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Use of a metagenetic approach to monitor the bacterial microbiota of "Tomme d'Orchies" cheese during the ripening process

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Highlights:

- Metagenetic analysis on artisanal cheese
- High number of non-inoculated species within the ripening microbiota of a raw milk cheese
- Bacterial diversity originates from the environment and ripening process

Keywords: Artisanal cheese, Illumina, 16S rDNA, dynamic microflora, environmental strain, Operational taxonomic units

Abstract

The study of microbial ecosystems in artisanal foodstuffs is important to complete in order to unveil its diversity. The number of studies performed on dairy products has increased during the last decade, particularly those performed on milk and cheese derivative products. In this work, we investigated the bacterial content of "Tomme d'Orchies" cheese, an artisanal pressed and uncooked French cheese. To this end, a metagenetic analysis, using Illumina technology, was utilized on samples taken from the surface and core of the cheese at 0, 1, 3, 14 and 21 days of ripening process. In addition to the classical microbiota found in cheese, various strains likely from environmental origin were identified. A large difference between the surface and the core content was observed within samples withdrawn during the ripening process. The main species encountered in the core of the cheese were *Lactococcus* spp. and *Streptococcus* spp., with an inversion of this ratio during the ripening process. Less than 2.5% of the whole population was composed of strains issued from environmental origin, as *Lactobacillales, Corynebacterium* and *Brevibacterium*. In the core, about 85% of the microbiota was attributed to the starters used for the cheese making. In turn, the microbiota of the surface contained less than 30% of these starters and interestingly displayed more diversity. The predominant genus was *Corynebacterium* sp., likely originating from the environment. The less abundant microbiota of the surface was composed of *Bifidobacteria*, *Brevibacterium* and *Micrococcales*. To summarize, the “Tomme d’Orchies” cheese displayed a high diversity of bacterial species, especially on the surface, and this diversity is assumed to arise from the production environment and subsequent ripening process.

1. Introduction

France is a country with a rich cheese history and according to a recent report from CNIEL, there are more than 1,200 varieties, among which 400 are commonly consumed by people (CNIEL 2016). The different varieties of traditional and industrial cheeses are known to be endowed with specific microbiota (Beresford *et al.*, 2001; Irlinger and Mounier, 2009). Cheeses

are nowadays inoculated with standard and marketed starter cultures such as *Lactococcus lactis*, *Streptococcus thermophilus, Debaryomyces hansenii* and other strains (Irlinger and Mounier, 2009). However, a secondary microbiota composed of bacteria, yeasts and moulds, originated from milk and the environment that is usually recovered from the cheese's surface and core. The starters and autochthonous microbiota could interact during the milk fermentation, curd maturation and storage, leading therefore to some cheese specificities such as biochemical changes and typical sensory properties (Irlinger and Mounier, 2009; Lavoie *et al*., 2012; Mei *et al*., 2014; Monnet *et al.*, 2015). Cheeses microbial ecosystems are complex and novel insights on their ecosystems are anticipated to help in improving the control of ripening, extending the shelf-life and providing a better safety policy (Beresford *et al.*, 2001; Bockelmann *et al.*, 2005; Dugat-Bony *et al.*, 2015; Montel *et al.*, 2014).

The bacterial population in the microbiota can be identified using the prokaryotic universal primer, or 16S ribosomal DNA (Bokulich and Mills, 2012; O'Flaherty *et al.*, 2011; Quigley *et al.*, 2012; Wolfe *et al.*, 2014). The next generation sequencing, or NGS, such as Illumina sequencing, allows high throughput sequencing of total DNA of a sample without prior cultures and available data on viable-but-not-cultivable strains (Bokulich and Mills, 2012). The Illumina method consists in the amplification of a fragment of interest, for all samples using primers with indexing adapters specific to Illumina technology. A DNA library is then made by mixing of each sample, previously tagged by short PCR primers with indexing tails and specific to each sample. The library is sequenced by using terminator nucleotides with fluorescent dye, during the sequencing PCR (O'Flaherty *et al.*, 2011). The use of a metagenomic approach to decipher the microbial communities is becoming a routine, due to its swiftness and ease of utilisation (Delcenserie *et al*., 2014; Franzosa *et al.*, 2015).

The "Tomme d'Orchies" is an uncooked pressed cheese using local raw milk of Holstein cows. It is produced and marketed in the north of France for less than 50 years. A recent culture-dependent study performed in our laboratory, permitted us to unveil the yeasts content of this cheese (Ceugniez *et al.*, 2015). This work aimed at studying the bacterial content of "Tomme

d'Orchies" cheese using the metagenetic approach on the samples taken at different periods of the ripening process. Consequently, this study enabled us to establish a signature of the "Tomme d'Orchies" bacterial content during the ripening process.

2. Materials and Methods

2.1 Cheese manufacturing and sampling

The "Tomme d'Orchies" is a cheese coming from the "G.A.E.C de la Motte" farm located in Nomain (North of France). Starter cultures are PAL114D (*Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis* biovar *diacetylactis*, *S. thermophilus*, *Lactobacillus helveticus* and *Lb. lactis*), *Propionibacterium* PAL25-2 (*P. freudenreichi* subsp. *shermanii*) and *D. hansenii* (PAL DH10), were purchased from Standa laboratory (Caen, France). The ripening is carried out at 12°C for 3 weeks. After three days of ripening, the "Tomme d'Orchies" cheese is rubbed with a mix of brown beer, rocou and chicory (Ceugniez et al., 2015)

For the dynamic microbiota analysis, five samples of "Tomme d'Orchies" in different cheeses were harvested at 0, 1, 3, 14 and 21 days of ripening process. The selling of cheese takes place after 21 days of ripening. For each time of ripening, samples were cut in two parts, with a sterile knife, to have surface and core separately. The surface corresponded to the first 2 mm starting from the upper part of the cheese. Each sample was suspended in 9 $g.L^{-1}$ NaCl leading to a concentration of 100 $g.L^{-1}$ with a Dilumat (BioMérieux, Craponne, France), and homogenized for 90s using a Stomacher BagMixer® (Interscience, St-Nom, France).

2.2 DNA extraction and purification

To extract total DNA, samples were centrifuged for 10 min at 5,000×g and the resulting pellets were treated for 1 hour at 37°C with an enzymatic lysis buffer containing Tris-HCl 20 mM, EDTA 2 mM, Triton X-100 1.2%, and Lysozyme 20 $g.L^{-1}$. Total DNA was isolated from each primary suspension with the DNeasy Blood & Tissue DNA extraction kit (Qiagen, Venlo, the Netherlands), following the manufacturer's recommendations. The DNA was eluted into

DNAse/RNAse-free water and its concentration and purity were evaluated by optical density using the NanoDrop ND-1000 spectrophotometer (Isogen, St-Pieters-Leeuw - Belgium). DNA samples were stored at -20°C until used for 16S rDNA amplicon sequencing analysis.

2.3 16S rDNA gene library construction and sequencing

16S rDNA PCR libraries were generated for each cheese sample using 16S rDNA universal primers with Illumina overhand adapters targeting V1-V3 hypervariable regions, forward (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3') and reverse (5'-GTVTVGTGGGCTCGGAGATGTGTATAAGAGACAG-3'). Each PCR product was purified with the Agencourt AMPure XP beads kit (Beckman Coulter, Pasadena, USA). A second PCR, for indexing, was performed using the Nextera XT index primers 1 and 2, specific to the Illumina technology. The PCR products were then purified as indicated above. Quantifications were made using the Quant-IT PicoGreen (ThermoFisher Scientific, Waltham, USA). To constitute a library, each PCR product, after quantification, was diluted to 10 $ng.\mu L^{-1}$ with Tris-HCl 10 mM Tween 20 0.05% and all PCR products were mixed together. An agarose gel (1%) was used to check if the library was free of unwanted bands, if not, a new purification with AMPure XP beads was made. A precise quantification, by qPCR, of each sample in the library was performed using the KAPA SYBR® FAST qPCR Kit (KapaBiosystems, Wilmington, USA) before normalization, pooling and sequencing on a MiSeq sequencer using v3 reagents (ILLUMINA, USA).

2.4 Bioinformatics analysis

Sequence reads processing were used as previously described by Rodriguez *et al*. (2015) using respectively MOTHUR software package v1.35, Pyronoise algorithm and UCHIME algorithm for alignment and clustering, denoising and chimera detection. The clustering distance for operational taxonomic unit (OTU) is 0.03. 16S rDNA reference alignment and taxonomical assignation were based upon the SILVA database (v1.15) of full-length 16S rDNA sequences, with an average length of 490 bp. All the biosample raw reads have been deposited at the

National Center for Biotechnology Information (NCBI) and are available under the Bioproject ID PRJNA300927.

3. Results

Next generation sequencing allows identification of OTUs at various taxonomic levels. OTUs could be identified from the species, genus, family or sometimes only at order level. Regarding the data given below, *species pluralis* (or spp.) was used to present strains belonging to the same genus without species identifications. In addition, each dual value given below is expressing a proportion of a strain from the beginning (0 day) to the end (21 days) of the ripening process.

The following study allowed the identification of 10 species, 5 genera, 1 family and 2 orders in the core of the cheese. About 0.40% of the core microbiota remained unidentified or at a too low level in order to be placed taxonomically. Moreover, 21 species, 8 genera, 3 families and 2 orders were found on the surface. The unidentified and uncited strains were in higher proportion on the surface compared to the core, with relative proportions of 18 to 24% (Table 1 and Table 2).

3.1 Analysis of the cheese core bacterial content

As shown in Figure 1, *Lactococcus* spp. and *Streptococcus* spp. were the main genera found in the core of the cheese. During the first period of ripening, 66.09% of the sequences were allocated to *Lactococcus* spp., and then a drastic drop occurred reaching 8.80% from the third day of ripening. On the other hand, sequences of *Streptococcus* spp. were 29.47% at the beginning and 88% at the end of ripening process. This increase started to occur from the third day of the ripening process. The other bacterial sequences were composed of *Streptococcaceae* (2.52% to 1.60%), *Lactobacillales* (0.70%) and *Lactobacillus* (0.63% to 0.30%). Other genera, including *Leuconostoc*, *Brevibacterium*, *Corynebacterium*, representing 0.58% to 0.41% of the core microbiota were also found (Table 1). It should be underlined that between the beginning (day 0) and the end (day 21) of the ripening process, the sequences corresponding to the starter

PAL 114D strains were fluctuating from 30.97% to 6.28% (*Lactococcus lactis* subsp. *lactis*), 24.96% to 1.96% (*L. lactis* subsp. *cremoris*) and 25.17% to 78.71% (*S. thermophilus*). The starter population has reached 81.11% even 86.95% of the core microbiota. Unidentified *Lactococcus* sp. (10.16% to 0.57%) decreased during the ripening process from day 0 to day 3, while *Streptococcus* sp. population has increased from 4.30% to 10.07%. The 1.92% to 1.31% remaining part of the microbiota was represented by some *Lactobacilli*, especially *Lactobacillus rhamnosus* (*Lb. rhamnosus*), *Lb. sakei* and *Lb. malefermentans*, and by *Corynebacterium* sp., *Leuconostoc* sp., unidentified *Micrococcales*, *Brevibacterium* sp. and other unspecified species (Table 1).

3.2 The bacterial diversity of cheese surface

As indicated in Figure 2, the surface diversity as investigated here revealed that 18.38% to 23.54% of the sequences correspond to an unassigned OTU and low proportion of genera, with less than 5% of sequences. The major part of the microbiota was composed of *Lactobacillus* spp. (24.13% to 1.70%), *Lactococcus* spp. (19.72% to 0.84%), *Corynebacterium* spp. (15.96% to 26.42%), *Micrococcales* (3.36% to 13.01%), and *Psychrobacter* spp. (0.68% to 25.44%). Besides these major species, we detected, to a less extent, the presence of *Brevibacterium* spp. (4.54% to 2.30%), *Lactobacillales* (around 1.25%), and *Bifidobacteriaceae* (1.02% to 0.07%). *Lactobacillus* spp., *Lactococcus* spp., *Streptococcus* spp., *Brevibacterium* spp. and *Bifidobacteriaceae* had a trend to decrease during the ripening and were replaced by populations of *Corynebacterium*, *Micrococcales* and *Psychrobacter*. Populations of *Lactobacillales* and other species were stable during the ripening. Starter strains sequences on cheese surface represented 34.00% at the beginning, and only 0.79% at the end of the ripening process. They were composed of the species as those found in the core, with the addition of *Lb. helveticus* and some *Propionibacteriaceae*. As indicated in Table 2, the microbiota also contained *Lactococcus* sp. (4.83% to 0.36%), *Corynebacterium* sp. (2.84% to 3.93%), *Corynebacterium variabile* (*C. variabile*) (8.74% to 19.76%), *C. flavescens* (4.36% to 1.32%), *C. casei* (0.03% to 1.41%),

Micrococcales (3.15% to 7.54%), *Psychrobacter celer* (0.05% to 12.98%), *Psychrobacter* sp. (0.63% to 12.46%), *Lb. capillatus* (3.54% to 0.02%), *Lb. rhamnosus* (1.23% to 0.00%), and other *Lactobacillales* (9.71% to 2.79%). Microbiota in lower proportion was composed of *Brevibacterium linens* (3.68% to 1.56%), *Brevibacterium* sp. (0.87% to 0.74%), *Micrococcales* (3.15% to 7.54%). Microbiota in the lowest proportion was composed of *Leucobacter chromiireducens* (0.18% to 5.46%), *Leuconostoc gelidum* (around 0.03%), *Peptostreptococcus russelli* (0.00% to 4.02%), *Bifidobacterium psychraerophilum* and other *Bifidobacteriaceae* (1.03% to 0.07%), *Streptococcaceae* (0.29% to 0.00%), *Staphylococcus* sp. (0.21% to 0.00%), and *Serratia* sp. (0.00% to 0.46%). The last portion was composed of *Acetobacteriaceae* (0.13% to 0.00%), *Propionibacteriaceae* (0.11% to 0.00%), *Brachybacterium tyrofermentans* (0.11% to 0.07%), *Carnobacteriaceae* (0.03% to 0.41%) and *Hafnia alvei* (0.00% to 0.07%; Table 2).

4. Discussion

This metagenomic study permitted us to provide insightful information on the bacterial content of a typical, local cheese named "Tomme d'Orchies", which is produced and marketed in the north of France. This first metagenomic analysis underpinned the dominance of the starter strains as *Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *Streptococcus thermophilus* in the core section of the cheese during the ripening process. Indeed, percentages ranging from 81.10% to 86.95% were detected at the beginning and the end of the ripening period. Besides the absence of *Lb. helveticus* in the core of the cheese, we observed that *Lactococcus* and *Streptococcus* contents progressed differently along the repining process. Indeed, the inversion of ratios observed at the end of ripening could be partly attributed to the salt concentration, which can inhibit the growth of *Lactococcus*. Holt *et al*. (1994) reported the inabilities of *Lactococcus* strains to grow at NaCl concentration higher than 0.5%, leading therefore to a decrease of its population within the total cheese microbiota. Moreover, lactic acid produced during the first three days of ripening process could also explain this decrease, as yet observed for acid sensitive strains (Beresford *et al.* 2001). Thus, the conditions encountered in

the core of the cheese appeared to offer a clear advantage to *Streptococcus* that could reach the highest percentage of the bacterial population (89.89%). On the other hand, "eyes" formation observed in the core of the Swiss cheeses (Beresford *et al.*, 2001; Dalmasso *et al.*, 2011) was associated with the development of bacteria belonging to *Propionibacterium* genus. Surprisingly, *Propionibacterium*, especially *P. freudenreichi* subsp. s*hermanii*, was almost non-existent in the core of the "Tomme d'Orchies" cheese, although it was incorporated as starter. The metagenetic approach used here did not permit detection of this species, in spite of its use as starter. Independently of the amount of starter and technology used, this genus is known to preferentially grow under anaerobic conditions. In our opinion, further analyses are required to determine whether this starter culture, has any prominent role during the manufacturing process.

Regarding the surface section of the cheese, we noticed that almost all the starter strains were present, mainly at the beginning of the repining as an average level of 33.4%, conversely at the end of the process only less than 1% was detected. This decrease could be explained by different factors including a plausible competition with the secondary microbiota, and antagonism resulting from the rubbing with the brine, brown beer (ethanol) and rocou, which is known to exert inhibitory activities against both Gram-negative and Gram-positive bacteria and also antibiotic-resistant strains (Fleischer *et al.*, 2003; Yasunaka *et al.*, 2005). It should be noted that in the core of the cheese, the secondary microbiota was represented about 1.84% to 1.31% of the microbiota along the ripening process. The secondary microbiota, designated also as the non-starter lactic acid bacteria (NSLAB) contained in the present analysis the following species: *Lactobacillus* sp., *Leuconostoc* sp., and *Streptococcus* sp. *Corynebacterium* strains. The NSLAB found in the core could have originated from milk (Vacheyrou *et al.* 2011). According to Irlinger and Mounier (2009), the decrease of the strains from raw milk observed progressively along the ripening process could be attributed to the nutritional competition or bacteriocins production.

Moreover, the *Corynebacterium* sp. and *Brevibacterium* strains found in the core of cheese were likely due to cross contaminations occurring during the manipulation, cutting and packaging of cheese samples. It should be noted that within the remaining strains, those

displaying stable levels during the ripening period were, for most of them, not identified strains, whereas the other ones were anticipated to be from the environment.

The low proportion of NSLAB and other strains can possibly be explained by their low levels in the raw milk initially used to produce the cheese, and/or to the unfavourable conditions faced for their growth. Moreover, the compression of the curd during the process increases the density of the product. This high density would limit therefore the microbial dispersion of environmental NSLAB strains in the core during the ripening. Furthermore, the secondary microbiota diversity observed on the surface cheese could be from the environment during ripening and from the manufacturing process, such as a contact with tools and ripening shelves. It should be pointed out that the upmost part of the genera highlighted in this study was also revealed in previous studies by Quigley *et al*, (2012) and Wolfe *et al*, (2014). However, in the above-cited studies, *Pseudoalteromonas* and *Vibrio* but not *Bifidobacterium* and *Pediococcus* were present in the rind of cow cheese, therefore differing from our study. This difference could potentially be explained by the raw cow milk and the cheese process. It was reported that indigenous milk bacteria found in low quantity including NSLAB such as *Leuconostoc, Streptococcus* sp., *Lactobacillus*, have a particular fate since their number started to decrease rapidly from the first day of ripening to almost non-detectable limits at the end of the ripening (Vacheyrou *et al.*, 2011). Nevertheless, *Leucobacter chromiireducens* increased to 5.46%. *Psychrobacter* sp. was present in the raw cow milk and was highly favoured by the low temperature in the ripening chamber (Montel *et al.*, 2014; Vacheyrou *et al.*, 2011). Analysis on the ripening shelf, or the brine used for rubbing, could be interesting to determine if *Psychrobacter* are present or not. Indeed, it was reported that *Psychrobacter celer* was isolated in seawater (Yoon *et al.*, 2005). This OTU would be able to grow in the cheese with high salt concentration possibly provided by the brine. The high proportion of this OTU could contribute to the aromatic properties of the cheese, as demonstrated in Herve Belgian cheese (Delcenserie *et al.*, 2014; Irlinger *et al.*, 2012).

The orange coloration of the cheese is usually attributed to the presence of *Brevibacterium linens* associated with yeast strains and some *Micrococcales* pigment producers as *Staphylococcus* or *Micrococcus* (Bockelmann *et al.*, 2005; Bockelmann and Hoope-Seyler, 2001; Irlinger and Mounier, 2009). In the case of "Tomme d'Orchies" cheese, most of the orange coloration was attributed to rubbing with rocou, a food dye coming from *Bixa orellana* (Barrozo *et al.*, 2013). *Bifidobacteriaceae* were present in low proportion (about 1%) at the beginning of the ripening period and then decreased to almost non-detectable presence by the end of the ripening. These strains can be considered native as recently reported Montel *et al*. (2014), or could originate from the annual treatment of the farm by spraying of probiotic strains (species not communicated by the producer) to avoid contaminations of the environment by pathogens such as *Salmonella* or *Listeria*. However, *Bifidobacteriaceae* could not grow in those cheeses, and remained at "trace" level from the third day of ripening. Other OTUs found in low proportion such as *Brachybacterium tyrofermentans*, *Carnobacteriaceae*, *Hafnia alvei* and *Serratia* sp. remained in stable proportions during the ripening. The origin of these OTUs was likely the raw cow milk, according to similar analysis (Montel *et al*., 2014).

Physico-chemical analyses of the cheese, such as determination of salt concentration, pH and a_w, could help to better understand the bacterial population dynamics. As this cheese was not involved in any public health case, its natural microbiota can be investigated to help in preventing food-borne pathogens despite the use of raw milk. The culture-dependent analysis of each strain will clearly highlight these species of interest according to their identification and their positive properties such as use as potential probiotics. In the core of "Tomme d'Orchies" cheese, the dominant presence of starter strains and the physicochemical conditions of the core have probably hampered the development of autochthonous strains from milk. Therefore, isolation and characterization of potential probiotics derived from *Corynebacterium variabile*, *Leucobacter chromiireducens*, *Peptostreptococcus russeli*, *Psychrobacter celer*, *Psychrobacter* sp., some *Micrococcales* and other unidentified strains using culture-independent methods, constitute our next research focus.

The bacterial microbiota of the “Tomme d’Orchies”, a washed, pressed, uncooked cheese, and revealed similarities with washed and smear cheeses such as the presence of *Psychrobacter* and *Corynebacterium* (Delcenserie *et al.*, 2014). The presence of *C. variabile* and *C. casei* in high proportion showed similarity with acid curd cheeses (Bockelmann *et al.*, 2005; Bockelmann and Hoope-Seyler, 2001). In conclusion, we determined that Tomme d'Orchies cheese contains the usual bacterial microbiota for cheeses. However, the bacterial content was not totally specific for its variety, pressed uncooked cheese, and showed high similarities with smear cheeses. No statistical analysis was undertaken in this study to detect the variability of bacterial content, but it would be interesting to study it at each time of ripening. Yeasts with antagonistic activity were identified in a previous study on this cheese and could influence the bacterial microbiota (Ceugniez *et al.*, 2015). Further investigations are required to provide insightful data on the indigenous bacterial strains and their beneficial properties.

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Figure caption

Figure 1. Bacterial genus distribution in the core of "Tomme d'Orchies" cheese, during the ripening process.

Figure 2. Bacterial genus distribution on the surface of "Tomme d'Orchies" cheese, during the ripening process.

Table 1. Bacterial microbiota distribution, at the species level, in the core of the "Tomme d'Orchies" cheese, according to the period of ripening. Grey shading correspond to strains added in the starter.

Strains		Ripening (Day)				
		0	1	3	14	21
Lactococcus	*lactis* subsp. *lactis*	30.97%	30.26%	7.12%	7.67%	6.28%
	lactis subsp. *cremoris*	24.96%	4.01%	0.74%	0.45%	1.96%
	sp.	10.16%	4.24%	0.57%	0.52%	0.56%
Streptococcus	*thermophilus*	25.17%	48.62%	78.54%	80.25%	78.71%
	sp.	4.30%	8.38%	10.07%	7.78%	9.58%
Streptococcaceae		2.52%	2.57%	1.55%	1.13%	1.60%
Lactobacillus	*rhamnosus*	0.42%	0.21%	0.09%	0.16%	0.09%
	sakei	-	-	0.02%	-	-
	malefermentans	-	-	-	-	0.02%
	sp.	0.21%	0.44%	0.15%	0.27%	0.19%
Lactobacillales		0.71%	0.74%	0.72%	1.42%	0.60%
Corynebacterium	*flavescens*	-	0.02%	-	-	-
	sp.	0.08%	0.02%	-	0.02%	0.02%
Leuconostoc	*gelidum*	0.02%	-	-	-	-
	carnosum	-	-	0.07%	-	-
Micrococcales		0.06%	0.02%	0.02%	-	-
Brevibacterium	*linens*	-	0.02%	-	-	0.02%
	sp.	0.02%	-	0.04%	0.05%	-
Others		0.40%	0.44%	0.28%	0.29%	0.37%

Table 2. Bacterial microbiota distribution, at the species level, on the surface of the "Tomme d'Orchies" cheese, according to the period of ripening. Grey shading correspond to strains added in the starter.

Strains		Ripening (Day)				
		0	1	3	14	21
Lactococcus	*lactis* subsp. *lactis*	12.29%	4.99%	0.51%	0.08%	0.46%
	lactis subsp. *cremoris*	2.60%	0.10%	0.02%	0.03%	0.02%
	sp.	4.83%	2.09%	0.41%	0.16%	0.36%
Corynebacterium	*variabile*	8.74%	23.45%	35.75%	12.01%	19.76%
	flavescens	4.36%	13.57%	10.35%	1.40%	1.32%
	casei	0.03%	1.58%	0.22%	2.08%	1.41%
	sp.	2.84%	9.01%	7.67%	2.34%	3.93%
Lactobacillus	*helveticus*	10.76%	2.12%	0.43%	-	0.02%
	capillatus	3.54%	0.29%	0.05%	-	0.02%
	rhamnosus	1.23%	0.12%	0.02%	-	-
	malefermentans	0.08%	0.10%	0.02%	-	0.02%
	sakei	-	-	-	-	0.02%
	sp	8.51%	2.05%	0.80%	0.49%	1.60%
Lactobacillales		1.13%	1.07%	0.46%	0.39%	1.15%
Streptococcus	*thermophilus*	8.24%	1.41%	10.04%	0.49%	0.31%
	sp.	1.97%	0.29%	3.26%	0.21%	0.19%
Streptococcaceae		0.29%	0.05%	0.02%	-	-
Brevibacterium	*linens*	3.68%	1.73%	1.18%	7.21%	1.56%
	sp.	0.87%	0.51%	0.19%	3.01%	0.74%
Leucobacter	*chromiireducens*	0.18%	0.56%	0.31%	4.33%	5.46%
Leuconostoc	*gelidum*	0.03%	0.02%	-	-	-
Peptostreptococcus	*russelli*	-	-	0.02%	0.80%	4.02%
Micrococcales		3.15%	2.83%	1.54%	8.17%	7.54%
Bifidobacterium	*psychraerophilum*	0.11%	0.10%	-	-	-
Bifidobacteriaceae		0.92%	0.63%	0.05%	0.03%	0.07%
Psychrobacter	*celer*	0.05%	3.56%	0.70%	2.62%	12.98%
	sp.	0.63%	3.73%	6.73%	25.95%	12.46%
Staphylococcus	sp.	0.21%	0.22%	-	-	-
Acetobacteraceae		0.13%	-	-	-	-
Propionibacteriaceae		0.11%	0.07%	-	-	-
Brachybacterium	*tyrofermentans*	0.11%	0.07%	0.07%	0.29%	0.07%
Carnobacteriaceae		0.03%	0.17%	0.07%	0.03%	0.41%
Hafnia	*alvei*	-	0.37%	0.05%	0.03%	0.07%
Serratia	sp.	-	0.22%	-	1.79%	0.46%
Others		18.38%	22.92%	19.03%	26.08%	23.54%

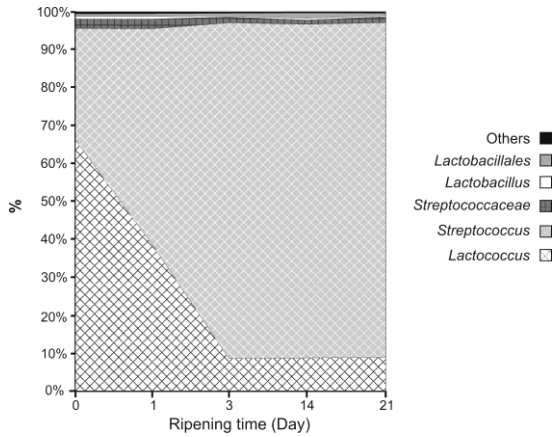

Figure 1

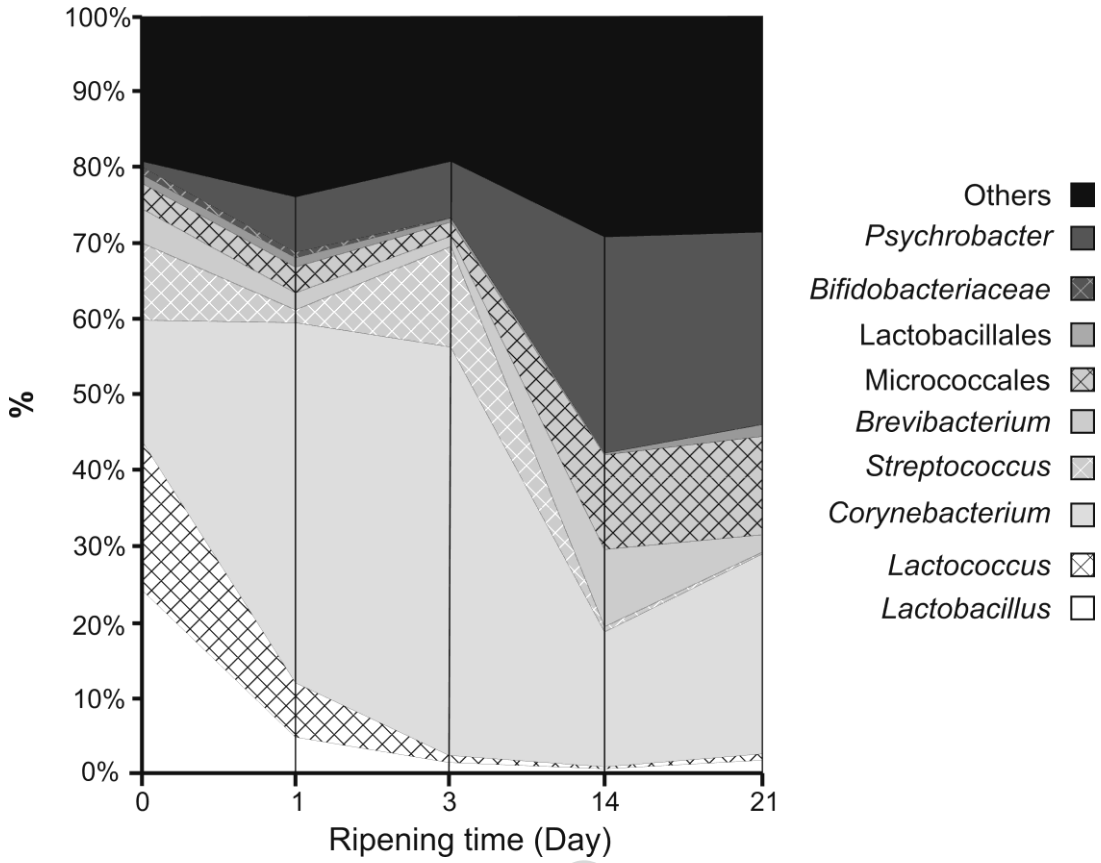

Figure 2