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Temporal differences in microbial composition of Epoisses cheese rinds during ripening and storage

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Interpretive summary:

Epoisses is a French Protected Designation of Origin smear-ripened cheese with an orange color and a strong flavor, both generated by surface microorganisms. We evaluated the microbial dynamics at the surface of Epoisses cheeses during ripening and post-manufacturing storage at low temperatures. Most of the bacterial species detected by amplicon-sequencing were Gram-negative species that were not deliberately inoculated during cheesemaking. These microorganisms may be of interest for the development of more efficient ripening cultures.

ABSTRACT

Epoisses is a Protected Designation of Origin (PDO smear-ripened cheese from the Burgundy region in France. It has an orange color and a strong flavor, both of which are generated by surface microorganisms. The objective of the present study was to investigate the microbial dynamics at the surface of Epoisses cheese during ripening and post-manufacturing storage at low temperatures. Rind samples were analyzed by enumeration on agar plates and by 16S rRNA gene and Internal Transcribed Spacer amplicon sequencing. During most of the ripening process, the counts of yeasts, which corresponded to the two species *Debaryomyces hansenii* and *Geotrichum candidum*, were higher than those of the aerobic acid-sensitive bacteria. *Debaryomyces hansenii* reached a level of about $3x10^8$ cfu/cm^2, and its viability strongly decreased at the late stage of ripening and during storage at +4°C. Two of the inoculated bacterial species, *Brevibacterium aurantiacum* and *Staphylococcus xylosus*, did not establish themselves at the cheese surface. At the end of ripening, among the 18 most abundant bacterial species detected by amplicon-sequencing, 14 were Gram-negative, mainly from genera *Psychrobacter*, *Vibrio*, *Halomonas* and *Mesonia*. It was hypothesized that the high moisture level of the Epoisses rinds, due the humid atmosphere of the ripening rooms and to the frequent washings of the curds, favored growth of these Gram-negative species. These species may be of interest for the development of efficient ripening cultures. In addition, since the orange color of Epoisses cheeses could not be attributed to the growth of *Brevibacterium*, it would be interesting to investigate the type and origin of the pigments that confer color to this cheese.

Key words: smear-ripened cheese, ripening, Epoisses, *Debaryomyces hansenii*, *Mesonia ostreae*

INTRODUCTION

Smear-ripened cheeses are covered by a layer of yeasts and bacteria that have a strong impact on appearance and flavor (Bockelmann and John, 2011). The yeasts dominate during the early stages of ripening because they are salt- and acid-tolerant, and subsequently increase the pH, thereby favoring the growth of aerobic acid-sensitive bacteria. Ripening takes place at high relative humidity (>95%) and the cheeses are washed several times with a dilute saline solution to spread any microcolonies of yeasts or bacteria that have developed on the surface. The beneficial yeasts and bacteria have to be competitive in order to prevent the growth of undesired microorganisms, and most smear-ripened cheese are inoculated with defined ripening cultures today (Bockelmann et al., 2005).

As for other food products, microbial analyses of smear-ripened cheeses traditionally relied on culture-based techniques, but the advent of culture-independent techniques based on the analysis of nucleic acids extracted from the food matrix was very useful to better characterize the structure of the microbial communities (De Filippis et al., 2018; Jonnala et al., 2018). However, only limited information is available about the microbial dynamics that occur during the manufacturing of smear-ripened cheeses. Such information may help to better understand the ecological relationships between cheese microorganisms and their adaptation to the cheese habitat. For example, population dynamic studies of Gubbeen cheeses revealed a progression of bacteria, with staphylococci dominating the early stages of ripening and *Actinobacteria* the later stages, and the commercial strains used for smearing the cheese were only present at a very low level early in ripening (Rea et al., 2007). In Tilsit cheeses, biodiversity increased during ripening and *Actinobacteria* were the most prominent group in the late phase.

The objective of the present study was to evaluate the microbial dynamics at the surface of Epoisses, a French Protected Designation of Origin (PDO) cheese (LEGIFRANCE, 2010), whose production level is approximately 1300 tons per year (CNIEL, 2020). This cheese is made from whole cow's milk produced by specified dairy breeds in the Burgundy region. Epoisses cheese can be manufactured from raw or from pasteurized milk and undergoes a dominant lactic type coagulation, whose duration is greater than 16 hours, and draining occurs spontaneously, without pressing. Salting is done using dry salt, and ripening time is at least 28

days, at a temperature of ~10-13°C. During ripening, the cheeses are washed one to three times per week with washing solutions supplied with increasing amounts of Marc de Bourgogne, a pomace brandy. Epoisses cheese has an orange color and a strong flavor, both generated by surface microorganisms. A 16S rRNA amplicon-sequencing analysis revealed that Epoisses rinds contain high levels of species adapted to cold aquatic and saline environments (Dugat-Bony et al., 2016). In order to better understand the microbial activity involved in the generation of the typical properties of Epoisses, as well as to provide information about ripening cultures that are suitable for this cheese variety, it was interesting to monitor the microbial composition during a typical manufacturing run and post-manufacturing storage at low temperatures.

MATERIALS AND METHODS

Cheese manufacturing and sampling

All the cheeses were taken from the same batch of cheese production of an industrial manufacturing unit in 2017. The factory produced the cheeses from pasteurized milk and in compliance with the Epoisses PDO specifications. Several commercial microbial cultures were inoculated during cheesemaking. At the beginning of the manufacturing process, milk was inoculated with a mesophilic lactic starter culture, with the yeasts *Debaryomyces hansenii* and *Geotrichum candidum* and with the aerobic ripening bacteria *Brevibacterium aurantiacum*, *Staphylococcus xylosus*, and *Glutamicibacter arilaitensis*. *Brevibacterium aurantiacum* was also added to the smearing solutions used after day 6. After ripening (12°C), the cheeses were packaged and transferred to a cold room at +4°C. Three different cheeses were analyzed at eight sampling times (day 6, day 9, day 21, day 24, day 28, day 33, day 40 and day 90). The cheeses were cut perpendicular to the surface in order to produce two equivalent parts. One part was used to measure the concentrations of lactose, lactate, free amino acids and non-protein fractions. The upper and lower sections (rinds) of the other part were removed with a knife (thickness ~2-3 mm), pooled, and used for cell counts, pH measurements and DNA extraction.

Microbiological analyses

One gram of cheese rind, sampled as described above, was mixed with 9 ml of physiological saline solution (9 g/L NaCl). After dispersion with a mechanical blender (Ultra-Turrax® model T25; Ika Labortechnik, Staufen, Germany) for 1 min at 14,000 rpm, 10-fold serial dilutions were performed in physiological saline solution and plated in triplicate on agar plates. After four days of incubation at 25°C, the aerobic ripening bacteria were counted on brain heart infusion agar (Biokar Diagnostics, Beauvais, France) supplemented with 50 mg/l amphotericin (Sigma Aldrich, Saint Louis, MO, USA), which inhibits the growth of fungi. The lactic acid bacteria were counted on MRS agar (Biokar diagnostics) supplemented with 50 mg/l amphotericin after three days of incubation at 30°C under anaerobic conditions. The yeasts were counted on yeast extract-glucose-chloramphenicol agar (Biokar Diagnostics) after

three days of incubation at 25 °C. *Geotrichum candidum* and *D. hansenii* could be selectively counted on this medium because they have distinct colony morphotypes.

Biochemical analyses

Biochemical analyses were performed for each of the three cheeses sampled at the eight sampling times. Non-protein nitrogen content of the cheeses was measured by the Kjeldahl method according to the NF EN ISO 8968 standard (Milk and milk products - Determination of nitrogen content). The levels of lactate and lactose were assayed using commercially available kits (Biosentec, Auzeville Tolosane, France) according to the manufacturer's instructions. Free amino acids were analyzed by HPLC, as previously described (Castellote et al., 2015). The pH was measured on the homogenized cheese rinds.

DNA extraction, 16S rRNA and ITS amplicon sequencing, and data analysis

DNA was extracted from three different cheeses at each of the eight sampling times, using the bead beating-based protocol detailed in a previous study (Dugat-Bony et al., 2015). Briefly, the cheese rind samples (~ 1 g) were diluted in 9 ml sodium citrate solution (20 g/l trisodium citrate dihydrate) and homogenized with an Ultra Turrax® model T25 mechanical blender at 24,000 rpm for 2 min. A second treatment was performed after 10 min of incubation at room temperature. The mixture was then centrifuged at 6,400 x g for 10 min at 4°C, and the supernatant was removed. The casein pellet (containing the microbial cells) was resuspended in 5 ml of a Triton X-100 aqueous solution (2.5% v/v), vigorously shaken, heated in a water bath at 70°C for 10 min, centrifuged at 6,400 x g for 10 min at 4°C, and rinsed twice with 10 ml of a physiological saline solution. The pellet was dissolved in a mixture of 270 µL of guanidium thiocyanate (4M) in Tris-HCl (pH 7.5, 0.1 M) and 30 µL of sodium lauroyl sarcosinate (100 g/l), and transferred to a 2-mL tube containing 250 mg of 0.1-mm-diameter zirconium beads and 250 mg of 0.5-mm-diameter zirconium beads (Biospec Products, Bartlesville, OK, USA). Proteinase K treatment, bead-beating, phenol-chloroform extraction, RNase treatment and ethanol precipitation were then performed as previously described (Leclercq-Perlat et al., 2013), and the DNA pellet was dissolved in 120 µl of Tris EDTA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Purified DNA was quantified with Qubit DNA assay kits on the Qubit 3.0 fluorometer (ThermoFischer Scientific, Villebon-sur-Yvette, France). PCR amplification of the bacterial V3-V4 region (16S rRNA gene) and of the fungal

ribosomal Internal Transcribed Spacer 2 (ITS2) region was done with the primer pairs F343/R784 and ITS3/ITS4_HYO1, as previously described (Dugat-Bony et al., 2016). The amplified products were used for Illumina paired-end library preparation and cluster generation, followed by 250 bp paired-end sequencing on an Illumina MiSeq instrument (INRAE, GeT-PLaGE platform, Toulouse, France). Paired-end reads (~2 x 25,000 sequences) were merged using Flash (Magoč and Salzberg, 2011), and the sequence data were processed using the FROGS pipeline (Escudié et al., 2018), according to the standard operating procedure. Briefly, operational taxonomic units (OTUs) were built using Swarm with a maximum number of differences allowed to group together two amplicons (aggregation distance parameter) of 3 (Mahé et al., 2014), and OTUs that accounted for <0.05% of the total set of sequences were discarded. Lastly, the OTU affiliations were checked using the EzBiocloud database (Kim et al., 2012) for the bacteria and the UNITE database (Nilsson et al., 2019) for the fungi. Alpha-diversity metrics were determined using the Phyloseq package (McMurdie and Holmes, 2013) implemented on the FROGS pipeline. Bacterial OTU-sample networks were computed using Gephi software (v. 0.9.1) (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. Raw sequence data were deposited at the Sequence Read Archive of the National Center for Biotechnology Information under the accession numbers SAMN17082512 to SAMN17082535 (bioproject PRJNA685310).

RESULTS

In order to investigate the microbial dynamics at the surface of Epoisses cheeses, samples corresponding to the same manufacturing run of a cheese factory were taken at various ripening stages and during storage at +4°C. A white covering became visible at the surface of the cheeses sampled at day 21, and a yellow/orange color, typical of this cheese variety, appeared afterwards, its intensity increasing during storage at +4°C (Figure 1A). The pH of the cheese surface increased from about 4.5 at day 6 to 6.5 at day 33, and continued to increase after packaging and storage at +4°C (Figure 1B). Lactose and lactate were consumed by the cheese microorganisms and were nearly exhausted in the cheeses at the end of ripening (Figure 2). During ripening, the non-protein nitrogen fraction of the cheeses increased, and it continued to increase during storage at +4°C. The production of non-protein nitrogen results from the degradation of proteins and amino acids.

Fungal growth at the cheese surface

At the cheese surface, the yeast *D. hansenii* reached about $3x10^8$ cfu/cm^2 at days 21 and 28, and cell numbers strongly decreased thereafter (Figure 3). *Geotrichum candidum* was not detected at days 6 and 9, but this may be due to the fact that it is not possible to distinguish *G. candidum* colonies on the agar plates when this species represents less than about 1% of *D. hansenii* because of the large colonies formed by the latter that rapidly cover the entire surface of the agar plates. In contrast to *D. hansenii*, the level of *G. candidum* was nearly constant from day 21 to day 90. The cheese rind contained lactic acid bacteria, whose level slightly decreased during ripening. The level of aerobic bacteria was about $3x10^4$ cfu/cm^2 at day 21 and reached $2x10^8$ cfu/cm^2 at day 33. This growth is probably the consequence of the increase in the cheese pH due to the activity of the yeasts, which favors the growth of acid-sensitive surface bacteria. Despite the use of orange-pigmented *B. aurantiacum* strains as ripening agents, orange colonies were not or almost never observed on the agar plates from cheeses sampled from day 6 to day 40 (<1% of the colonies enumerated on brain heart infusion agar), and represented only about 2% of the colonies at day 90.

ITS amplicon-sequencing analyses revealed a lower level of *G. candidum* sequences in comparison to *D. hansenii* at the beginning of ripening (Figure 4A). From day 21 to day 90,

the changes in the proportion of the ITS sequences of the two yeasts was different than what was observed by counting on agar plates (Figure 5). Indeed, after day 21, there was only a slight increase in the proportion of the *G. candidum* ITS sequences, whereas a large increase was observed for the proportion of *G. candidum* living cells. This may be explained by the large decrease of *D. hansenii* viability that was observed after day 24. Except for *G. candidum* and *D. hansenii*, no other fungi were detected in the cheeses (cutoff was set at 0.05% relative abundance of the ITS sequences).

Bacterial growth at the cheese surface

The 16S rRNA amplicon-sequencing analyses showed the presence of two sequence clusters of lactic acid bacteria (Figure 4B and Supplemental Table S1). The first corresponds to *L. lactis* and the second (referred to as *Lactococcus* group *chungangensis*) to *L. chungangensis* and *L. laudensis*. The proportion of the lactic acid bacteria 16S rRNA sequences considerably decreased during ripening, especially after day 24, as the consequence of the growth of the aerobic acid-sensitive bacteria. At the late stage of ripening, there was a large increase of 16S rRNA sequences corresponding to *G. arilaitensis, Psychrobacter aquimaris, Psychrobacter* group *nivimaris, Psychrobacter cibarius, Vibrio litoralis, Halomonas* group *venusta* and *Mesonia ostreae*. Among these bacteria, only *G. arilaitensis* was deliberately inoculated as a ripening agent. The other inoculated ripening bacteria, *B. aurantiacum* and *S. xylosus*, did not establish themselves at the surface of the cheese. The 16S rRNA sequences of *S. xylosus* were not detected (0.05% cutoff) and those of *B. aurantiacum* represented less than 0.5% of the average relative abundance, except in one cheese sample at day 6 (1.0%) and in one cheese sample at day 90 (2.2%). At day 24, *P. aquimaris* was the most abundant non-inoculated species that grew at the surface of the cheeses and represented between 7 and 13% of the 16S rRNA sequences. 16S rRNA sequences from this species were also abundant in one of the cheeses at day 6 and one at day 21, showing that there were some differences in cheeses sampled at the same ripening time. At the end of ripening at day 33, lactic acid bacteria represented less than 10% of the 16S rRNA sequences, and, in addition to *P. aquimaris*, the most abundant OTUs were *G. arilaitensis*, *Psychrobacter* group *nivimaris, V. litoralis, Halomonas* group *venusta* and *M. ostreae.* The bacterial composition changed during storage at 4°C. The proportion of *G. arilaitensis* increased, whereas there was a decrease for *P. aquimaris*, *Psychrobacter* group *nivimaris*, *V. litoralis* and *M. ostreae*. Eight minor bacterial groups ("Other" category in Figure 4B) representing an average relative abundance of

between 0.05 and 0.3% were detected by 16S rRNA amplicon-sequencing: *Halomonas titanicae*, *Halomonas glaciei*, *Psychrobacter proteolyticus*, *Psychrobacter namhaensis*, *Pseudoalteromonas* group *issachenkonii*, *Marinomonas polaris*, *Cobetia* group *marina* and *Sphingomonas* group *insulae* (Supplemental Table S1). Shannon and Inverse Simpson alpha-diversity indices showed that the highest bacterial diversity was at days 28 and 33 and decreased during storage at +4°C (Supplemental Figure S1).

Since a previous study concerning the bacterial composition of Epoisses cheese rinds from three different factories in 2014 was available (Dugat-Bony et al., 2016), we recovered the corresponding 16S rRNA sequencing data (accession number SRP071345 of the Sequence Read Archive of the National Center for Biotechnology Information) and processed them using the bioinformatic pipeline described in the Materials and Methods section. The same protocol for DNA extraction and amplicon sequencing was used in the two studies. An OTU-sample network was built in order to visualize differences between the samples (Figure 6). The cheese samples that were investigated in the present study were produced by Factory 1. The absence of *Brevibacterium* and the dominance of Gram-negative species such as *Psychrobacter* are observed in all of the samples. In comparison to the cheeses produced by the Factory 1 in 2014, the cheeses produced in 2017 (present study) contained lower levels *of P. cibarius* and *Pseudoalteromonas* group *issachenkonii*, but higher levels of *G. arilaitensis*, *V. litoralis*, *M. ostreae* and *Psychrobacter* group *nivimaris.*

DISCUSSION

One key feature of Epoisses cheese is the type of coagulation, which is mostly of the lactic type. Coagulation takes place for 16 to 24 hours, and the pH at the beginning of molding is about 4.5. Mesophilic starter cultures are used for the manufacturing of this cheese variety, which explains the dominance of lactococci at the beginning of ripening. Interestingly, in addition to *L. lactis*, there was a high level of *L. chungangensis* or *L. laudensis*. Little is known about the presence and activity of these species in cheeses, but there are at least three publications in which their occurrence in cheeses has been reported (Masoud et al., 2012; Frétin et al., 2018; Park et al., 2019). It would be interesting to investigate their potential functional properties relevant for cheese manufacturing.

Debaryomyces hansenii was the predominant yeast at the beginning of ripening. This can be explained by its deliberate inoculation at the beginning of manufacturing, its acid- and salt-tolerance, and its ability to grow at 5-10°C. In addition, lactose and lactate, which constitute two important energy compounds for this species (Fröhlich-Wyder et al., 2019), were present at the beginning of ripening. *Debaryomyces hansenii* reached about $3x10^8$ cfu/cm^2 in the Epoisses cheeses, which represents a high level, considering that in smear-ripened cheeses, yeasts typically reach around 10^6 - 10^8 cfu/cm^2 (Cogan et al., 2014; Fröhlich-Wyder et al., 2019). Smear-ripened cheeses are also referred to as "bacterial smear surface-ripened cheeses" because bacteria are considered to be the dominant microorganisms in these cheeses typically representing 10-100 times higher colony counts than yeasts (Corsetti et al., 2001; Cogan et al., 2014). This trend was not observed for the Epoisses cheeses investigated in the present study since the maximum count of surface bacteria was nearly the same as for the yeasts. One possible explanation could be the low pH of the Epoisses curd at the beginning of ripening, which delays the growth of the acid-sensitive aerobic bacteria. At least until day 21, the increase in pH (the pH value was 5.7 at day 21) can be attributed to the activity of the yeasts since the level of aerobic bacteria was very low. The yeasts contribute to the increase in the pH of the cheese surface by transforming lactate to CO_2 and also by producing ammonia from amino acids. Both colony counts and ITS amplicon-sequencing analyses revealed that growth of *G. candidum* occurred later than that of *D. hansenii*. This is probably the consequence of the high salt content of the cheese surface at the beginning of ripening due to the salting procedure with dry salt. This favors the growth of *D. hansenii* since it is more tolerant to salt

than *G. candidum* (Boutrou and Gueguen, 2005). Interestingly, the fungal diversity in the Epoisses cheeses was very low since no species other than the two inoculated fungi *D. hansenii* and *G. candidum* could be detected. This is consistent with a previous study in which Epoisses cheeses from three different producers were analyzed by amplicon high-throughput sequencing (Dugat-Bony et al., 2016). Consequently, there is no evidence that adventitious fungal species are common in Epoisses cheeses. Several factors may be involved in the large decrease of *D. hansenii* viability that occurred during the last stage of ripening and during storage at low temperatures. One of these is lactose or lactate exhaustion, which has been proposed to explain *D. hansenii* lysis in experimental cheeses (Leclercq-Perlat et al., 1999). Other possible factors are the lower oxygen supply after cheese packaging or the cheese washing solution, which is supplemented with increasing amounts of Marc de Bourgogne during the ripening process. It is possible that the decrease of *D. hansenii* viability is a key feature of Epoisses cheese manufacturing since it may also correspond to proteolytic and lipolytic enzymes liberated in the cheese curd by cell lysis (Kumura et al., 2002).

The present study also highlights the absence or poor growth of some ripening culture components at the cheese surface, as already observed in other studies (Brennan et al., 2002; Feurer et al., 2004; Mounier et al., 2005, 2006; Goerges et al., 2008; Cogan et al., 2014). The inoculated *B. aurantiacum* and *S. xylosus* strains were outcompeted by adventitious strains belonging to other species. It is noteworthy that among the 18 bacterial OTUs detected (0.05% relative abundance cutoff), 14 corresponded to Gram-negative species. At the end of ripening, Gram-negative species accounted for about 70% of the 16S rRNA sequences, with the most abundant genera being *Psychrobacter*, *Halomonas*, *Mesonia*, and *Vibrio*. One possible factor explaining the dominance of Gram-negative species is the high moisture level of the Epoisses rinds due the humid atmosphere of the ripening rooms and to the frequent washings of the curds (1 to 3 washings per week). Indeed, in a previous study of the community composition of 137 cheese rinds (Wolfe et al., 2014), a positive correlation was observed between rind moisture and some *Proteobacteria* genera such as *Psychrobacter* and *Vibrio*, whereas there was a negative correlation for the Gram-positive genera *Brevibacterium* and *Staphylococcus*, which correspond to the inoculated genera that did not establish themselves at the surface of the Epoisses cheeses. One interesting feature of the cheese samples investigated in the present study is the presence of *M. ostreae*. To our knowledge, *Mesonia* has never been identified in cheese, except in one of the three Epoisses cheese brands analyzed in 2014 (Dugat-Bony et al., 2016), and which was produced by the same

manufacturing unit as in the present study. The presence of *M. ostreae* is thus a specific signature of the cheese manufacturing unit considered here. *Mesonia* strains have mostly been isolated from marine environments, including seawater and seaweed; they are considered to be salt-tolerant and some members are able to form biofilms (Lee et al., 2012; Huan et al., 2019), which may constitute useful properties for the growth on the cheese surface. The cheeses from the present study were manufactured from pasteurized milk, which is why the presence of high levels of non-inoculated Gram-negative species probably resulted from the facility-specific "house" microbiota, and especially environment microorganisms that were present on processing surfaces, materials and airflows (Bokulich et al., 2016). The large presence of these microorganisms in the final product also raises the question about the efficiency of the inoculated ripening cultures used for manufacturing Epoisses cheeses. The function of these cultures is to outcompete undesired microorganisms and to generate the typical sensory properties. It may be considered that the design of more efficient cultures requires the presence of Gram-negative strains belonging to the genera *Psychrobacter*, *Halomonas*, *Vibrio* or *Mesonia*, which are currently not used as ripening culture components. Interestingly, most Gram-negative bacteria of dairy origin investigated in a recent safety assessment study were considered to be safe (Imran et al., 2019). Interestingly, *B. aurantiacum*, the orange-pigmented bacterium that is used to give an orange color to cheeses, represented only a very small minority of the bacterial population. This species is thus probably not responsible for the typical color of Epoisses cheeses. In addition, HPLC profiles of the carotenoids from Epoisses cheese rinds revealed the presence of yellow carotenoids produced by yellow bacteria such as *Glutamicibacter arilaitensis*, but not of orange carotenoids (Galaup et al., 2007). Furthermore, several studies reported the absence or presence at only very low levels of *Brevibacterium* in some orange smear-ripened cheeses (Brennan et al., 2002; Feurer et al., 2004; Bockelmann et al., 2005; Bockelmann and John, 2011; Delcenserie et al., 2014). It has also been suggested that several factors such as the proteolytic activity of smear bacteria may also be important for the typical color development of smear-ripened cheeses (Bockelmann and Hoppe-Seyler, 2001). Thus, it would be interesting to investigate the type and origin of the pigments that confer color to Epoisses cheeses.

Another noteworthy observation was that during storage at +4°C, between the packaging stage and the "best-before" date (day 90), there was a considerable change in bacterial composition of the Epoisses cheeses. This may also occur in other cheese varieties and it

would be interesting to investigate microbial growth and activity during the storage of cheeses at low temperatures and its relationship to the shelf life of the product. In addition, the present study also revealed some changes in the microbial composition of cheeses produced by the same factory over a three year interval. Whether this is common and possibly impacts the sensory properties of the final product is not known, but a previous study reported major changes in the relative importance of the bacterial species present at the surface of Gubbeen cheeses manufactured in the same plant (Rea et al., 2007).

CONCLUSIONS

As observed at the surface of other smear-ripened cheeses, the acid-tolerant yeasts grow before the acid-sensitive aerobic bacteria in Epoisses cheeses. However, in this cheese variety, the yeast *Debaryomyces hansenii* reaches a high level during ripening, and there is subsequently a large decrease in its cell viability, which possibly impacts the organoleptic properties of the final product. Most of the growth of the acid-sensitive bacteria occurs during the last week of ripening, and some changes in bacterial composition also occur during post-manufacturing storage at +4°C. The high moisture level probably favors Gram-negative species, which are the dominant bacteria at the end of ripening. Since these species are able to outcompete part of the inoculated ripening bacteria, it might be interesting to devise ripening cultures containing typical Gram-negative species present in this cheese variety and with previously assessed safety. The orange color of Epoisses cheeses does not seem to be due to the presence of orange-pigmented bacteria, and it would thus be interesting to investigate the type and origin of the pigments present in this cheese.

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FIGURES

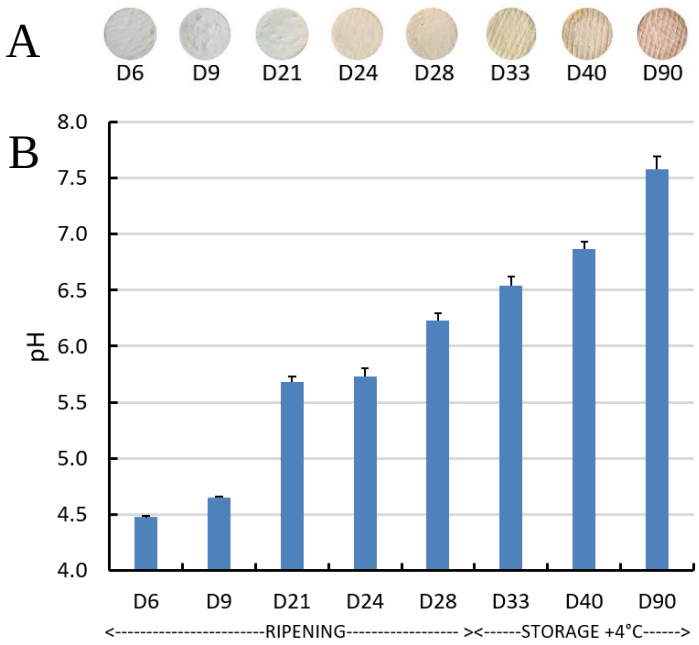

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Figure 1. (A) Appearance of the Epoisses cheeses during their manufacturing and storage. (B) Changes in the pH values at the surface of the cheeses. Bars show mean ± sd of three replicates. The samples D6, D9, D21, D24, D28, D33, D40 and D90 correspond to cheeses sampled from 6 to 90 days after the beginning of manufacturing.

Figure: 8.9 cm wide

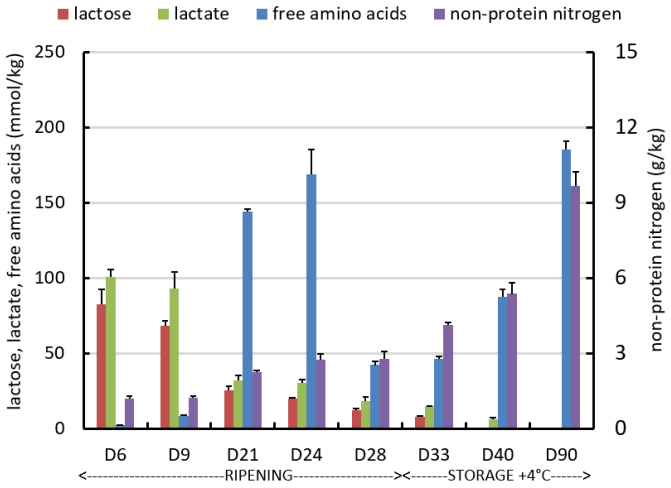

Figure 2. Concentration of lactose, lactate, free amino acids and non-protein nitrogen fractions during the manufacturing and storage of Epoisses cheeses. Bars show mean ± sd of three replicates. The samples D6, D9, D21, D24, D28, D33, D40 and D90 correspond to cheeses sampled from 6 to 90 days after the beginning of manufacturing.

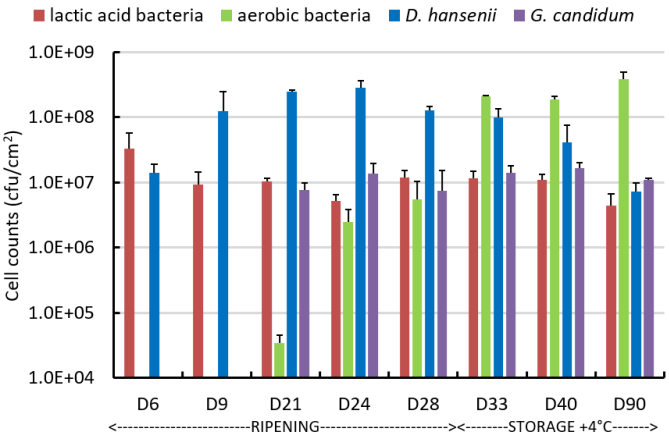

Figure 3. Bacterial and yeast counts at the surface of the Epoisses cheeses during their manufacturing and storage. Bars show mean ± sd of three replicates. The samples D6, D9, D21, D24, D28, D33, D40 and D90 correspond to cheeses sampled from 6 to 90 days after the beginning of manufacturing.

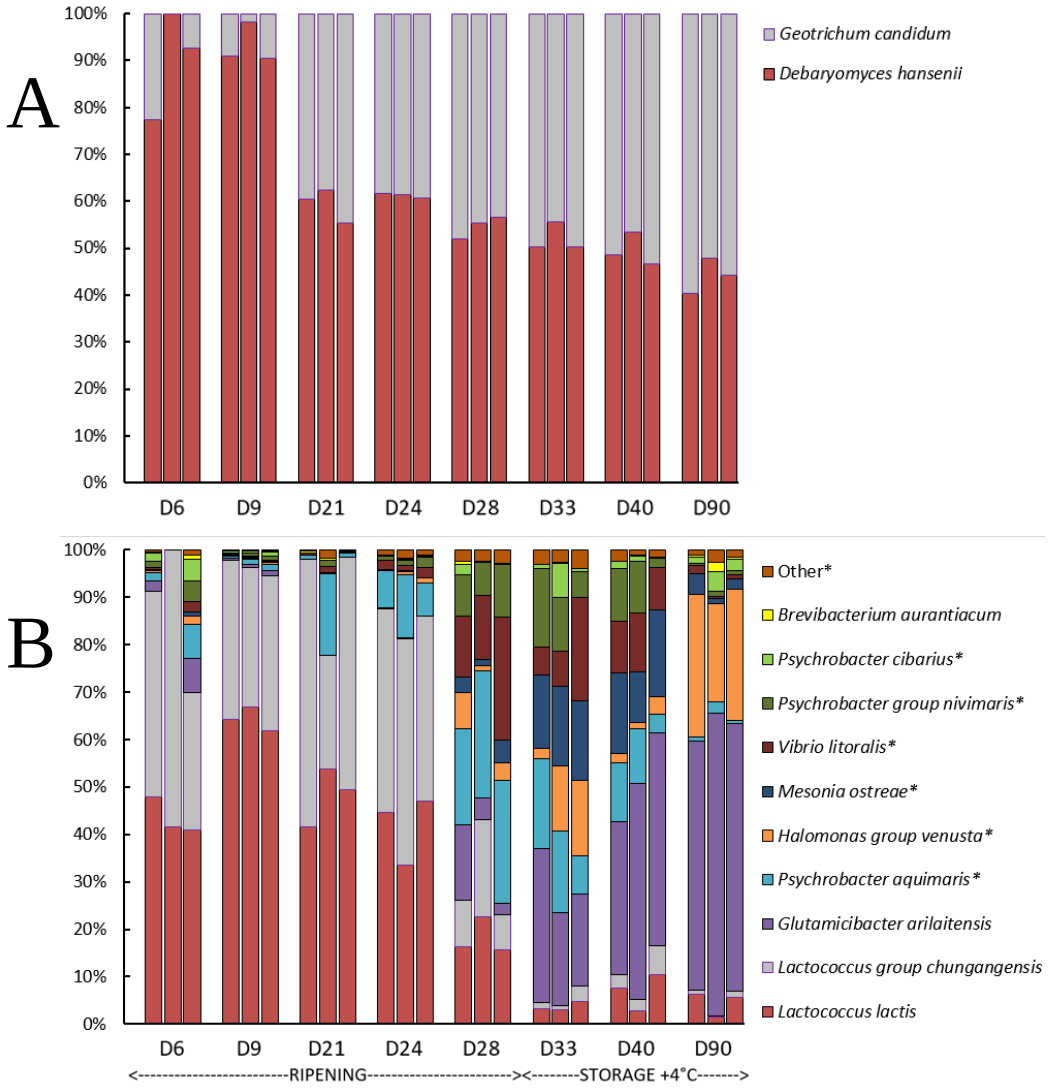

Figure 4. Relative abundances of the fungal (A) and bacterial (B) species at the surface of the Epoisses cheeses during their manufacturing and storage. Abundances were estimated by 16S rRNA and ITS marker gene analysis. Microorganisms that were not inoculated during cheesemaking are indicated by an asterisk. The "Other" category corresponds to the subdominant species *Halomonas titanicae*, *Halomonas glaciei*, *Psychrobacter proteolyticus*, *Psychrobacter namhaensis*, *Pseudoalteromonas* group *issachenkonii*, *Marinomonas polaris*, *Cobetia* group *marina* and *Sphingomonas* group *insulae*. Three separate cheeses were analyzed at each sampling time. The samples D6, D9, D21, D24, D28, D33, D40 and D90 correspond to cheeses sampled from 6 to 90 days after the beginning of manufacturing.

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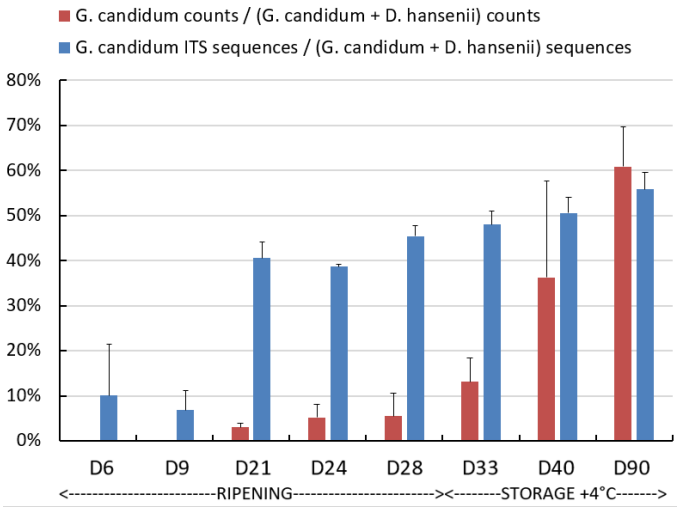

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639 **Figure 5**. Proportion of *G. candidum* among the yeasts at the surface of the Epoisses cheeses
640 during their manufacturing and storage. Abundances were estimated by colony counting and
641 by ITS marker gene analysis. The samples D6, D9, D21, D24, D28, D33, D40 and D90
642 correspond to cheeses sampled from 6 to 90 days after the beginning of manufacturing.

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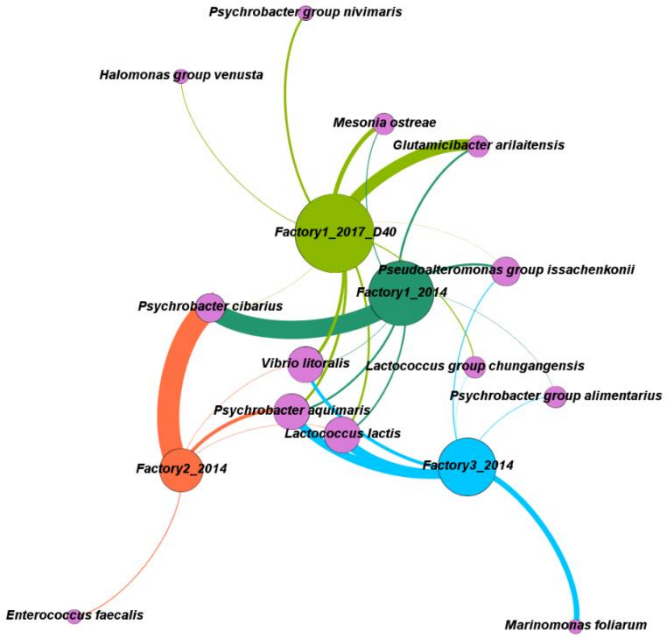

Figure 6. OTU-sample network summarizing the relationships between bacterial species of the cheese rinds from the present study at day 40 (sampled in 2017) and of cheeses sampled in 2014 and that were manufactured by the same factory (Factory 1) or by two other Epoisses factories (Factories 2 and 3). Nodes represent OTUs and cheeses. Connection lines indicate the detection of OTUs in the cheeses. Only lines corresponding to OTUs detected at >0.5% relative abundance in the cheese rinds (mean of three samples) are shown. For the cheese nodes, different colors (green, dark green, orange and blue) are used to differentiate cheese factories, and sizes are proportional to bacterial richness (i.e., number of connected OTU nodes per sample). For OTU nodes, sizes are proportional to the number of occurrences in the cheese rinds. The thickness of the connecting edges is proportional to the relative abundance of the OTU in the considered cheese.

SUPPLEMENTARY MATERIAL

Supplemental Table S1 Proportion of bacterial or fungal operational taxonomic units (OTUs) in the samples (%)

bacterial OTUs	D6a	D6b	D6c	D9a	D9b	D9c	D21a	D21b	D21c	D24a	D24b	D24c	D28a	D28b	D28c	D33a	D33b	D33c	D40a	D40b	D40c	D90a	D90b	D90c	Mean
Lactococcus lactis	47.877	41.522	40.862	64.167	66.859	61.761	41.494	53.752	49.335	44.684	33.590	46.964	16.265	22.653	15.644	3.159	2.963	4.793	7.673	2.910	10.520	6.251	1.545	5.668	28.871
Lactococcus group chungangensis	43.383	58.404	29.107	33.667	29.416	32.743	56.576	23.981	49.034	42.969	47.629	39.024	9.949	20.509	7.331	1.311	0.915	3.314	2.816	2.301	5.938	0.958	0.233	1.365	22.620
Glutamicibacter arilaitensis	2.251	0.000	7.080	0.491	0.625	1.228	0.004	0.100	0.022	0.051	0.357	0.015	15.728	4.461	2.577	32.447	19.712	19.382	32.158	45.543	44.966	52.428	63.823	56.264	16.738
Psychrobacter aquimaris	1.646	0.005	7.309	0.387	1.089	1.275	0.901	17.065	0.908	7.857	13.269	6.979	20.360	26.845	25.908	19.089	17.153	8.092	12.346	11.475	3.815	0.927	2.420	0.842	8.665
Halomonas group venusta	0.386	0.000	1.760	0.117	0.267	0.281	0.030	0.172	0.027	0.287	0.632	1.118	7.604	1.221	3.537	2.223	13.599	15.883	2.031	1.337	3.774	30.068	20.572	27.627	5.606
Mesonia ostreae	0.323	0.000	0.902	0.061	0.125	0.233	0.004	0.023	0.000	0.051	0.065	0.030	3.305	1.167	4.811	15.440	16.785	16.800	17.090	10.672	18.250	4.396	1.138	2.225	4.746
Vibrio litoralis	0.435	0.005	2.115	0.135	0.369	0.343	0.354	1.434	0.135	1.810	1.119	2.192	12.745	13.590	26.108	5.806	7.603	21.779	10.839	12.412	8.956	1.736	0.556	0.696	5.553
Psychrobacter group nivimaris	1.285	0.000	4.328	0.357	0.486	0.790	0.060	1.321	0.153	0.917	1.101	2.251	8.734	6.827	10.929	16.646	11.258	5.433	11.104	10.930	2.020	0.358	1.090	1.051	4.143
Psychrobacter cibarius	1.688	0.005	4.528	0.291	0.461	0.980	0.009	0.032	0.004	0.009	0.194	0.005	2.305	0.151	0.135	0.768	7.113	0.601	1.507	1.164	0.186	1.363	3.933	2.188	1.234
Brevibacterium aurantiacum	0.373	0.034	0.988	0.200	0.157	0.214	0.286	0.335	0.112	0.194	0.301	0.236	0.689	0.281	0.117	0.039	0.327	0.092	0.121	0.054	0.073	0.436	2.160	0.528	0.348
Pseudoalteromonas group issachenkonii	0.066	0.000	0.188	0.013	0.012	0.010	0.004	0.344	0.013	0.079	0.047	0.044	0.350	0.708	0.674	0.421	0.290	0.301	0.892	0.431	0.432	0.187	1.515	0.550	0.316
Marinomonas polaris	0.025	0.000	0.078	0.017	0.007	0.019	0.000	0.005	0.000	0.000	0.000	0.005	0.249	0.124	0.400	1.820	0.649	1.164	0.866	0.286	0.708	0.345	0.143	0.378	0.304
Cobetia group marina	0.037	0.000	0.147	0.013	0.025	0.019	0.043	0.742	0.072	0.556	0.697	0.552	0.294	0.383	0.426	0.113	0.154	0.370	0.031	0.016	0.104	0.000	0.000	0.005	0.200
Halomonas titanicae	0.000	0.000	0.016	0.004	0.007	0.005	0.004	0.036	0.000	0.030	0.108	0.094	0.260	0.065	0.400	0.080	0.450	1.310	0.067	0.027	0.076	0.317	0.446	0.150	0.165
Psychrobacter namhaensis	0.017	0.000	0.114	0.004	0.022	0.014	0.009	0.194	0.036	0.282	0.404	0.079	0.424	0.605	0.591	0.231	0.279	0.085	0.166	0.102	0.041	0.025	0.018	0.023	0.157
Sphingomonas group insulae	0.021	0.020	0.045	0.039	0.025	0.019	0.218	0.425	0.148	0.218	0.400	0.291	0.350	0.383	0.222	0.055	0.082	0.108	0.081	0.043	0.062	0.028	0.023	0.045	0.140
Halomonas glaciei	0.087	0.000	0.147	0.013	0.030	0.014	0.004	0.032	0.000	0.007	0.069	0.123	0.203	0.022	0.174	0.132	0.338	0.432	0.152	0.172	0.073	0.137	0.170	0.341	0.120
Psychrobacter proteolyticus	0.100	0.005	0.286	0.022	0.017	0.052	0.000	0.009	0.000	0.000	0.017	0.000	0.186	0.005	0.017	0.220	0.330	0.062	0.058	0.124	0.007	0.040	0.215	0.055	0.076
fungal OTUs	D6a	D6b	D6c	D9a	D9b	D9c	D21a	D21b	D21c	D24a	D24b	D24c	D28a	D28b	D28c	D33a	D33b	D33c	D40a	D40b	D40c	D90a	D90b	D90c	Mean
Debaryomyces hansenii	77.428	99.887	92.624	90.852	98.276	90.489	60.520	62.480	55.353	61.711	61.524	60.679	51.859	55.442	56.476	50.204	55.634	50.271	48.493	53.323	46.538	40.436	47.793	44.199	63.020
Geotrichum candidum	22.572	0.113	7.376	9.148	1.724	9.511	39.480	37.520	44.647	38.289	38.476	39.321	48.141	44.558	43.524	49.796	44.366	49.729	51.507	46.677	53.462	59.564	52.207	55.801	36.980

Supplemental Figure S1 Alpha-diversity indices of bacterial communities during cheese-making and storage