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**Highlighting the microbial diversity of 12 French cheese varieties**

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**ABSTRACT**

Surface-ripened cheeses host complex microbial communities responsible for the transformation of milk into cheese as well as the development of important properties in terms of texture, color and sensory perception. In this study, we used high-throughput amplicon sequencing to decipher the bacterial and fungal diversity of 60 cheeses belonging to 12 popular French cheese varieties. Using this approach, 76 bacterial and 44 fungal phylotypes were identified. Major differences were observed between rind and core samples and also according to cheese varieties and manufacturing processes. Occurrence analysis revealed the presence of widespread taxa as well as operational taxonomic units (OTUs) specific to one or several cheese varieties. Finally, we observed patterns specific to the cheese production facility, supporting the importance of indigenous microorganisms for the microbial assemblage of cheese microbiota.

**Highlights:**

- Deciphering the microbial diversity of 12 French cheese varieties.
- Sample clustering according to cheese varieties and manufacturing processes.
- Patterns specific to the cheese production facility.

**Keywords:** cheese, microbial diversity, amplicon sequencing.

## 1. Introduction

Cheese is one of the oldest fermented food products. The cheese fermentation process is deeply related to culture and tradition, especially in rural households and village communities. Cheese has been produced and consumed for thousands of years and has been adapted to the technical, social and economic conditions in various parts of the world. Moreover, the technological processes associated with the manufacturing environment shape the chemistry and microbiology of local cheeses, which subsequently develop the visual, odor and texture characteristics that express and embody the identity of traditional cheese. There are over 1400 traditional cheese varieties identified around the world, displaying an amazing diversity of textures, aromas and flavors. France alone represents almost 1,000 different cheese varieties, including 45 registered under the Protected Designation of Origin (PDO), a European protection program that covers traditional cheese practices (Donnelly, 2013). One of the main characteristics of PDO cheeses produced, processed and prepared from raw or heat-treated milk is that they harbor a complex microbial community. Through their growth and metabolic activities during the cheese-making process and ripening period, these microbial communities contribute to the transformation of milk into final products that are recognized, appreciated and envied by consumers worldwide (Montel et al., 2014). Thus, microbial communities appear to be major players in the expression and perception of cheese organoleptic properties as well as in terms of safety since they act as a biopreservative shield against microbial pathogenic and spoilage populations (Grattepanche et al., 2008; Marcelino, 2013).

Over the past 15 years, microbial diversity studies of several varieties of European cheeses that combine both genotypic and phenotypic approaches have partly described the complexity of such communities (Irlinger et al., 2015). It has been shown that each cheese has a specific, dense microbiota ( $2-3 \times 10^9$  cells/g cheese) composed of a few to several dozen species. Importantly, more than half of the microorganisms detected were not deliberately inoculated as starters but originated from the milk itself and/or the manufacturing environment (Feurer et al., 2004; Goerges et al., 2008). The molecular approaches based on the use of metagenomics combined with high-throughput sequencing (HTS) technologies now offer the unprecedented opportunity to profile dominant as well as subdominant cheese microbial populations on a large scale (Ercolini, 2013; Parente et al., 2016). For example, Wolfe et al. (2014) and Quigley et al. (2012) used such approaches to reveal the microbial composition of 137 cheese rind communities sampled in ten different countries and from 62 Irish artisanal cheeses, respectively. These pioneer studies allowed, for example, the correlation between abiotic

factors such as moisture and rind type (natural, bloomy and washed) with microbial community composition. Such an approach was also used for identifying possible microbial reservoirs in two American cheese production facilities (Bokulich and Mills, 2013). Most HTS studies limit taxonomic affiliation to the genus level due to technological (e.g., sequence length) and/or data processing issues (e.g., sequence database availability). Currently, there is a need for increasing taxonomic resolution in order to study possible relationships that exist between cheese species composition and technological/environmental parameters.

The objective of this study was to investigate the specificity and diversity of cheese microbiota associated with 60 cheeses belonging to 12 traditional French cheese varieties manufactured from cow's milk, and including nine PDO cheese varieties. These varieties were selected from among uncooked washed-rind and natural-rind cheeses and covered a wide range of technological processes (**Fig. 1**). We used high-throughput amplicon sequencing (V3-V4 region of the 16S rRNA gene and ITS2) to characterize both the bacterial and fungal community composition and to propose sequence affiliation down to the species level whenever possible. Specifically, we aimed to determine whether microbial profiles can be used as a signature for a technological process, a cheese variety or even a cheese factory.

## 2. Material and Methods

### 2.1. Sample collection and DNA extraction

Ready-to-consume cheeses were purchased from a master cheese ripener (Fromagerie Beaufils, Paris, France, <http://www.fromagerie-beaufils.com/>) who is directly supplied by different carefully-selected cheese producers, from local producers or from the local supermarket. A total of 60 cheeses were collected, encompassing 12 distinct cheese varieties and diverse manufacturing processes (**Fig. 1**). Regarding the Saint-Marcellin, Maroilles, Soumaintrain, Langres, Pont l'Evêque, Reblochon, Abbaye de Cîteaux and Mont d'Or varieties, three independent cheeses were purchased at the same date. Rind was gently separated from the core using sterile knives and only the rind fraction was analyzed. Regarding Epoisses, Munster, Livarot and Saint-Nectaire, which were chosen to cover four contrasted manufacturing processes (coagulation mostly due to lactic acidification, mixed or mostly due to rennet action and for the last one without pressure or with high pressure), the sampling effort was greater. For each variety, three independent cheeses were purchased at the same date from three different producers (named Factories 1, 2 and 3; different factories were selected for each cheese variety), for a total of nine cheeses per variety, in order to

evaluate variation observed within cheese varieties. Rind was gently separated from the core using sterile knives and both rind and core fractions were analyzed in order to obtain a more detailed picture of the microbial diversity for those cheeses. Cheese samples were diluted 1:10 (w/v) in sterile distilled water and homogenized with an Ultra Turrax® (Labortechnik) at 8000 rpm for 1 min. DNA extraction was performed on 0.5 g of the mixture using the bead beating-based protocol detailed in a previous study (Dugat-Bony et al., 2015). DNA concentration was determined using a Qubit fluorometer (Life Sciences) according to the Broad Range DNA assay kit protocol.

### *2.2. Bacterial 16S rRNA gene amplification and sequencing*

PCR amplification, library preparation and sequencing were performed at the GeTPlaGe platform (Toulouse, France). Briefly, the V3-V4 region of the 16S rRNA gene (nearly 460 bp long) was amplified from 10 ng of purified genomic DNA using the primers F343-TACGGRAGGCAGCAG (Liu et al., 2007) and R784-TACCAGGGTATCTAATCCT (Andersson et al., 2008), as previously described (Lazuka et al., 2015). The resulting libraries were purified using Ampure beads (Beckman Coulter Genomics) and loaded onto the Illumina MiSeq cartridge according to the manufacturer's instructions. Each paired-end sequence was assigned to its corresponding sample according to a previously integrated index.

### *2.3. Fungal ITS2 amplification and sequencing*

PCR amplification, library preparation and sequencing were performed at Genoscreen (Lille, France). Briefly, the Illumina sequencing MiSeq ITS2 libraries were prepared by PCR from 10 ng of purified genomic DNA according to the GenoScreen protocol, using the primers ITS3-GCATCGATGAAGAACGCAGC (White et al., 1990) and ITS4\_KYO1-TCCTCCGCTTWTTGWTWTGC (Toju et al., 2012), generating amplicons with a size ranging from 240 to 400 bp. According to Illumina (personal communication), this difference (< 200 bp) don't bias the results. The resulting libraries were purified using Ampure beads (Beckman Coulter Genomics) and loaded onto the Illumina MiSeq cartridge according to the manufacturer's instructions. Each single-end sequence was assigned to its corresponding sample according to a previously integrated index.

### *2.4. Data analysis*

For bacteria, paired-end reads were merged using Flash (Magoč and Salzberg, 2011) prior to further analyses. FASTQ files were then quality-filtered and analyzed using QIIME v.1.9.0

(Caporaso et al., 2010b). For bacteria, any sequences  $< 400$  bp and  $> 500$  bp were discarded. For fungi, the expected sequence length ranged from 180 to 210 bp. Furthermore, sequences with an average Phred quality score  $< 25$ , containing at least one ambiguous base or exhibiting at least one error in primer sequences, were discarded. Chimeric sequences were identified using USEARCH (Edgar, 2010) implemented in QIIME's pipeline using both the reference mode against either the SILVA LTP database (release 119) (Yarza et al., 2008) for bacteria or the UNITE fungal ITS database (version 7.0) (Abarenkov et al., 2010) for fungi and, subsequently, the *de novo* mode. Operational taxonomic units (OTUs) were clustered at 97% identity with QIIME's *de novo* OTU-picking pipeline using UCLUST (Edgar, 2010). Any OTU comprising less than 200 reads (almost 0.005% of total sequences for each run) were removed, as recommended by Bokulich et al. (2013). Representative sequences for each OTU were classified taxonomically using BLAST (Altschul et al., 1990) against the same databases used for chimera checking, and assignments were controlled using the EzTaxon database (Kim et al., 2012). When multiple affiliations were possible at the species level, we chose one representative species and added the term "group" in the final affiliation. Alpha-diversity metrics, corresponding to variables describing diversity within samples, were computed using QIIME. Bacterial 16S rRNA gene sequences were aligned using PyNAST (Caporaso et al., 2010a) and the resulting alignment was used to build a phylogenetic tree using FastTree (Price et al., 2010). UniFrac distances (Lozupone et al., 2011) were calculated for community comparison between samples (referred to as beta diversity). For fungi, the non-phylogenetic Bray-Curtis distance was used for sample comparison because it was not possible to align ITS2 sequences. Bacterial OTU-sample networks were computed using Gephi software 0.9.0 (Bastian et al., 2009). For a better visualization, only edges corresponding to the presence of OTUs with a relative abundance  $> 0.5\%$  in the samples were represented on the graph.

### 2.5. Statistical analysis

The Pearson correlation was used to evaluate the relationship between pH values and alpha-diversity metrics or the relative abundance of certain microbial groups. The effect of categorical variables (e.g., sampling zone, manufacturing process) on alpha-diversity metrics was evaluated using ANOVA. Pairwise comparisons of alpha-diversity metrics between rind and core samples from the same cheese (set of three replicates) were performed using the paired Student's t-test and a confidence level of 0.95. Comparisons of alpha-diversity metrics between sample groups of different sizes were performed using the Mann-Whitney U-test and

a confidence level of 0.95. Regarding beta-diversity metrics (weighted UniFrac distances for bacteria and Bray-Curtis distances for fungi), MANOVA with 999 permutations was used to test significant differences between sample categories using the Adonis function implemented in QIIME.

### 2.5. Sequence accession numbers

Raw sequence data were deposited at the Sequence Read Archive of the National Center for Biotechnology Information under the accession numbers SRP071345 and SRP071552.

## 3. Results

### 3.1. Structure of cheese microbiota

A total of 3,683,334 16S rRNA (V3-V4 regions) and 2,187,729 ITS2 sequencing reads were generated from the 96 cheese samples, covering 12 different cheeses varieties (Mont d'Or, Livarot, Munster, Epoisses, Langres, Saint-Nectaire, Saint-Marcellin, Reblochon, Maroilles, Pont l'Evêque, Abbaye de Citeaux and Soumaintrain). An average of 22,000 fungal and 35,000 bacterial sequences per sample were obtained after quality filtering and chimera removal. These sequences were clustered into 44 fungal and 76 bacterial OTUs for which taxonomic assignment was possible down to the species level (or group of species sharing almost identical sequences) in most cases (**Fig. 2, Tables S1 and S2**). These OTUs belonged to four different bacterial phyla (Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes) and three fungal phyla (Ascomycota, Zygomycota and Basidiomycota).

Little variation was observed regarding the fungal community composition over cheese varieties and sample types (rind or core) (**Fig. 2A**). Indeed, most samples were dominated by OTUs affiliated with *Geotrichum candidum*, *Debaryomyces hansenii* and *Candida sake* (> 90% combined sequence relative abundance), with minor detection of other fungal species. It is important to note that three OTUs representing non-negligible sequence proportions in Saint-Nectaire and Soumaintrain cheeses displayed no similarity with fungal ITS2 sequences present in the UNITE database (listed as unknown species and highlighted in black in **Fig. 2A**).

Bacterial community composition was much more variable among the samples (**Fig. 2B**). Eleven bacterial OTUs, affiliated with *Corynebacterium* group *casei*, *Leucobacter aridiculis*,

*Arthrobacter arilaitensis*, *Enterococcus faecalis*, *Lactobacillus group casei*, *Lactococcus lactis*, *Streptococcus thermophilus*, *Halomonas variabilis*, *Psychrobacter aquimaris*, *P. cibarius* and *Pseudoalteromonas prydzensis* were detected in almost all of the cheese samples (core microbiota). Their relative abundance was systematically high in several samples and at least > 1% in every sample. Conversely, few OTUs were detected in only one sample, and for most cases, their relative abundance were never > 3%. It is noteworthy to mention the high presence (14%) of *Myroides injenensis* on the surface of Soumaintrain. A large number of bacterial OTUs, namely those affiliated with the *Brevibacterium*, *Corynebacterium*, *Arthrobacter*, *Bavariicoccus*, *Vagococcus*, *Psychrobacter* and *Pseudomonas* genera, were mostly detected in rind samples, ranging from 20% to 55% of the total number of reads. On the contrary, OTUs affiliated with Lactic Acid Bacteria commonly used as starters (mostly *Lactococcus lactis* and *Streptococcus thermophilus*) clearly dominated all of the cheese cores as well as the rind samples from Langres and Saint-Marcellin, distinguishing them from the other cheese varieties.

### 3.2. Community richness and diversity among the 12 cheese varieties

Community richness and diversity were assessed for each cheese variety using five alpha-diversity metrics (observed OTU, Chao1, Dominance, Equitability and Shannon entropy). The data are presented in **Table 1** for rind samples and **Table 2** for core samples, respectively. The number of observed bacterial OTUs and the Chao1 estimator were much lower in Saint-Marcellin, Langres and Epoisses rinds than in other cheese varieties. For those cheese varieties, we also observed a very high dominance associated with a low equitability and Shannon entropy, indicating a low-diverse bacterial community composition with few dominant populations. Mont-d'Or and Soumaintrain exhibited the richest surface bacterial communities (45 and 44 observed bacterial OTUs on average, respectively) and showed high equitability and Shannon entropy values.

Regarding fungal communities, Saint-Nectaire showed the highest observed OTU number (> 21 on average), whereas Abbaye-de-Citeaux showed the lowest (< 11 on average). The Maroilles fungal community exhibited a very high dominance index and a very low equitability, which can be explained by the high proportion of sequences affiliated with *Debaryomyces hansenii* (**Fig. 2**). No significant correlation was observed between bacterial and fungal OTU number (Pearson correlation,  $r^2 = 0.010$ ,  $P = 0.337$ ) (**Fig. S1**).

Bacterial richness (observed OTUs) was significantly higher and dominance was significantly lower in rind samples than in core samples ( $P < 0.001$ , analysis of variance (ANOVA)).



Comparison of each set of three replicates indicated that these differences were observed for most Livarot, Munster and Saint-Nectaire cheeses (**Fig. 3**). In contrast, Epoisses cheeses exhibited similar bacterial diversity between rind and core samples. We did not observe significant differences between rind and core regarding the fungal diversity index.

Analysis of the alpha-diversity data (**Tables 1 and 2**) also revealed a significant effect of the cheese manufacturing process on the observed OTU number and dominance index for both bacterial and fungal communities ( $P < 0.05$ , analysis of variance (ANOVA)). In rind samples, cheeses produced by dominant lactic acidification (i.e., Saint-Marcellin, Maroilles, Soumaintrain and Epoisses) had a less diverse bacterial surface microbiota than those produced by enzymatic coagulation and accelerated draining, (i.e., Pont l'Evêque, Livarot, Mont d'Or, Abbaye de Citeaux, Reblochon and Saint-Nectaire) ( $P < 0.05$ , Mann-Whitney test). Similarly, cheeses produced by dominant lactic acidification or mixed coagulation (i.e., Saint-Marcellin, Maroilles, Soumaintrain, Epoisses, Langres and Munster) had a less diverse fungal community than those produced by enzymatic coagulation and accelerated draining (i.e., Pont l'Evêque, Livarot, Mont d'Or, Abbaye de Citeaux, Reblochon and Saint-Nectaire) ( $P < 0.05$ , Mann-Whitney test).

Finally, significant correlations were observed between pH values and observed OTU numbers and the dominance index for bacteria (Pearson correlation,  $r^2 = 0.086$ ,  $P < 0.005$ ;  $r^2 = 0.267$ ,  $P < 0.0001$ , respectively) but not for fungi (**Fig. S2**).

### *3.3. Comparison of community structure among the 12 cheese varieties*

Comparison of community structure between samples was performed using beta-diversity metrics, i.e., abundance-weighted UniFrac for bacterial communities and Bray-Curtis for fungal communities. Principal coordinate analysis (PCoA) on the distance matrix revealed a strong influence of cheese sampling area (rind vs. core) on the bacterial community structure (**Fig. 4A**) but not on the fungal community structure (**Fig. 4B**). Rind samples were clustered by cheese variety (**Fig. 4C-D**) and manufacturing process (**Fig. 4E-F**) regarding both bacterial and fungal data, and this was supported by the Adonis statistical test ( $P < 0.001$ ). Collectively, these results suggest that cheesemaking practices shape cheese surface microbiota.

Pearson correlation analyses were computed to examine possible relationships between pH values and relative abundances of the different phyla or bacterial groups (**Fig. S3**). Relative abundances of Actinobacteria, Proteobacteria and the lactic acid bacteria (LAB) groups were significantly correlated with cheese pH ( $P < 0.0001$ ). Indeed, relative abundances of the LAB group decreased with pH (**Fig. S3D**) and, conversely, relative abundances of Actinobacteria

and Proteobacteria increased with pH (**Fig. S3A-B**). In contrast, NSLAB-HALAB group abundance showed no significant relationship with cheese pH (**Fig. S3C**).

#### 3.4. Specific taxa observed in some cheese varieties or cheese factories

As mentioned above, fungal communities observed in the 12 cheese varieties were highly dominated by a few OTUs. It is noteworthy that OTUs affiliated with *G. candidum* and *D. hansenii* were detected in all cheese varieties (**Fig. 2A, Table S1**). On the contrary, OTUs affiliated with *Mucor lanceolatus* and *M. racemosus* were only detected in Saint-Nectaire cheeses. *M. lanceolatus* was detected in Saint-Nectaire cheeses collected from the three factories, whereas *M. racemosus* was only detected in cheeses collected from Factories 1 and 2.

Rind bacterial communities were more complex (**Fig. 2B, Table S2**). We therefore decided to focus our attention on four cheese varieties, i.e., Epoisses, Livarot, Munster and Saint-Nectaire, and to build an OTU-sample network in order to visualize whether specific OTUs were possibly associated with one or several cheese varieties and/or specific factories (**Fig. 5**). This analysis confirmed the clustering of the samples according to the cheese varieties, already observed using beta-diversity metrics (**Fig. 4, Table S3**). Furthermore, among the four tested varieties, we observed that Livarot has the widest variation in microbiota between factories. Interestingly, simplified sub-networks computed for each cheese variety (**Fig. S4**) clearly showed that within varieties, samples were clustered according to the production facility (data supported by weighted UniFrac distances, **Table S4**).

Among the most abundant OTUs, some were shared by several cheese varieties such as those affiliated with the common starters (e.g., *Lactococcus lactis* and *Streptococcus thermophilus*) or ripening cultures (e.g., *Arthrobacter arilaitensis* and *Corynebacterium group casei*), as well as more surprising taxa such as *Psychrobacter aquimaris*. On the contrary, other OTUs were preferentially found in one or two cheese varieties. For example, the OTU corresponding to *Brevibacterium aurantiacum* was highly abundant in all Saint-Nectaire cheeses, regardless of the facility, but was not detected - or in very little abundance - in other cheese varieties. The OTU affiliated with *Halomonas variabilis* was more specifically detected in Livarot cheeses. Similarly, the OTU affiliated with *Vagococcus salmoninarum* was essentially detected in both Munster and Livarot cheeses.

Finally, specific OTUs could be clearly distinguished according to the factory within each cheese variety (**Fig. S4**). For example, OTUs affiliated with *Psychroflexus halocasei*, *Marinilactibacillus* group *psychrotolerans*, *Brochotrix thermosphacta*, *Marinomonas foliarum* and *Pseudoalteromonas prydznensis* were OTUs that distinguished cheeses produced in different facilities within the Munster variety.

#### 4. Discussion

Cheese microbial communities are rich and diverse, and provide ideal model systems for microbial ecology studies (Irlinger et al., 2015; Wolfe et al., 2014). With the recent development of next generation sequencing technologies, characterization of microbial communities from cheeses collected around the world is currently an active field of research (Delcenserie et al., 2014; Ercolini et al., 2012; Parente et al., 2016; Quigley et al., 2012; Riquelme et al., 2015; Wolfe et al., 2014). In this work, we contributed to this effort by investigating the microbial communities of 96 cheese samples covering 12 French cheese varieties among the most appreciated by consumers and belonging to natural and washed-rind cheeses made from cow's milk. Bacterial communities were mainly composed of known taxa. Indeed, all the 76 bacterial OTUs detected were unambiguously assigned to the genus level, and the great majority (68) down to the species or group of species level. This finding confirmed that closely-related sequences obtained from cultivated representative strains are already present in public repositories. Analyzing fungal diversity highlighted that some cheese varieties such as Saint-Nectaire and Soumaintrain still hosted unknown species (illustrated by three OTUs with representative sequences that did not match any other sequences from the UNITE database) that may account for a large proportion of the total community. Microbial isolation targeting such fungal species should thus now be performed in order to obtain new information about these unknown microbes.

The composition of microbial communities varies according to the cheese variety, corroborating the results already observed by Quigley et al. (2012) in a similar study performed on Irish cheeses. In their large-scale study, Wolfe et al. (2014) highlighted the importance of rind type and moisture on the structure of cheese microbial communities. Here, by focusing mainly on washed rind and natural rind cheeses, we also observed a great influence of the cheese manufacturing process, either with respect to alpha and beta-diversity metrics. This finding suggests that other parameters, such as the coagulation type (lactic

versus rennet) or the draining method, also play a role in shaping microbial communities. Specific studies should now be designed to evaluate each of these parameters in an individual manner and with more details.

As expected, cheese surface microbiota was generally much more diverse and equitable than that of the cheese core, except for Epoisses cheese. A positive correlation was also observed between bacterial diversity and pH, but not for fungi that are generally known to be tolerant to this factor (Masoud and Jakobsen, 2005). Furthermore, a positive correlation was found between pH and the relative abundance of certain bacterial groups such as Actinobacteria and Proteobacteria and a negative correlation was observed with LAB. In fact, the surface-ripened cheese manufacturing process classically involves the early development of LAB at low pH, followed by deacidification by yeasts and/or fungi, allowing the growth of acid-sensitive bacteria including Actinobacteria and Proteobacteria (Larpin et al., 2006; Larpin-Laborde et al., 2011; Mounier et al., 2008).

We did not observe any correlation between fungal and bacterial richness across the 12 cheese varieties studied in this work, suggesting that cheese varieties with rich bacterial communities do not necessarily have rich fungal communities. In contrast with our study, a significant correlation between fungal and prokaryotic richness was reported by Wolfe et al. (2014), who applied high-throughput amplicon sequencing to 137 rind samples collected from cheeses produced worldwide. The authors hypothesized that species interactions or environmental factors select for communities with similar compositions. This can probably be partially explained by changes in pH that can affect microbial community structure by either promoting or inhibiting the growth of acid-sensitive organisms. In other words, whereas yeast metabolism causes deacidification that favors the growth of less-acid-tolerant bacterial strains, the presence or absence of particular acid-sensitive prokaryotic groups (Actinobacteria and Proteobacteria) often implies the presence or absence of corresponding fungal taxa (Mounier et al., 2008). In this work, this relationship was not observed probably because we kept reads matching against known LAB starters (belonging to *Streptococcus*, *Lactobacillus* and *Lactococcus* genera) in our analysis of rind samples, which was not the case in the study of Wolfe et al. (2014). The presence of this group, known to inhabit environments under a broad range of pH conditions, is not conditioned by fungal presence and activities.

On the basis of microbial diversity profiles, Saint-Marcellin and Langres can be easily distinguished from the other cheese varieties. They exhibited poor microbial communities

highly dominated by yeasts (mainly *G. candidum*) and LAB (mainly *L. lactis*) - microorganisms traditionally inoculated into this type of cheese - and resembling what one can observe when looking at the core of several cheese varieties. On the contrary, some cheese varieties had more diverse and equitable communities, such as Saint-Nectaire, Mont d'Or, Munster and Livarot. In those cheese varieties, a wide array of Actinobacteria, Proteobacteria, NSLAB and HALAB were detected on the rind in addition to the common LAB starters, confirming previous work on cheese surface bacterial communities (reviewed in Irlinger et al., 2015).

This study provides new insights into the prevalence of psychrophilic bacteria in cheese products. Indeed, according to our results, microbial species belonging to *Psychrobacter*, *Pseudomonas*, *Pseudoalteromonas*, *Halomonas* and *Psychroflexus* genera are very common in traditional French cheeses and sometimes very abundant. For example, dominant bacterial OTUs detected in Epoisses cheeses sampled in this work were affiliated with *Psychrobacter cibirius* and/or *P. aquimaris*. Such species are generally adapted to cold aquatic and hypersaline environments including marine water, permafrost and sea ice (Rodrigues et al., 2009) but their detection has already been reported in many cheese products worldwide (Delcenserie et al., 2014; Mounier et al., 2009; Quigley et al., 2012; Wolfe et al., 2014), and in other food products as well (Chaillou et al., 2015). The high prevalence of psychrophilic bacteria in ready-to-consume food products, particularly in cheese, might be due to storage conditions applied for transportation and distribution to grocery stores (temperature of 4-8°C for days or weeks) that can favor their development while limiting the growth of other mesophilic microbial species. Their origin in cheeses is not clearly identified at the moment, but milk (de Oliveira et al., 2015; Quigley et al., 2013) and the processing environment including working surfaces, materials, water, brine, sea salt, shelves and airflows (Bokulich and Mills, 2013; Stellato et al., 2015) constitute the most probable sources of such bacteria.

Some microbial species were detected in several cheese varieties and can thus be considered as being well adapted to the cheese habitat. Among eukaryotes, we can mention *G. candidum*, *D. hansenii*, *Kluyveromyces* species, *Yarrowia lipolytica*, *Candida* species, *Clavispora lusitania*, *Cyberlindnera jadinii*, *S. cerevisiae* and *Torulasporea delbrueckii*, which are commonly detected in many beverages and food products including cheeses (Irlinger et al., 2015), and some others that seem more specific to dairy products such as *Penicillium camembertii* (identified as *P. commune* because of identical ITS sequences), *Trichothecium domesticum*, *Mucor lanceolatus* and *M. racemosus* (Hermet et al., 2014, 2012; Ropars et al.,

2012). *Mucor* species, responsible for the 'cat-hair' characteristic in cheese products (Hermet et al., 2012), were only detected in Saint-Nectaire cheese (natural rind) in our survey. Regarding bacterial diversity, we were able to identify some OTUs that were specific to one or few cheese varieties (e.g., *Desemzia incerta* in Mont d'Or, *Myroides sp.* in Soumaintrain). But one of the central findings of our study is that we were also capable to shed light onto OTUs specific to one factory among certain varieties (e.g., *Arthrobacter alpinus* and *Lactococcus formosensis* in Saint-Nectaire from Factory 2), revealing potential signatures that can help producers to better understand and monitor microbial assemblages in their products.

Large-scale microbial inventories in food-related habitats provide the unique opportunity to identify microbial taxa without reference to food isolates. Targeted isolation of these microbes along with genome sequencing is now critical to facilitate functional and quantitative analysis of future meta-omic data.

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### Figure legends

**Figure 1.** Classification of the 12 studied cheese varieties according to their manufacturing process. For all of the cheese varieties, three independent cheeses were sampled and the rind was analyzed. For four cheese varieties, highlighted in red, three independent cheeses were sampled in three different factories and both rind and core were analyzed. Rind type is written in blue.

**Figure 2.** Microbial composition of the 12 cheese varieties. Vertical bars indicate the average relative abundance of sequences corresponding to fungi (A) and bacteria (B) at the species level. Fungal sequences that could not be assigned are colored in black and labeled as "unknown species". Cheese varieties were ordered based on weighted UniFrac distances computed on the bacterial dataset.

**Figure 3.** Comparison of bacterial diversity metrics between rind and core samples for four cheese varieties sampled in different factories. The number of observed OTUs (97% identity level) is shown in A and the dominance index in B. \* $P < 0.05$ , \*\* $P < 0.005$  (paired t-test testing alternative hypothesis "greater" or "less" for the number of observed OTUs and the dominance index, respectively). F1, F2 and F3 represent the different factories sampled for each cheese variety.

**Figure 4.** Differences in cheese microbial community composition highlighted by principal coordinate analysis; A and B: comparison of rind vs. core samples; C and D: comparison based on the rind samples from the 12 cheese varieties; E and F: comparison based on the rind

samples classified according to the manufacturing process; A, C and E: data from the bacterial 16S rRNA gene study (weighted UniFrac distance matrix); B, D and F: data from the fungal ITS2 study (Bray-Curtis distance matrix). P values obtained for permutational MANOVA analysis (Adonis statistical test) indicate significance between sample groups.

**Figure 5.** OTU-sample network summarizing the relationships between bacterial taxa and 36 cheese rind samples belonging to four cheese varieties (Munster, Epoisses, Livarot and Saint-Nectaire). Nodes represent OTUs and cheese samples. Connecting edges indicate the detection of OTUs in the samples. Only edges corresponding to OTUs detected at 0.5% relative abundance in the samples are shown. For sample nodes, different colors (brown, orange, blue and pink) are used to differentiate cheese varieties (Munster, Epoisses, Livarot and Saint-Nectaire, respectively) and sizes are made proportional to bacterial richness (i.e., number of connected OTU nodes). For OTU nodes, different colors (gray, purple, green and greenish-blue) are used to differentiate bacterial phyla (Bacteroidetes, Proteobacteria, Firmicutes and Actinobacteria, respectively) and sizes are made proportional to degree (i.e., for OTUs, this measures the total occurrence of an OTU in the whole dataset).

**Table 1:** Alpha-diversity metrics characterizing cheese rind samples and inferred from the sequencing of 16S V3-V4 and ITS2 amplicons. Sequences were clustered at 97% identity and rarefied to 6450 sequences for bacteria and 12290 for fungi per sample. Letters (a, b, c, etc.) indicate significantly different means at  $P < 0.05$  (analysis of variance (ANOVA)).

Cheese variety	Manufacturing process	Bacteria					Fungi				
		Observed OTUs	Chao1	Dominance	Equitability	Shannon	Observed OTUs	Chao1	Dominance	Equitability	Shannon
<b>Epoisses</b>	Lactic, slow draining	17.44 e	19.41 e	0.44 b	0.44 b	1.79 de	12.81 b	14.70 ab	0.54 bc	0.29 b	1.05 abc
<b>Maroilles</b>	Lactic, slow draining	34.19 bc	37.34 bc	0.25 bcd	0.54 ab	2.76 bcd	11.80 b	13.21 ab	0.95 a	0.07 c	0.24 c
<b>Soumaintrain</b>	Lactic, slow draining	44.22 a	48.27 ab	0.10 d	0.72 a	3.90 a	12.63 b	14.08 ab	0.70 ab	0.26 bc	0.89 bc
<b>Saint-Marcillin</b>	Lactic, slow draining	16.85 e	23.16 de	0.78 a	0.16 c	0.66 f	10.93 b	12.43 ab	0.68 ab	0.23 bc	0.77 bc
<b>Langres</b>	Mixed, slow draining	19.54 e	23.92 de	0.42 bc	0.40 b	1.71 e	12.03 b	12.80 ab	0.53 bc	0.31 b	1.10 abc
<b>Munster</b>	Mixed, slow draining	29.77 bcd	33.43 cd	0.23 bcd	0.57 ab	2.79 bcd	11.34 b	12.97 ab	0.74 ab	0.21 bc	0.76 bc
<b>Livarot</b>	Rennet, cutting, stirring	35.12 bc	39.98 abc	0.16 d	0.64 a	3.30 abc	15.38 ab	18.52 ab	0.66 ab	0.26 bc	1.01 abc
<b>Pont l'Eveque</b>	Rennet, cutting, stirring	21.11 de	23.61 de	0.22 cd	0.58 ab	2.54 cde	13.10 b	14.66 ab	0.73 ab	0.19 bc	0.71 c
<b>Mont d'or</b>	Rennet, cutting, stirring, slight pressure	45.70 a	50.28 a	0.14 d	0.66 a	3.65 ab	11.30 b	12.23 ab	0.53 bc	0.30 b	1.06 abc
<b>Abbaye de Citeaux</b>	Rennet, cutting, stirring, high pressure	25.41 cde	29.65 cde	0.33 bcd	0.53 ab	2.47 cde	10.17 b	11.22 b	0.33 c	0.52 a	1.72 a
<b>Reblochon</b>	Rennet, cutting, stirring, high pressure	39.19 ab	45.59 ab	0.17 d	0.59 ab	3.15 abc	14.77 ab	21.42 ab	0.69 ab	0.22 bc	0.85 bc
<b>Saint-Nectaire</b>	Rennet cutting, stirring, high pressure	32.41 bc	38.01 bc	0.29 bcd	0.52 ab	2.63 bcde	21.02 a	23.89 a	0.51 bc	0.37 b	1.60 ab

**Table 2:** Alpha-diversity metrics characterizing cheese core samples and inferred from the sequencing of 16S V3-V4 and ITS2 amplicons. Sequences were clustered at 97% identity and rarefied to 6450 sequences for bacteria and 12290 for fungi per sample. Letters (a, b, c, etc.) indicate significantly different means at  $P < 0.05$  (analysis of variance (ANOVA)).

Cheese variety	Manufacturing process	Bacteria					Fungi				
		Observed OTUs	Chao1	Dominance	Equitability	Shannon	Observed OTUs	Chao1	Dominance	Equitability	Shannon
<b>Epoisses</b>	Lactic, slow draining	15.28 b	19.5 1 b	0.69 a	0.24 b	0.97 b	12.84 b	15.00 b	0.57 a	0.29 b	1.06 b
<b>Munster</b>	Mixed, slow draining	20.78 b	25.06 b	0.62 a	0.26 b	1.13 b	12.18 b	15.27 b	0.63 a	0.28 b	1.03 b
<b>Livarot</b>	Rennet, cutting, stirring	27.06 a	32.88 a	0.44 b	0.37 a	1.77 a	15.46 b	18.35 b	0.74 a	0.19 b	0.74 b
<b>Saint-Nectaire</b>	Rennet, cutting, stirring, high pressure	16.14 b	21.53 b	0.75 a	0.19 b	0.74 b	21.22 a	25.78 a	0.39 b	0.43 a	1.88 a



## Supplementary material

**Figure S1.** Correlation analyses between fungal and bacterial richness (Pearson correlation).

**Figure S2.** Correlation analyses between pH and fungal (A) or bacterial (B) OTU richness and fungal (C) or bacterial dominance (D) in cheese samples (Pearson correlation).

**Figure S3.** Relationships between pH values and relative abundance for different bacterial phyla and groups: Actinobacteria (A), Proteobacteria (B), Non Starter Lactic Acid Bacteria (NSLAB) – HALAB (Halophilic and Alkaliphilic Lactic Acid Bacteria) group (C) and Lactic Acid Bacteria (LAB) group (D). Different groups and phyla are composed of the following genera. Actinobacteria: *Actinomyces*, *Agrococcus*, *Arthrobacter*, *Brachybacterium*, *Brevibacterium*, *Citricoccus*, *Corynebacterium*, *Leucobacter*, *Microbacterium*. Proteobacteria: *Alcaligenes*, *Arcobacter*, *Enterobacter*, *Hafnia*, *Halomonas*, *Klebsiella*, *Marinomonas*, *Morganella*, *Pantoea*, *Parapusillimonas*, *Proteus*, *Providencia*, *Pseudoalteromonas*, *Pseudomonas*, *Psychrobacter*, *Serratia*, *Vibrio*. NSLAB- HALAB: *Bavariicoccus*, *Brochothrix*, *Carnobacterium*, *Desemzia*, *Enterococcus*, *Facklamia*, *Macroccoccus*, *Marinilactibacillus*, *Staphylococcus*, *Vagococcus*, *Weissella*. LAB: *Lactobacillus*, *Lactococcus*, *Streptococcus*.

**Figure S4.** OTU-sample sub-network summarizing the relationships between bacterial species and nine cheese rind samples belonging to one cheese variety (A: Munster; B: Epoisses; C: Saint-Nectaire; and D: Livarot). Nodes represent OTUs and cheese samples, and connecting edges indicate the detection of OTUs in the samples. Only edges corresponding to OTUs detected at 0.5% relative abundance in the samples are shown. For sample nodes, different colors (orange, blue and green) are used to differentiate the three different factories (F1, F2 and F3) and sizes are made proportional to bacterial richness (i.e., number of connected OTU nodes). The color purple is used for OTU nodes, and sizes are made proportional to the degree (i.e., for OTUs, this measures the total occurrence of an OTU over the whole dataset).

**Table S1:** Fungal OTU table.

**Table S2:** Bacterial OTU table.

**Table S3:** Weighted UniFrac distances between rind bacterial communities among four cheese varieties.

**Table S4:** Weighted UniFrac distances between factories for each cheese variety (Epoisses, Livarot, Munster and Saint-Nectaire).

ACCEPTED MANUSCRIPT

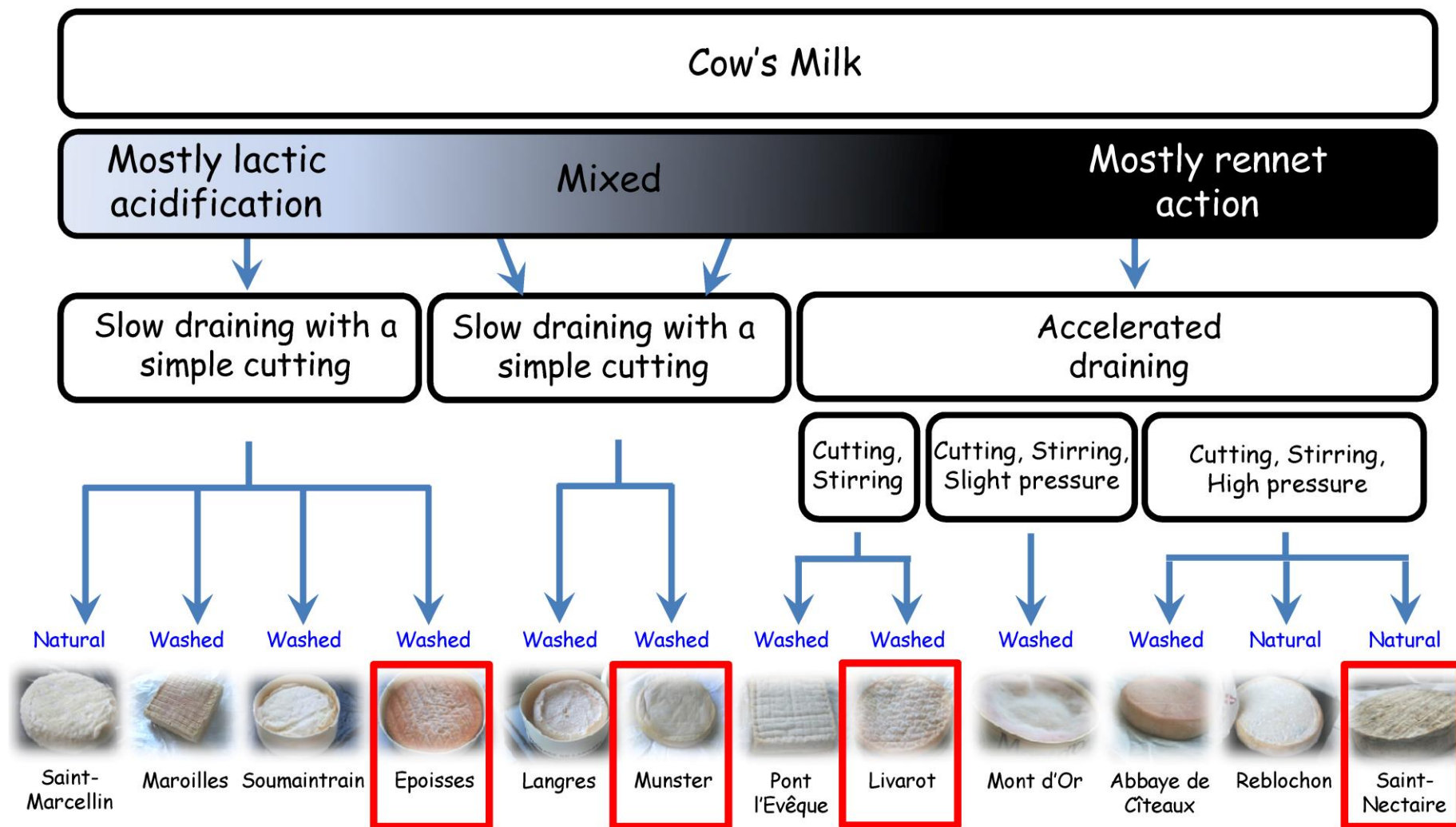


Figure 1

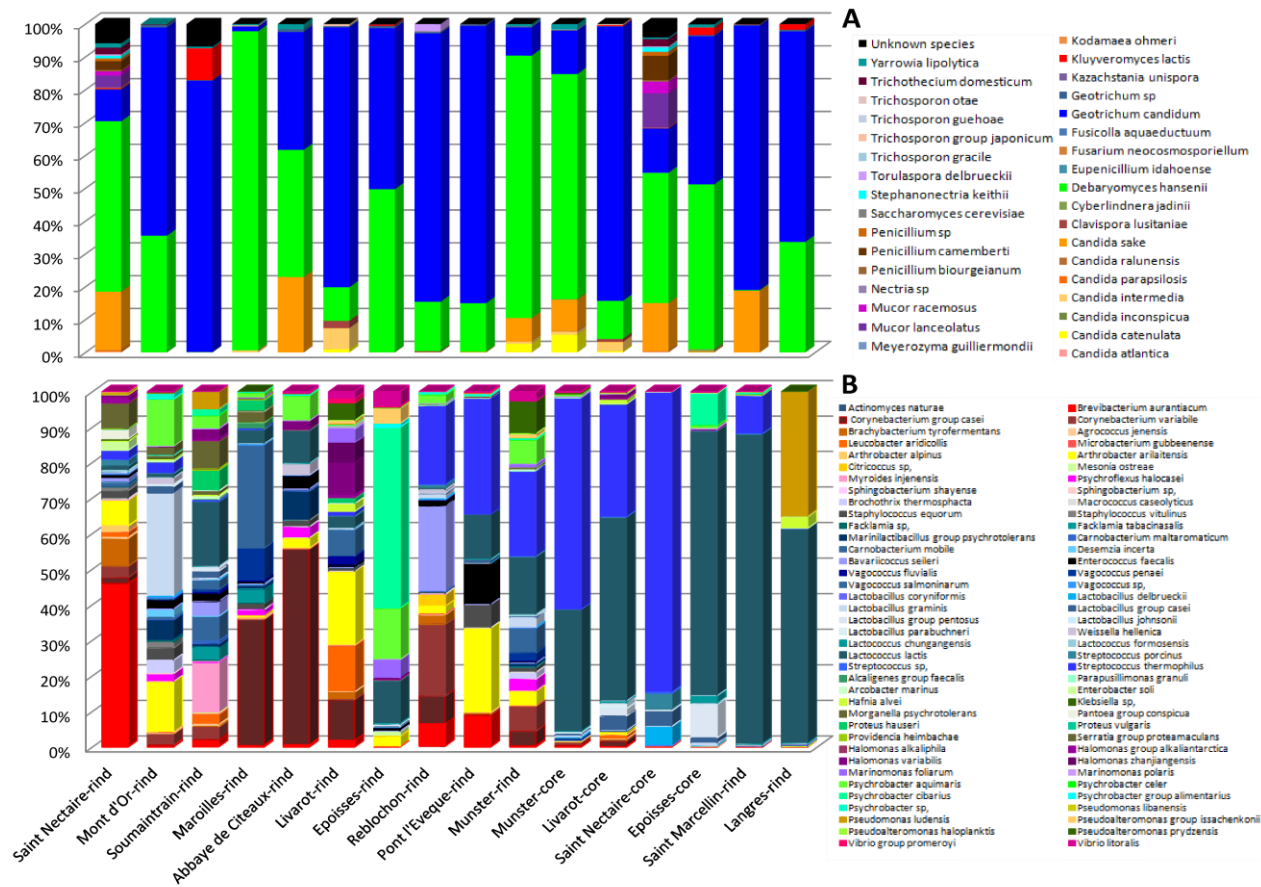


Figure 2

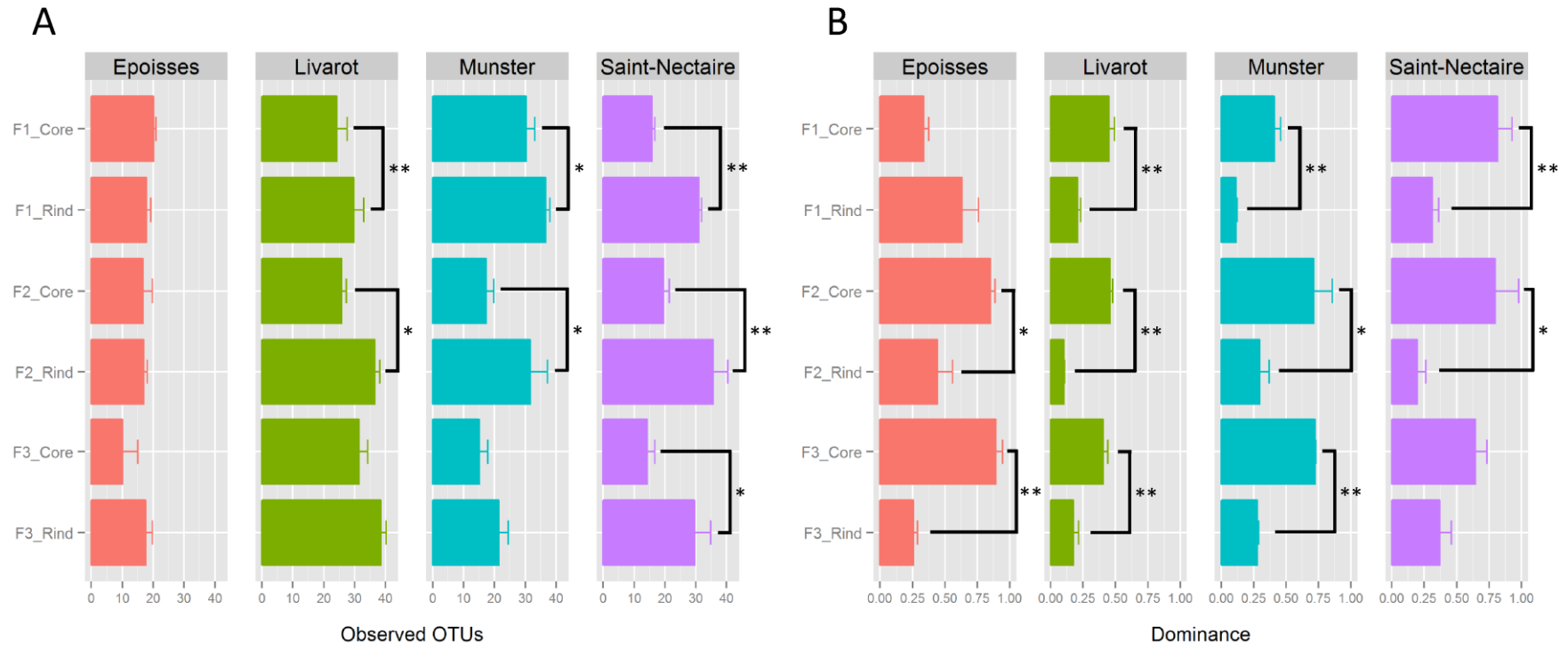


Figure 3

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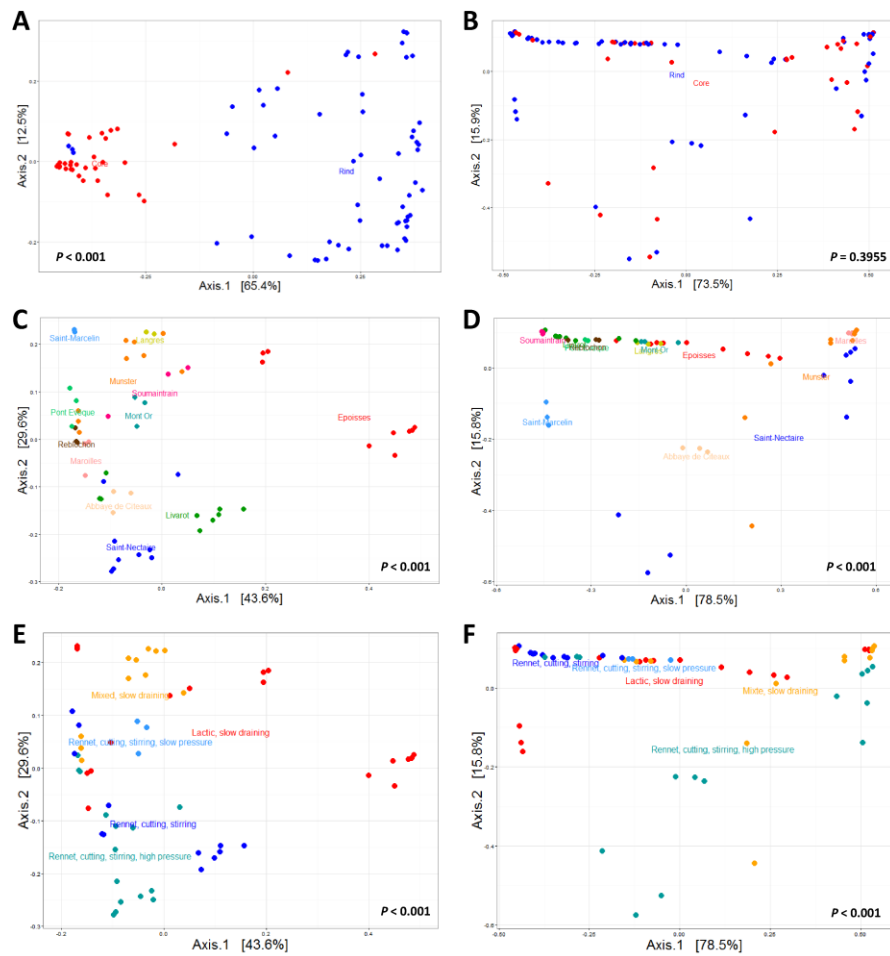


Figure 4

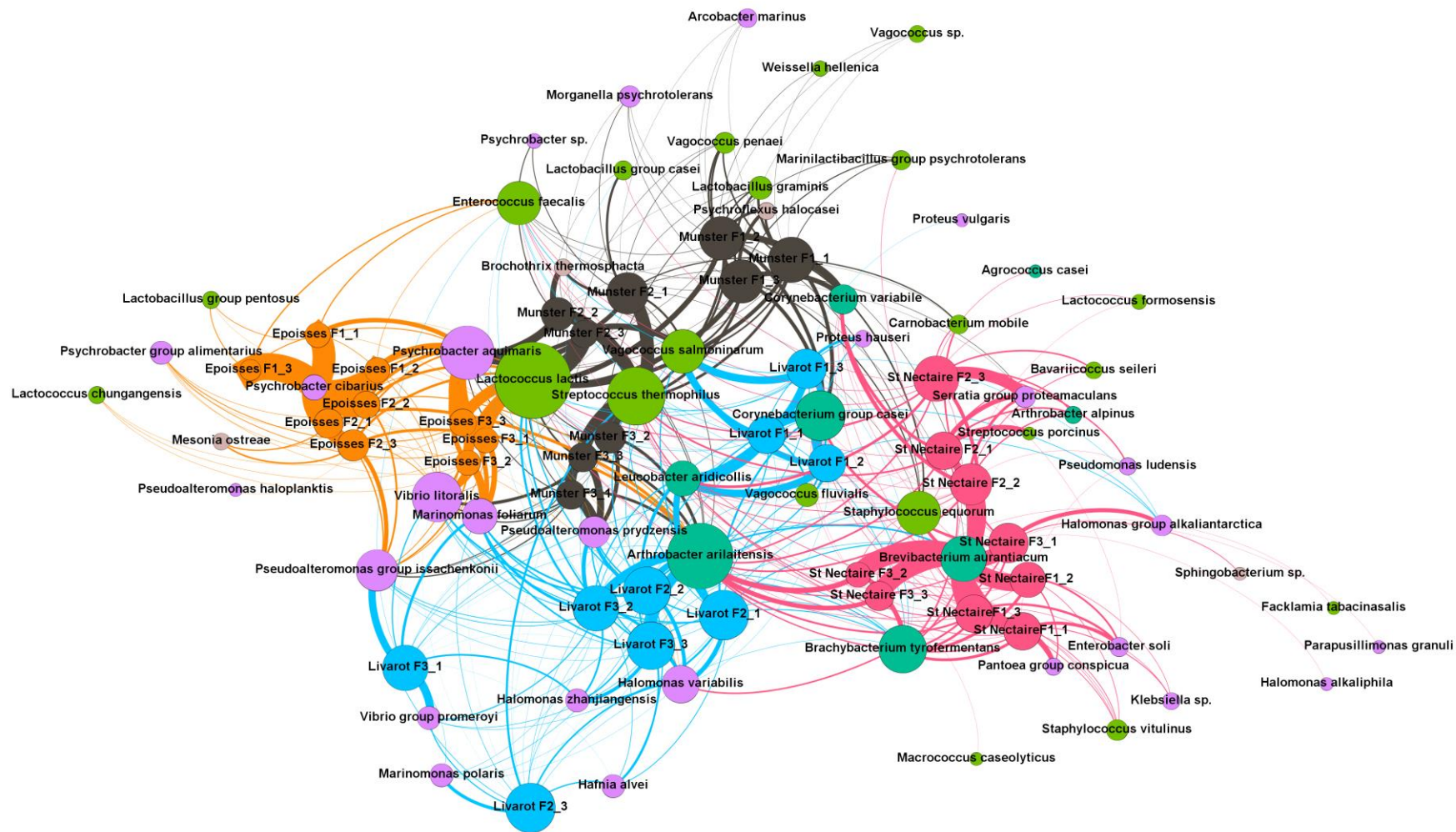


Figure 5

**Highlights:**

- Deciphering the microbial diversity of 12 French cheese varieties.
- Sample clustering according to cheese varieties and manufacturing processes.
- Patterns specific to the cheese production facility.

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