in Celle les Bordes and Coubertin farm in Saint-Rémy-lès-Chevreuse respectively. Each medium was prepared following the same procedure: 1L of milk was mixed with 62.5g of Danone brand yogurt, heated for 5 hour at 43°C and stored in a fridge before use. A subset of 300g of this preparation was used with 16g of agar powder, 8g of NaCl, 4 drops of blue food dye and filled up with deionized water to reach 800mL.

Growth in different conditions and different media

Petri dishes were inoculated with 10µL of the 1.10^6 cells/mL in a 10% glycerol solution. Inoculated Petri dishes were wrapped with plastic film before letting them grow in the dark. A millimeter rule was used to measure two opposite diameters of fungal colonies to estimate their growth. Means of these two measures were used for statistical analyses.

To test media and temperature effect on growth, *G. candidum* strains were grown on minimal, YPD and cheese media. We took pictures and measured their growth at seven, 11 and 14 days for minimal, YPD and cheese media at 10°C (ripening cellar temperature), at seven and 11 days for the cheese medium at 15°C and at seven days for minimal, YPD and cheese media at 25°C (Figure S8).

To test salt tolerance, *G. candidum* strains were grown at 10°C on cheese media of different salt concentrations: unsalted media, 1% salt as St Nectaire and cream cheeses, 2% as Camembert and goat cheeses and 4% as Roquefort blue cheeses. We took pictures and measured colony diameters after 14 days of growth.

To test adaptation of *G. candidum* populations to different milk origins, growth was measured on different yogurt media made from goat, sheep and cow raw milk for seven days at 25°C.

To test lipolytic and proteolytic activities of *G. candidum* populations, we grew strains on tributyrin agar and caseinate agar, respectively. Each strain was inoculated in triplicate Petri dishes that were let grown at 25°C for 14 days. The radius of lysis was measured and the mean between triplicates was used for the analysis.

Pictures were taken using a Scan 1200 (Interscience). Petri dishes grown on cheese were analyzed using IRIS (Kritikos et al., 2017) which measured Integral opacity scores, defined as the sum of the brightness values for all the pixels within the colony bounds.

Volatile compounds analysis using Gas-chromatography mass-spectrometry (GC-MS)

Volatile compounds produced by *G. candidum* were analyzed using gas-chromatography mass-spectrometry (GC-MS). Compounds were extracted and concentrated by using a dynamic headspace (DHS) combined with a thermal

desorption unit (TDU). Strains were grown for 21 days at 10°C (minimum Camembert ripening time) on a cheese agar medium made with Camembert-type curds. After 21 days, each Petri dish content, with its medium and *G. candidum* mycelium, was mixed with a fork for one minute, gathered in vials and immediately frozen in liquid nitrogen. For each sample, three grams of frozen cultured media were weighted and stored in vials with septum caps at -80°C. Sixteen hours before analysis, samples were stored at 4°C. The Cheese_2 population was not tested in this experiment because population delineation was not known at this time.

Dynamic headspace (DHS) conditions were as follows: Inert gas: He; Incubation: 30°C for 3min; Needle temperature: 120°C; Trap: nature tenax, 30°C, 450 mL He; He flow: 30 mL/min; Dry purge : temperature 30°C, 850 mL He, He flow 50 mL/ min. Thermal Desorption Unit (TDU) conditions were as follows: inert gas : He; Initial temperature: 30°C, then 60°C/min until 290°C kept for 7 minutes; Transfer temperature: 300°C. Cool Injection System (CIS) conditions were as follows: inert gas : He; Initial temperature: -100°C, then 12°C/s until 270°C kept for 5 minutes. Gas chromatograph (brand Agilent 7890B) was used with a polyethylene glycol (PEG) type polar phase column (HP-Innowax, ref. Agilent 19091N-116I, 60mx0.32mm, 0.25µm film thickness). Helium flow was set at 1.6mL/min. Samples were injected in splitless mode with a holding time of 1 minute. To optimize separation of compounds, a specific program of the gas chromatography oven was used, with initial temperature at 40°C for 5 minutes, rising temperature from 40°C to 155°C with a slope of 4°C/min, rising temperature from 155°C to 250°C with a slope of 20°C/min and then temperature was kept at 250°C for 5 minutes. A single quadrupole mass spectrometer was used to determine m/z of sample molecules (Agilent, référence 5977B MSD). Molecules were identified using NIST libraries (NIST 2017 Mass Spectral Library).

Competition experiments

To test the abilities of *G. candidum* populations to exclude other fungi by secreting molecules or volatile compounds, we compared the growth of competitors when grown alone and on a lawn of an already grown *G. candidum* mycelium. We inoculated a cheese medium with 150µL of a *G. candidum* calibrated spore solution (1.10^6 spores/mL), spread evenly on the Petri dish. After 24h of growth, we inoculated 10µL of a competitor spore solution (1.10^6 spores/mL) in a single spot, in

the middle of the Petri dish. We used as competitors the following species and strains: *Penicillium biforme* (ESE00018, ESE00023, ESE00125, ESE00222), *Penicillium roqueforti* (ESE00645, ESE00925, LCP06040), *Scopulariopsis asperula* (ESE00044, ESE00102, ESE00835, ESE01287, ESE01324) and *Debaryomyces hansenii* (ESE00284, ESE00561, ESE00576; Table S15). For each competitor, two Petri dishes were inoculated without any *G. candidum* as controls for measuring growth without a lawn.

We took pictures of the Petri dishes at 6 days, when the competitor mycelium grown alone was near the Petri dishes border; we measured colony size at 7 days for *P. biforme* and *P. roqueforti* and at 19 days for *D. hansenii*, which grows more slowly.

To test the abilities of *G. candidum* populations to exclude other microorganisms by producing volatile compounds, we set up an experiment with splitted Petri dishes where only air can be shared between the two parts. In one part of the Petri dish, we spread 75µL of a *G. candidum* spore solution (1.10^6 spores/mL) and let it grow during 24 hours before adding on the other part of the Petri dish a drop of 5µL of a competitor spore solution (1.10^6 spores/mL). For each competitor, two Petri dishes were inoculated without any *G. candidum* as controls. We used as competitors the following species and strains: *Penicillium biforme* (ESE00018, ESE00023, ESE00125, ESE00222, ESE00423), *Penicillium roqueforti* (ESE00250, ESE00631, ESE00640, ESE00925) and *Scopulariopsis asperula*(ESE00044, ESE00102, ESE00835, ESE01287, ESE01324; Table S15). Petri dishes were grown at 10°C, measured and pictured at 11 days for *P. biforme* and *P. roqueforti* and 19 days for *Scopulariopsis asperula*.

Graphics and statistical analyses

Plots and statistical analyses were made using *ggplot2* (Wickham, 2016), *rstatix* and *ggpubr* packages in the R environment. For ANOVAs, we used standard linear models in which all explanatory variables were discrete, with explained variables being radial growth for growth conditions (for media, temperature, salt content and adaptation to milk experiments), integral opacity score (for opacity experiment), relative proportions of volatiles compounds (for volatile compounds experiment) and radial growth of the competitor (for competition experiments). The explanatory variable common for all analyses was the ‘population’ of *G. candidum*. The variables ‘medium’, ‘day’ and ‘temperature’ were explanatory variables specific to the growth

analysis. The ‘competitor species’ variable was specific to competition analyses. All variables and all interactions between them were implemented in the ANOVA and non-significant interactions were subsequently removed before performing post-ANOVA Tukey’s honest significant difference (HSD) tests. The data normality of residuals was checked; when residues deviated from normality (only for the salt content experiment), we also ran non-parametric tests (Wilcoxon ranking tests) using R. Radius of lysis for lipolytic and proteolytic activities experiments was often discrete, strains either showing lytic activity or not at all. This is why we decided to transform these data into qualitative discrete data in order to fit a generalized linear model with a binomial function as logit. Growth time (7, 14 and 21 days) and temperature (15 and 25°C) were taken as random variables because no fit could be achieved with little data and we wanted to test for population effect. Tukey contrasts were used to compare population means of populations when population effect was significant.

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Author contributions

J.R. designed and supervised the study, and obtained funding. B.B., V.P., J.R. and A.S. generated the data. C.G. provided strains from the CIRM-Levures INRAE collection. B.B., J.-P.V., R.C.d.l.V. and S.O. analyzed the genomes. B.B., S.H., J.R. and A.S. performed the experiments. B.B. analyzed the data from laboratory experiments. M.H.L. and St.L. supervised the lipolysis and proteolysis analyses.

B.B., So.L. and A.-C.P. performed the volatile compound experiment. T.G. contributed to interpretation and writing; B.B. and J.R. wrote the manuscript, with contributions from all the authors.

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Figure legends

Figure main:

Figure 1: Phylogenetic relationships and population structure of 98 strains of *Geotrichum candidum,* based on whole-genome data

(A) Neighbor-net analysis based on a single nucleotide polymorphism (SNP) distance matrix. The scale bar represents 0.01 substitutions per site for branch lengths.

(B) Genetic relationships among strains and population structure in *G. candidum* based on 699,755 SNPs. a) Maximum likelihood tree showing genetic relationships among the 98 isolates used in this study. All nodes are supported by bootstrap support >98% (bootstrap analysis with 1000 resampled datasets). The scale bar represents 0.05 substitutions per site for branch lengths. We used the midpoint rooting method to root the tree. The "$" symbol pinpoints commercial starter strains and "*" the PacBio sequences. Genomes used as reference are written in bold. b) Population subdivision inferred for K = 5. Colored bars represent the coefficients of membership in the five gene pools based on SNP data.

(C) Principal component analysis (PCA) based on 699,755 SNPs and 98 strains. Genetic clusters are represented by the same colors on all panels: light blue for Cheese_1, dark blue for Cheese_2, pink for Cheese_3, light grey for the mixed-origin population and dark grey for the wild population. Borders of points were colored in red when multiple points were overlapping due to clonal lineages (with a threshold set at <90,000 SNP for defining clonal strains). The shape of points represents the environment from which strains were sampled: circle for cheese/dairy, square for food and triangle for wild environment.

(D) PCA based on the 323,385 SNPs when the dataset was restricted to the 78 strains from the cheese clade

Figure 2: Synteny of the beta lactamase-like genes lost in the cheese clade of *G. candidum*

Synteny of the scaffold QQZM01000080.1 of the LMA-244 wild strain against the scaffold CCBN010000010.1 of the cheese CLIB 918 strain. The two scaffolds were

shortened according to the range of nucleotide position on the right of each sequence. Beta-lactamase like genes are annotated in red while other genes are displayed in blue. Black triangles indicate positions where repeated sequences were detected by tandem repeats finder (Benson, 1999). All strains from the cheese clades and 5 from the mixed-origin populations (LMA-317, ESE00274, MUCL8652 and ESE00540) carried the 20kb deletion containing the g5112 and g5113 genes, both encoding for beta-lactamase like.

Figure 3: Heatmap of different repeats that expanded in the cheese populations

From the repeat database based on the LMA244 (wild strain), the copy number of each repeated element was estimated by aligning illumina reads of each strain. Only repeated elements that were at least in a 5-fold copy relative to the LMA244 genome were kept on the heatmap. The total number of copies is written in the center of each cell and filled with different shadings of grey to red depending on the relative expansion from the smallest copy number.

Figure 4: Differences in growth, opacity and volatile compounds among the five populations of *Geotrichum candidum*

Each point represents a strain, horizontal dotted lines and vertical lines represent the mean and the standard deviation of the phenotype in the population, respectively. The number *n* at the bottom of plots indicates the number of strains used per population for measuring the corresponding phenotypes. The pairwise Tukey tests performed to assess whether there were mean differences between populations are indicated with brackets and their p-values are given.

(A) Mean radial growth of the three cheeses, mixed-origin and wild populations on cheese (1% salt), yeast peptone dextrose (YPD) and minimal media at 25°C.

(B) Differences in opacity between the three cheese populations, mixed-origin and wild populations on cheese medium (1% salt) at 10°C. Integral opacity is defined as the sum of the brightness values for all the pixels within the fungal colony bounds and measures the whiteness and density of the mycelium.

(C) Petri dish of a strain (ESE00182) from the Cheese_2 population showing the fluffiness of the colony

(D) Principal component analysis (PCA) of *G. candidum* strains based on their

relative proportions of the different volatile compounds produced. Strains are plotted using the first two PCA axes.

(E) Contribution of each volatile compound to the first two PCA axes. Compounds contributing the most to the differentiation were colored in red and labeled (i.e., those distant from 0 by an Euclidean distance >=0.1).

Figure 5: Competitive abilities of the different populations of *G. candidum* against *Penicillium biforme, P. roqueforti* and *Scopulariopsis asperula* challengers.

(A) At the top, radial growth abilities of the competitors on lawns of *G. candidum* belonging to different populations (the three cheese, the mixed-origin and the wild populations). Each point represents a combination of the growth of a competitor strain on a lawn of a *G. candidum* strain. Horizontal dotted lines and vertical lines represent the mean and the standard deviation of the competitor growth in the population, respectively. The number *n* at the bottom of plots indicates the number of combinations of competitor-mat used per population. The competitor was inoculated in a central point 24h later on a lawn of *G. candidum.* At the bottom, from left to right, are shown pictures of *P. biforme* ESE00023 on a *G. candidum* ESE00186 lawn and without any lawn, *P. roqueforti* ESE00645 on a *G. candidum* ESE00186 lawn and without any lawn, and *S. asperula* ESE01324 on a *G. candidum* ESE00198 lawn and without any lawn, all on a salted cheese medium.

B: At the top, radial growth abilities of competitors, with various *G. candidum* strains belonging to different populations being grown on the other side of splitted Petri dishes. The competitor was inoculated in a central spot on one side and the *G. candidum* strain was spread on the other side of the splitted Petri dish (a picture is shown as example below the figure). Media is not contiguous between the two sides of Petri dishes, so that inhibition can only occur by volatile compounds. *Penicillium biforme* and *P. roqueforti* were grown for 11 days while *S. asperula* was grown for 19 days at 25°C.

Each point represents a combination of the growth of a competitor strain on a *G. candidum* strain. Horizontal dotted lines and vertical lines represent the mean and the standard deviation of the competitor growth in the population, respectively. The number *n* at the bottom of plots indicates the number of combinations of competitor-mat used per population.

Figure sup:

Figure S1: Population structure of *Geotrichum candidum*.

Population subdivision inferred for *K* population ranging from two to six. Colored bars represent the coefficients of membership in the K gene pools based on genomic data. Each bar represents a strain, its name being indicated at the bottom of the figure. The new color for each *K* increment is indicated on the right part. The second order rate of change in the likelihood (ΔK) peaked at *K*=6. However the additional population distinguished at *K*=6 only encompassed two strains that were not that differentiated in the splistree (MUCL 14462 and CBS 9194; ; FIGURE 1).

Therefore, we chose to set the number of populations to five, the *K* value at which the structure was the strongest and the clearest, with three cheese populations, several admixed or hybrid cheese strains, a population of mixed origins and a wild population. These populations are indicated on the last rows.

Below the admixture plot, two rows are indicating milk from which the strains was sampled and the population delineation.

Figure S2: Pairwise identity between admixed and other strains, averaged by population of *Geotrichum candidum.*

In order to see traces of introgression from different populations of *G. candidum* in some hybrid strains we computed the pairwise identity along the genome. Only the first scaffold of the CLIB 918 genome is shown in this figure. Values were averaged by population and when admixed strains had the same genetic background (name of strains are above each subplot) across 30kb overlapping sliding windows with 5kb steps. If no introgression happened, we expect the admixed strain to be equally distant to the different populations along the genome. Introgression results in genomic regions being atypically closer to a single population. All admixed strains within the cheese clades had introgression imprints while the other three strains (CBS 9194^{T}, MUCL 14462 and ESE01080) did not, meaning that they were either from different genetic backgrounds or introgressed with genetic backgrounds different from the five populations of *G. candidum*.

Figure S3: Linkage disequilibrium against distance between SNPs for the five *Geotrichum candidum* populations.

The r^2 (square of the correlation coefficient between two indicator variables) varies between 0 when two markers are in perfect equilibrium and 1 when they provide identical information. Under recombination, we expect r^2 to decrease exponentially with the distance between two SNP while in non recombining lineages linkage disequilibrium remains flat. All *G. candidum* populations r^2 decay curve behaved as recombining populations except the Cheese_2 population.

Figure S4: Density of transposable elements copy number relative to the LMA-244 strain

To better show the fat tail distribution, the y-axis (density) was cut at 25%. A red dashed line indicates the threshold of five times more copy number than the LMA-244 strain. This threshold was set to identify repeats expansion related to small peaks on the density curve

Figure S5: Distribution of absolute divergence d_{xy} values for different pairwise populations tested.

Distribution of absolute divergence (d_{xy}) values for each pairwise population comparison from the genomic scan analysis. Density is given as an overlapping window number for a specific value of d_{xy}, each window being 7.5kb wide with a step of 5kb. A black vertical line indicates the threshold of 1% highest values kept for the enrichment test.

Figure S6: Genomic scan of within-population genetic diversity and between-population differentiation in geotrichum candidum.

Genomic of the nucleotide diversity π, watterson's theta θ_w, absolute divergence d_{xy} and fixation index F_{ST} along the scaffold 1 of the CLIB 918 reference genome. At the bottom a guide indicates genic regions in black and non genic regions in white. 7.5kb overlapping windows with a step of 5kb. On the bottom of the figure, genic regions are shown in black (not positively selected) or red (positively selected in the MK test analysis) rectangle. On the first panel (nucleotide diversity π), 5% lowest π values in the three cheese populations were highlighted by black dots. On the third panel (absolute divergence d_{xy}), 1% highest values of d_{xy} of each pairwise comparison

were highlighted by back dots. These outliers were checked for gene presence and functions that could be involved in cheese adaptation and subsequently tested for enrichment within this subset of outliers compared to the whole genome

Figure S7: SNPs inducing a premature stop codon for each *Geotrichum candidum* strain.

In order to keep genes that carried a single nucleotide polymorphism (SNP) inducing a premature STOP codon in most of a population, we only showed STOP-inducing SNP that were at least in more than three strains. Columns represent strains and strains are ordered following the ML tree. Cells are colored in black when the corresponding SNP induces a premature stop codon, white when there is no substitution for this site compared to the reference genome and grey when the SNP status could not be attributed during SNP calling, substitution that induces other effects on the protein sequence were not present in this subset of STOP-inducing sites. Each row is a site, when multiple SNPs induced stop codons in the same gene, the corresponding rows were grouped and separated from other genes by black lines. The analysis was done using either the CLIB 918 (Cheese_3) genome (A) or the LMA-244 (Wild) genome (B) as a reference.

Figure S8: Differences in growth among the five populations of *Geotrichum candidum* populations for different media and temperature

Mean radial growth of the three cheese populations, mixed-origin and wild populations on cheese (1% salt), yeast peptone dextrose (YPD) and minimal media at 10, 15 and 25°C for 7, 11 and 14 days.

Each point represents a strain, horizontal dotted lines and vertical lines represent the mean and the standard deviation of the population respectively. The number *n* at the bottom of plots indicates the number of strains per population used for measuring these phenotypes. To assess difference in means between populations, significant pairwise Tukey tests are indicated with brackets and p-values.

Figure S9: Differences in lipolytic and proteolytic activity among the five populations of *Geotrichum candidum* populations for different growing time and temperature

A: Lipolytic activity of *G. candidum* at 15 and 25°C and grown for 7, 14 and 21 days.

B: Proteolytic activity of *G. candidum* at 15 and 25°C and grown for 7, 14 and 21

days.

Each point represents a strain, horizontal dotted lines and vertical lines represent the mean and the standard deviation of the population respectively. The number *n* at the bottom of plots indicates the number of strains per population used for measuring these phenotypes. To assess difference in means between populations, significant pairwise Tukey tests are indicated with brackets and p-values. Length of lysis was measured as a radius between the center of the colony and the limit of the lysis area when lysis happened under the colony or measured as the thickness of the lysis area when there was no lysis under the colony.

Figure S10: Differences in salt tolerance, growth on different milk and volatile compounds in details among the five populations of *Geotrichum candidum.*

Each point represents a strain, horizontal dotted lines and vertical lines represent the mean and the standard deviation of the phenotype in the population, respectively. The number *n* at the bottom of plots indicates the number of strains used per population for measuring the corresponding phenotypes. The pairwise Tukey tests performed to assess whether there were mean differences between populations are indicated with brackets and their p-values are given.

(A) Mean radial growth at 10°C of the three cheese, mixed-origin and wild populations of *G. candidum* on cheese agar medium with different salt concentrations: unsalted, 1% salt for mimicking St Nectaire and cream cheeses, 2% salt for mimicking Camembert and goat cheeses and 4% salt for mimicking blue cheeses.

(B) Mean radial growth at 10°C of the three cheese populations, the mixed-origin and the wild populations on yogurt agar media made with raw cow, goat and sheep milks.

(C) Relative proportions of major volatile compounds in Cheese_1, Cheese_3, mixed-origin or wild populations of *G. candidum.* The volatile compounds shown were those contributing the most to the two first PCA axes or that are important for cheese ripening. For each compounds, the related corresponding descriptor from thegoodscentscompany.com was added.

Table sup:

Table S1: Description of the origin, phylogenetic assignation and phenotype tested of *G. candidum* strains used in this study

Table S2: Population genetics statistics related to genetic differentiation (fst, dxy) in the five G. candidum populations and other cheese fungi

Table S3: Proportions of fixed, shared and private SNP for each pairwise combination of G. candidum population and other cheese fungi

Table S4: F3 test performed on each combination of G. candidum populations using admixr package of R. In case of introgression we expect negative value of the F3 test

Table S5: Population genetics statistics related to genetic diversity (π, watterson's θ) in the five G. candidum populations and other cheese fungi

Table S6: Distribution of mating type in each population and proportion test of the deviation to 1:1 ratio

Table S7: Phi test of each population of G. candidum using the first scaffold

Table S8: Test for enrichment of copy number variant that differentiated G. candidum populations

Table S9: Repeat copy number for the different strains of *G. candidum*

Table S10: Table of proteins tested for function enrichment that were detected either by keeping 1% highest of dxy between cheese and wild strains or 5% lowest Pi in the cheese population (GeoA) but not in the wild population (GeoC). Only functions related to lactose, lipid, protease were kept

Table S11: Results of the MK test for positive selection

Table S12: Table of SNP count and percentage by impact, functional class, effect and genomic regions for each G. candidum populations

Table S13: Anova table of all phenotypic linear model and post-hoc test table

Table S14: Anova table and post-hoc test of lipolysis and proteolysis analysis

Table S15: Description of the origin and species of strains used in the competition experiment

Figure 1

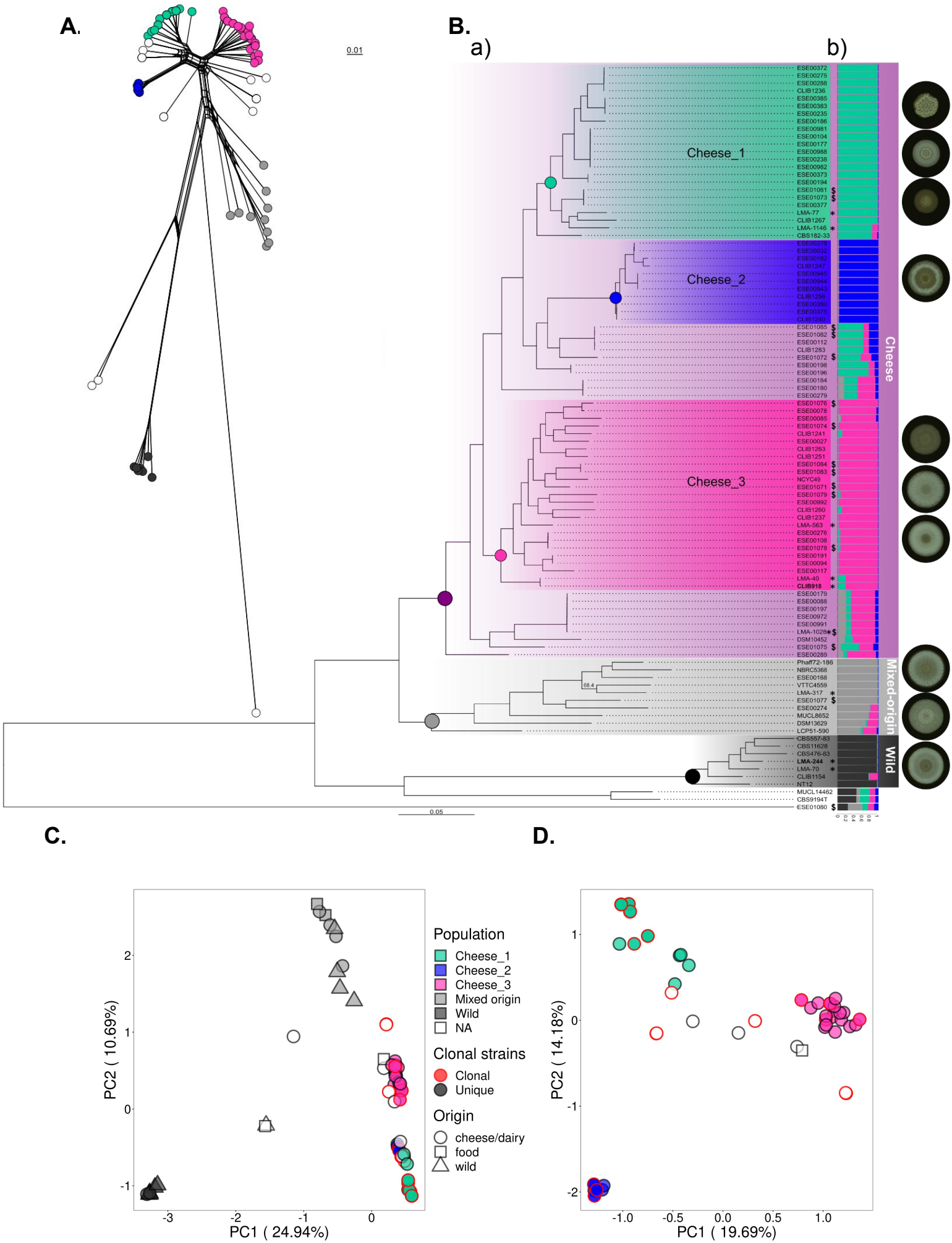

A.
0.01
B.
a)
b)
Cheese_1
Cheese_2
Cheese_3
Cheese
Mixed-origin
Wild
0.05
C.
D.
Population
Cheese_1
Cheese_2
Cheese_3
Mixed origin
Wild
NA
Clonal strains
Clonal
Unique
Origin
cheese/dairy
food
wild
PC2 (10.69%)
PC1 (24.94%)
PC2 (14.18%)
PC1 (19.69%)

Figure 2

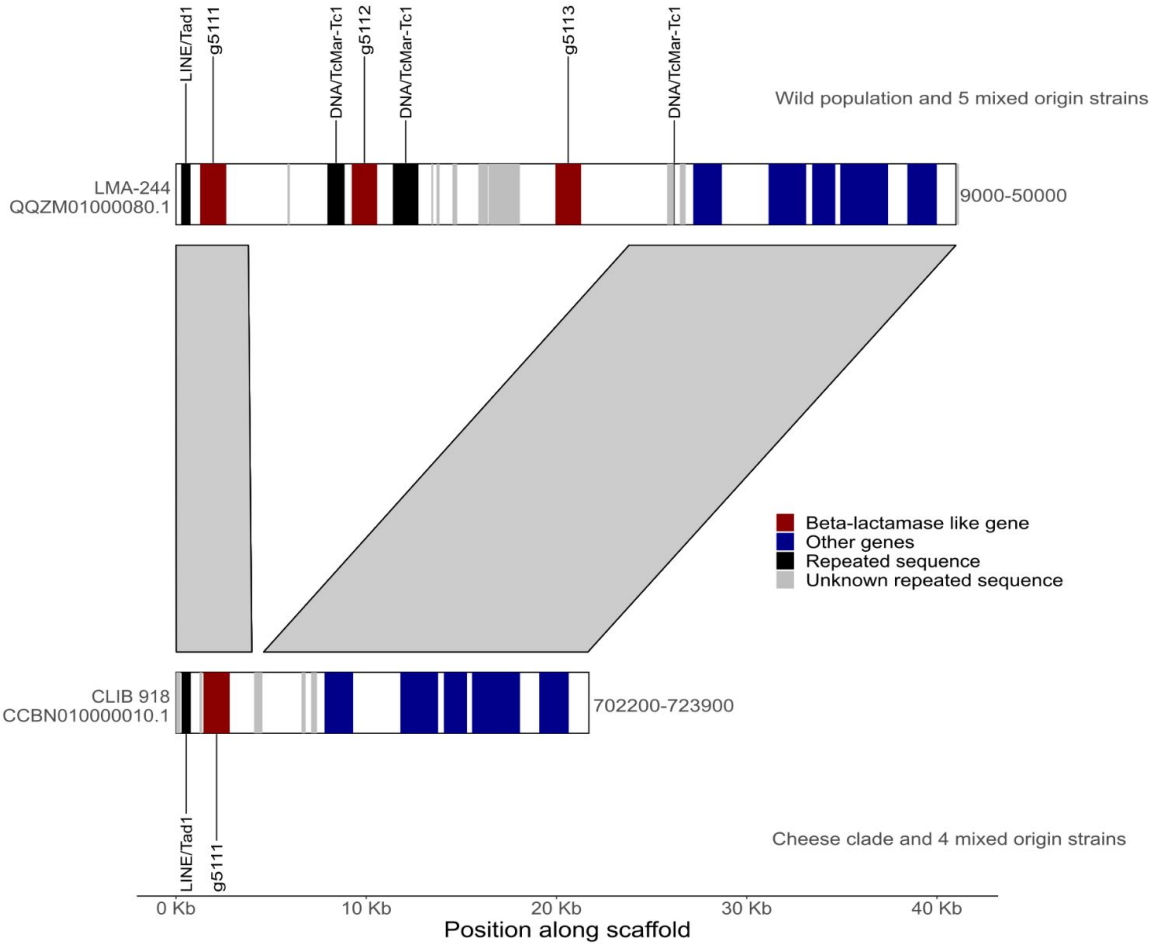


Figure 3

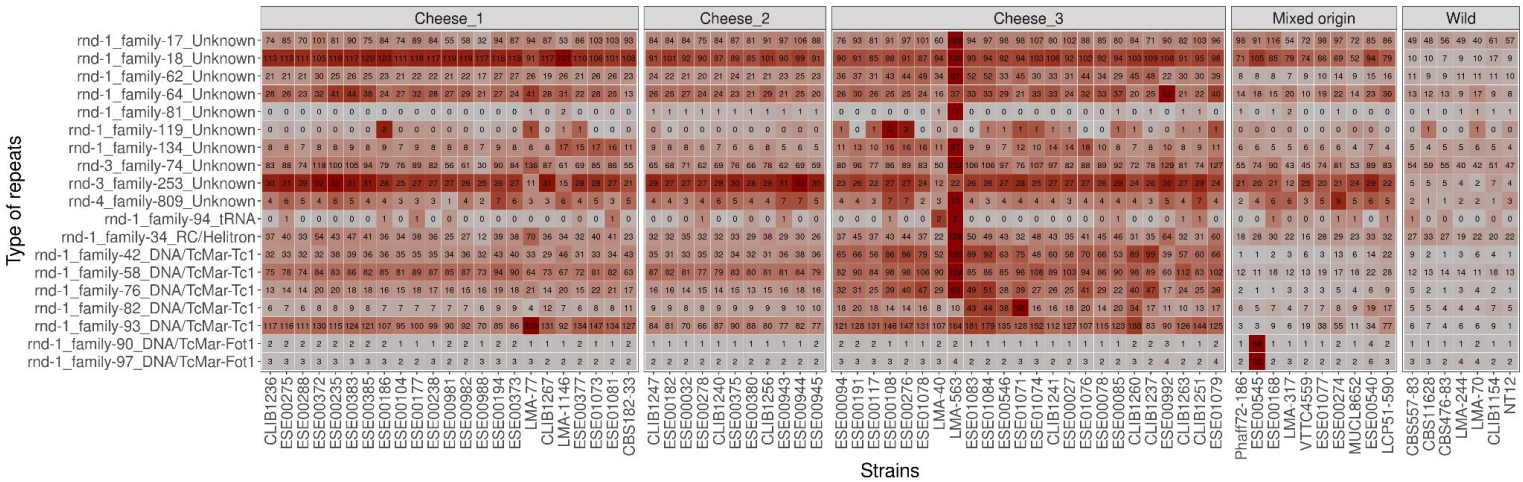
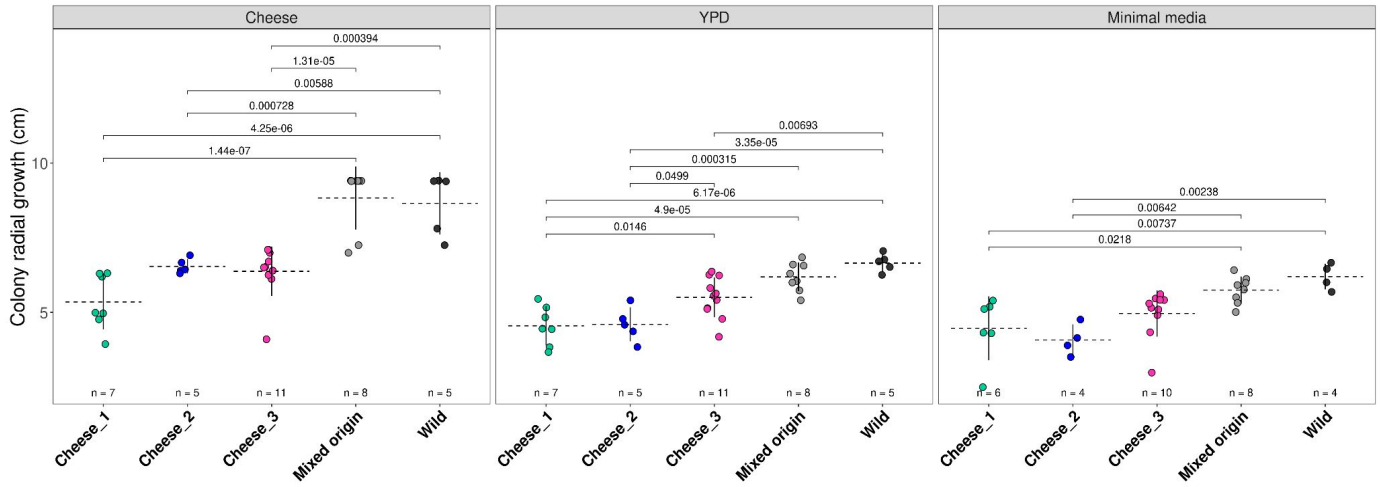
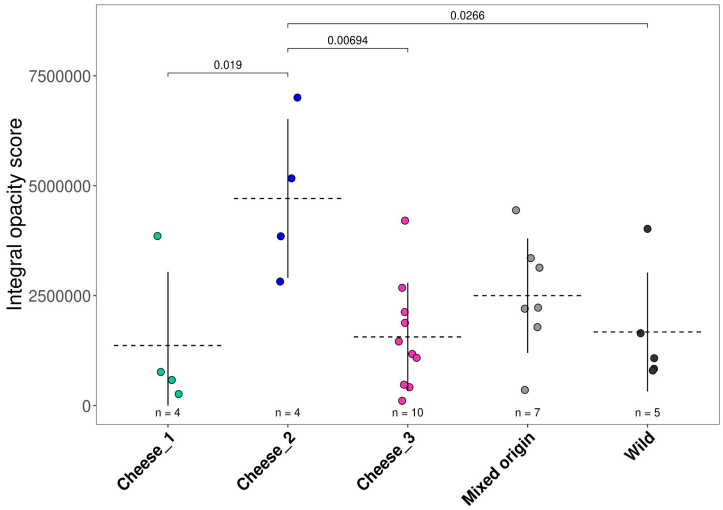


Figure 4

A.

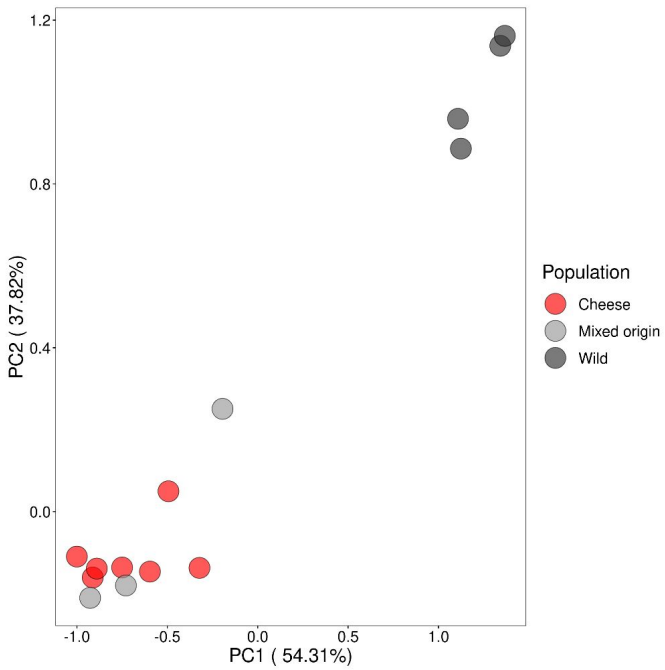


B.



C.

D.



E.

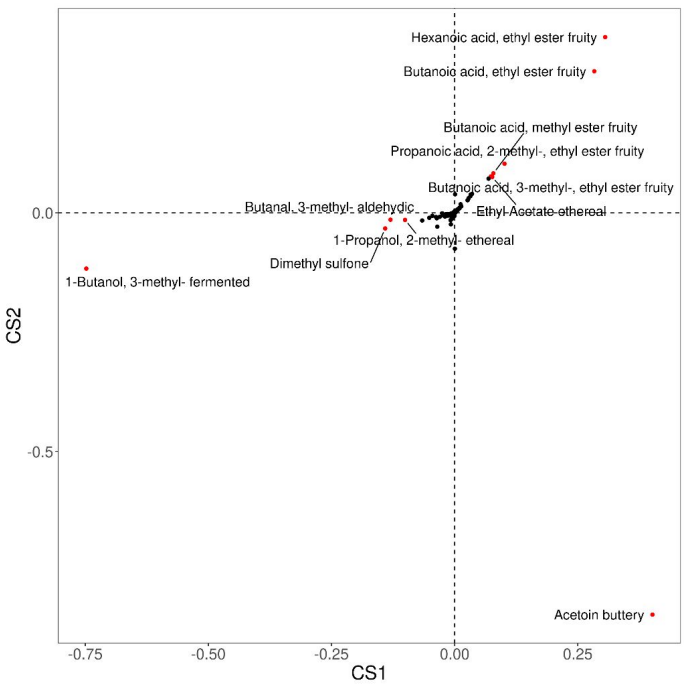
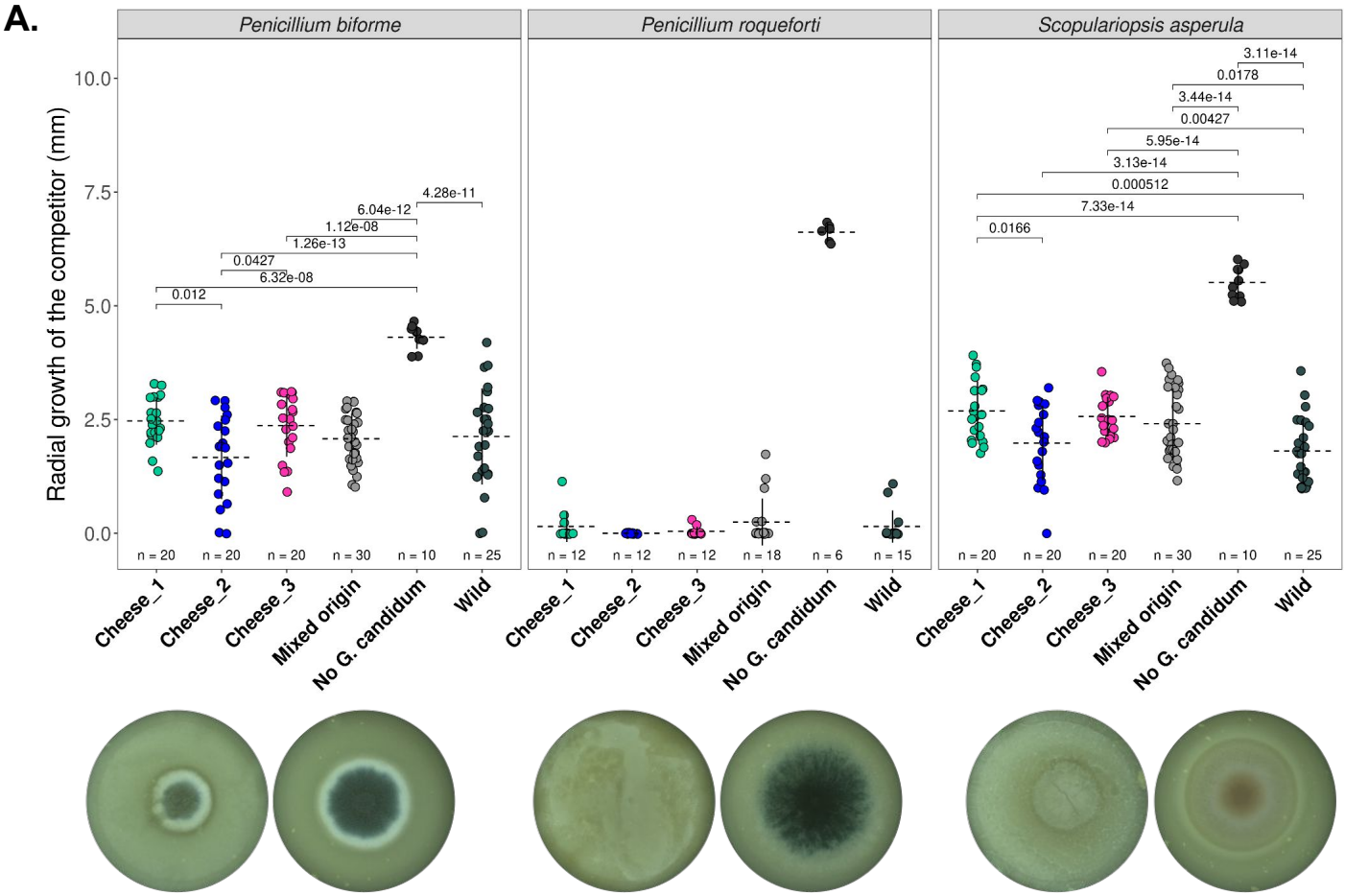
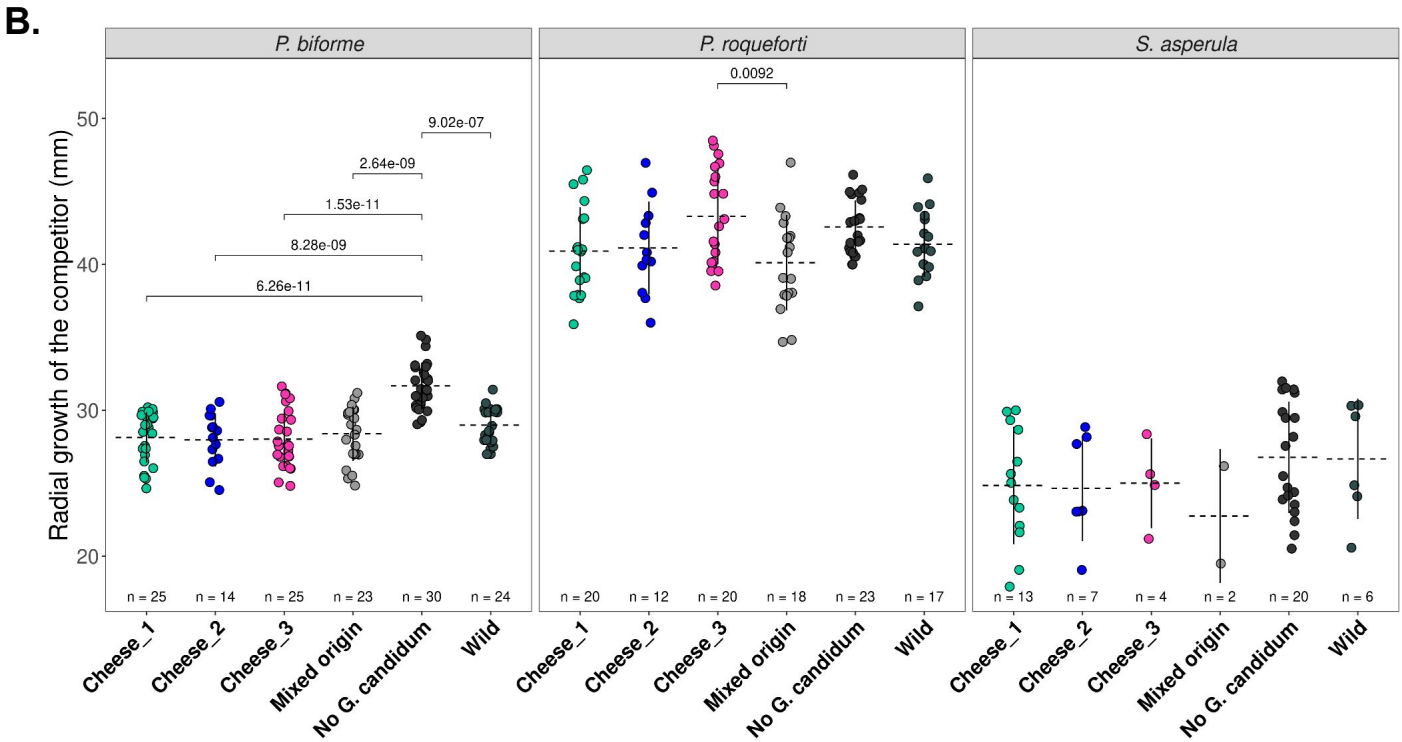
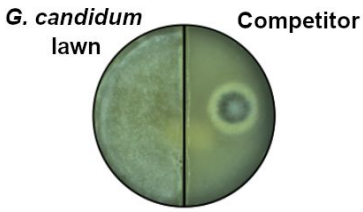


Figure 5


A.
Penicillium biforme
Penicillium roqueforti
Scopulariopsis asperula
Radial growth of the competitor (mm)
10.0
7.5
5.0
2.5
0.0
0.012
0.0427
6.32e-08
1.26e-13
1.12e-08
6.04e-12
4.28e-11
0.0166
7.33e-14
0.000512
3.13e-14
5.95e-14
0.00427
3.44e-14
0.0178
3.11e-14
n = 20
n = 20
n = 20
n = 30
n = 10
n = 25
n = 12
n = 12
n = 12
n = 18
n = 6
n = 15
n = 20
n = 20
n = 20
n = 30
n = 10
n = 25
Cheese_1
Cheese_2
Cheese_3
Mixed origin
No G. candidum
Wild


B.
P. biforme
P. roqueforti
S. asperula
Radial growth of the competitor (mm)
50
40
30
20
6.26e-11
8.28e-09
1.53e-11
2.64e-09
9.02e-07
0.0092
n = 25
n = 14
n = 25
n = 23
n = 30
n = 24
n = 20
n = 12
n = 20
n = 18
n = 23
n = 17
n = 13
n = 7
n = 4
n = 2
n = 20
n = 6
Cheese_1
Cheese_2
Cheese_3
Mixed origin
No G. candidum
Wild


G. candidum
lawn
Competitor