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The Composition of Camembert Cheese-Ripening Cultures Modulates both Mycelial Growth and Appearance

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The fungal microbiota of bloomy-rind cheeses, such as Camembert, forms a complex ecosystem that has not been well studied, and its monitoring during the ripening period remains a challenge. One limitation of enumerating yeasts and molds on traditional agar media is that hyphae are multicellular structures, and colonies on a petri dish rarely develop from single cells. In addition, fungi tend to rapidly invade agar surfaces, covering small yeast colonies and resulting in an underestimation of their number. In this study, we developed a real-time quantitative PCR (qPCR) method using TaqMan probes to quantify a mixed fungal community containing the most common dairy yeasts and molds: *Penicillium camemberti*, *Geotrichum candidum*, *Debaryomyces hansenii*, and *Kluyveromyces lactis* on soft-cheese model curds (SCMC). The qPCR method was optimized and validated on pure cultures and used to evaluate the growth dynamics of a ripening culture containing *P. camemberti*, *G. candidum*, and *K. lactis* on the surface of the SCMC during a 31-day ripening period. The results showed that *P. camemberti* and *G. candidum* quickly dominated the ecosystem, while *K. lactis* remained less abundant. When added to this ecosystem, *D. hansenii* completely inhibited the growth of *K. lactis* in addition to reducing the growth of the other fungi. This result was confirmed by the decrease in the mycelium biomass on SCMC. This study compares culture-dependent and qPCR methods to successfully quantify complex fungal microbiota on a model curd simulating Camembert-type cheese.

The surface fungal microbiota of bloomy-rind cheeses, such as Camembert, forms a complex ecosystem. The most common commercial ripening strains used to produce Canadian Camembert-type cheese from pasteurized milk are *Penicillium camemberti*, *Geotrichum candidum* (teleomorph, *Galactomyces candidus*), *Kluyveromyces lactis*, and *Debaryomyces hansenii*. These ripening fungi are the major contributors to the technological and sensory properties of bloomy-rind cheeses, such as Camembert (22, 33, 38). The mycelium development of *G. candidum* and *P. camemberti* is responsible for the bloomy aspect of Camembert-type cheese. Moreover, their proteolytic and/or lipolytic activity leads to the typical sensory properties of this cheese (5, 28). Because *D. hansenii* can accelerate surface alkalinization and enhance cheese flavor, cheese makers may inoculate this yeast directly into the milk, brine, or washing solution (35). Because *D. hansenii* is ubiquitous and highly salt tolerant, it may be present at the surface of manufactured cheeses, even in cases where the yeast is not added deliberately. In those cases, *D. hansenii* originates from the dairy environment, particularly from milk or brine (1, 21, 35, 40).

To date, microbiological enumeration has been the preferred method to monitor these species, but this approach is laborious and was shown to be inadequate and inaccurate for filamentous fungi (19, 23, 24). Given the multicellular structure of some fungi and the filamentous phenotype of molds, colonies on traditional agar media rarely represent single cells (20, 29). Moreover, molds quickly invade the medium surface, overlapping or merging with other colonies, making enumeration difficult. As a result, researchers have proposed other strategies to monitor fungal growth in cheese. Biomass estimation using mycelium dry weight determination was attempted (5), but it was inadequate for complex environments because the contribution of each species in the cheese could not be determined (29). Because of the challenges, quantitative real-time PCR (qPCR) has been widely proposed for fungal and bacterial enumeration, such as for ripening or spoilage fungi in dairy products (8, 24), and could provide an accurate estimate of the surface microbiota of Camembert-type cheese. Among others, a SYBR green-based qPCR method showed that *G. candidum* dominates the surface of a red-smeared cheese during ripening, while *K. lactis* appeared only in the first days (17). Comparison of microbiological and molecular methods showed a 10- to 100-fold underestimation of the total microbiota when the classical medium counting method was used.

Different properties or behaviors of cheese microorganisms can be evaluated using cheese models (16, 18, 24, 32, 33, 38). The most common application of these models has been to study the microbial interactions between starters and/or spoilage microorganisms, pathogen inhibition, and the production of metabolites (18, 24, 32, 33). Cheese agar mediums were used to describe fungal interactions between Camembert starters (*P. camemberti*, *Penicillium nalgiovese*, *Penicillium roqueforti*, and *G. candidum*) and spoilage microorganisms (33) or between yeasts (*D. hansenii* and *Yarrowia lipolytica*) and *P. roqueforti*. A soft red-smeared model cheese (close to Livarot) was inoculated with a cheese microbiota to demonstrate the interactions between yeasts (*G. candidum*, *D. hansenii*, and *Y. lipolytica*) and bacteria (32). The association of cheese models and qPCR was reported recently, as a SYBR green qPCR method was developed to quantify *P. camemberti* on Coulommiers and Carré cheeses (24). This soft-cheese model was compared to natural cheeses provided by a French industrial

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TABLE 1 Target genes for qPCR assays and primers used for gene sequencing

Microorganism	Targeted gene	Primers (5′→3′)	Length (bp)	Accession no.[a]
K. lactis	β-Galactosidase (*lac4*)	Forward primer: GAG GGT GAC TTG AAC GCT TTG Reverse primer: GCC GTG TTG AAA GAT GAT GCT	603	JN426962
D. hansenii	Phosphatidylinositol-4-kinase (*pik1*)	Forward primer: CCA CGT CGC ACA GTG AAC AT Reverse primer: CAC CGA ACC AAA CAC AGG C	1,001	JN426963
G. candidum	Cystathionine γ-lyase (*cgl*)	Forward primer: CGG TGG TAC CCA CAG ATA CTT T Reverse primer: GCC GGT AAC GAG AAC TCC AT	231	JN426964
P. camemberti	β-Tubulin (*tub*)	Forward primer: TTA CAG GCA AAC CAT CTC TGG Reverse primer: AGT CCG GTG CTG GTA ACA ACT	370	JN426965

[a] Accession numbers are for GenBank.

cheese maker. For both, the active growth phase of *P. camemberti* was observed until day 11 (3, 24) and the qPCR method was shown to be an accurate mold quantification method without the bias of traditional microbiological methods (19, 23, 24).

The aim of the present study was to develop a qPCR method to accurately enumerate individual members of a common Camembert cheese-ripening culture while providing a soft-cheese model curd (SCMC) that allows characterization of Camembert cheese microbiota behavior.

MATERIALS AND METHODS

Biological material. Yeast and mold inocula were prepared separately from lyophilized commercial cultures of *K. lactis* LMA-437, *G. candidum* LMA-436, *P. camemberti* LMA-435, and *D. hansenii* LMA-1019. The cultures were rehydrated in water containing 0.1% (wt/vol) peptone and stored at 4°C overnight before use.

Soft-cheese model curd production. The SCMC is intended to mimic Camembert-type cheese composition using controlled conditions. For this reason, neither salt nor ripening culture was added during processing, but both were added at the rehydration step. The SCMC was produced using 160 liters of pasteurized milk (18 s at 72°C), held at 33°C and inoculated with 1% (vol/vol) Flora Danica (DK-2970; Chr. Hansen, Hørsholm, Denmark); 0.26 ml/liter of a $CaCl_2$ solution (Cal-Sol; 45% wt/vol) was added. When the milk reached pH 6.3 (after 80 to 100 min), Chy-Max rennet (containing 520 mg/liter of chymosin; Chr. Hansen) was added to 0.1 ml/liter. After a 13-min coagulation time followed by a 26-min hardening, the cheese curd was cut and shaped in polyurethane molds (diameter, 110 mm; height, 107 mm). Molds were inverted three times, 30 min, 1 h, and 3 h after molding. They were placed in a ripening chamber with a relative humidity greater than 85% and incubated at 23°C overnight. No salting step was performed. The curd was ground by hand, freeze dried using a Lyo-Tech apparatus (Lyo-Tech, Lachute, Quebéc, Canada), and shredded using a Quadro Comil instrument (Quadro Engineering, Waterloo, Ontario, Canada). The final product was a fine, dry powder. The powder was sealed in plastic bags under vacuum in 1-kg fractions and stored at −20°C, away from light.

SCMC preparation and ripening conditions. The rehydration of SCMC was designed to match the standard physicochemical condition of cheese. The freeze-dried cheese curd powder was hydrated in sterile water containing 2% (wt/wt) NaCl and the fungal ripening strains to be studied. *K. lactis*, *G. candidum*, *D. hansenii*, and *P. camemberti* were inoculated following typical inoculation methods for commercial strains in Camembert cheese. Hydrated and inoculated curds (55 g) were distributed in 250-ml glass jars (Bernardin; Jarden Branded Consumables, Richmond Hill, Ontario, Canada) and sealed with cheese wrapping paper (Amcor Kirkland, Kirkland, Quebec, Canada). Hydrated curds contained 57% moisture and a 3.5% salt-in-moisture ratio (S/M). The initial pH of the hydrated curd was 4.6. For all experiments, the ripening chamber was held at 90% relative humidity (RH) at 14°C for 9 days and then cooled to 4°C (90% RH) and maintained at this temperature for 31 days of ripening.

SCMC sampling and pH measurement. Three biological repetitions were performed for each experiment. The analysis included pH measurement, fungi enumeration, fresh and dry weight determinations, and DNA isolation. Sampling was done in triplicate at days 0, 4, 6, 9, 14, 18, 23, and 31. The pH of the rind was measured using a pH meter (Accumet basic AB15 instrument; Fisher Scientific, Ottawa, Ontario, Canada) with a calibrated electrode (Probe pH spear tip; Sper Scientific, Taiwan, China). To measure pH, we placed the electrode just beneath the rind. Three readings were recorded for each sample, and three samples were analyzed at each sampling day.

Fungi enumeration and dry weight measure. For enumeration of fungi, 12.5 cm^2 of the rind was scraped using a sterile surface template and scalpel. The entire 12.5 cm^2 was weighed, ground with a mortar and pestle, and then diluted (1:9) in 2% sodium citrate and homogenized in a stomacher instrument (Stomacher 400 circulator; Seward) for 2 min at 250 rpm. Serial dilutions in peptone water (0.1%) were spread on acidified potato dextrose agar (PDA; pH 3.5 medium). Colonies were counted after an incubation of 4 days at 25°C. Moreover, 1 ml of each 10-fold dilution used for counting CFU was centrifuged in preweighed 1.5-ml tubes, and the pellet was dried for 4 days at 65°C and weighed.

DNA isolation and design of real-time qPCR probe and primers. Another 12.5-cm^2 sample of mycelia (prepared and ground as described above) was frozen in liquid nitrogen (13), and the total DNA was isolated from a 20- to 25-mg aliquot using the method optimized by Al-Samarrai and Schmid (4). The qPCR target genes are listed in Table 1; the sequence of each target gene was determined and confirmed for all strains. Based on these sequences and data available in GenBank, we designed probes and primers using Primer Express v2.0 software (Applied Biosystems (ABI); Life Technologies Corporation, Carlsbad, California).

Optimization of real-time qPCR assays. TaqMan qPCR assays were individually optimized for quantification. A large range of oligonucleotide concentrations (from 200 μM to 900 μM) was tested, and the combination of 200 μM each primer and 200 μM the TaqMan probe was chosen in each case to maximize qPCR efficiency, detection limit, and fluorescence level. The efficiency of the qPCR with each target gene ranged from 93% to 101% ($R^2 > 0.99$), and no cross-amplification or negative control amplification was observed (Table 2). For each gene, a standard curve was obtained using dilutions of recombinant plasmids containing cloned target genes. Under these conditions, a dynamic range from 10^3 to 10^9 gene copies/reaction was established as accurate for precise quantification. Finally, DNA isolation was optimized for ready-to-eat Camembert cheeses in order to maximize DNA yield per mg of mycelium. DNA extractions using 1, 5, 10, 21, 40, and 60 mg of ground mycelium were performed to determine the optimal mycelium mass to be used. Of the six quantities tested, 21 mg showed the highest DNA yield (950.7 ng of DNA/mg of ground mycelium) and was selected for further experiments.

TABLE 2 Primers and probes selected for qPCR quantification and reaction efficiency

Species	Target gene	Primers and probes (5′→3′)[a]	qPCR efficiency (%)	R^2
K. lactis	*lac4*	Primer 1: GCG CAC GGA TCT GTA ACA ATC Primer 2: GTT TAT CGA CAA GGA CAC GGG Probe: 5′FAM-CAA ATA AGC ACG ACT TCA TTA CGA CAG ACC ACT T-TAMRA3′	101.4	0.994
D. hansenii	*pik1*	Primer 1: AGA GCG CCG ATA GTA CTT CTA AGG Primer 2: AAG CTT GGA CAC ATG GGA AGA Probe: 5′FAM-ACT GCG CAA GCT TCA AAA TTC GGC TA-TAMRA3′	100.4	0.995
G. candidum	*cgl*	Primer 1: CGC TTT CAC CAA CTC CAT TGA Primer 2: CTT GTC TGG ATC GAG TCT CCC T Probe: 5′FAM-CCG AGC TCC GTG ACA TCA TCA CTG A-TAMRA3′	93.1	0.995
P. camemberti	*tub*	Primer 1: CCG TTC TCG TCG ATT TGG A Primer 2: CTT TCG GCA AGC TTT TCC G Probe: 5′FAM-TGG TAC CAT GGA CGC TGT CCG CT-TAMRA3′	94.3	0.993

[a] FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

Real-time qPCR standard curve construction. Recombinant plasmids were created to be used as quantification standards for all qPCR assays. Each target gene was PCR amplified using sequencing primers (Table 1), cloned into the PCR8 vector using a Topo-TA cloning kit (Invitrogen; Life Technologies Corporation, Carlsbad, CA), and transformed in *Escherichia coli* TOP10. The recombinant plasmid DNA was purified using a Megaprep kit (Qiagen, Toronto, Ontario, Canada) and was sequenced. Plasmid DNA concentration was measured with a Nanodrop (ND-1000) spectrophotometer (Thermo Scientific, Wilmington, NC). Ten-fold dilutions of plasmid (between 10^3 and 10^9 gene copy) were used as a standard curve for absolute qPCR quantification of our samples.

Real-time qPCR assays. Real-time qPCR assays were performed using an ABI 7500 Fast system (ABI). Each 25-μl reaction mixture contained 2.5 μl of the appropriate dilution of each DNA sample, 12.5 μl of TaqMan Fast universal PCR master mix (2×) No AmpErase UNG (ABI), and 200 μM (each) primers and probe. PCR plates were set up using an epMotion 5075 VAC automated pipetting system (Eppendorf, Mississauga, Ontario, Canada). The PCR temperature cycles included an initial denaturation at 95°C for 20 s, followed by 40 cycles of denaturation at 95°C for 3 s and annealing/extension at 60°C for 30 s. Quantification of each sample was possible by comparing the threshold cycle with the standard curve. All qPCR data are expressed as gene copies per cm^2 of rind.

Comparison of real-time qPCR and microbiological quantification. For comparison of the results obtained using real-time qPCR and microbiological counts, individual fungal strains were inoculated on SCMC and quantified using both methods. Each model curd was ripened at 14°C for 10 days. Dry mycelium weight, microbiological counts, and qPCR assays were performed to compare the results and the accuracy of the molecular quantification method used for further experiments.

Study of a dynamic fungal community during ripening. The qPCR method was used to study the dynamics of a simple ripening microbiota composed of three fungal strains: *K. lactis*, *G. candidum*, and *P. camemberti*. Inoculation of these fungal strains provided an initial count of approximately 4×10^3 CFU of *G. candidum* LMA-436, 4×10^3 CFU of *P. camemberti* LMA-435, and 1.6×10^2 CFU of *K. lactis* LMA-437 per ml of milk, corresponding to what is normally observed in a Camembert cheese at the beginning of the ripening period. This experiment was performed in triplicate. To address the impact of the addition of *D. hansenii* to this ecosystem, another set of experiments was performed in which the composition of the ripening microbiota was the same except that *D. hansenii* LMA-1019 was added at approximately 1×10^4 CFU/ml of milk.

RESULTS

Soft-cheese model curd as a tool to study surface microbiota of a Camembert-type cheese. We evaluated the use of SCMC in combination with real-time qPCR as tools to study surface microbiota of a soft cheese. We validated the growth of a simple microbiota composed of three species, *K. lactis*, *G. candidum*, and *P. camemberti*, under typical Camembert-type cheese-ripening conditions over 31 days. The alkalinization of the rind was observed from day 0 to day 14 (Fig. 1) and corresponded to what is generally reported for Camembert-type cheese (11, 19, 25). The initial rind pH of 4.54 increased to 8.10 at day 14 and remained stable until the end of ripening at day 31. The microbiota development on the surface of SCMC was evaluated by applying the real-time qPCR technique using TaqMan probes in combination with traditional microbiological methods. At day 0, since no rind was observed, each fungal

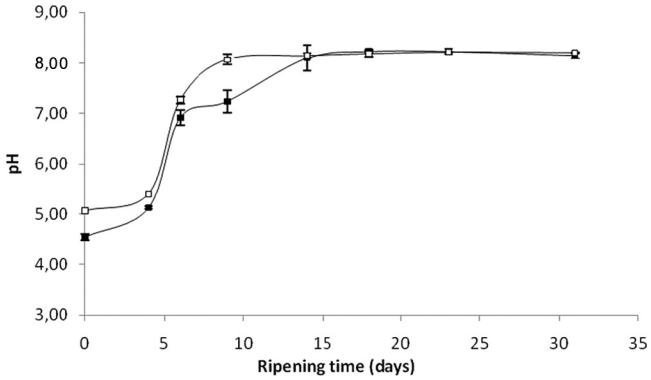

FIG 1 pH evolution of a model cheese curd over 31 days of ripening. The ripening culture was a mixture of *K. lactis* LMA-437, *G. candidum* LMA-436, and *P. camemberti* LMA-435 (■) and of *K. lactis* LMA-437, *D. hansenii* LMA-1019, *G. candidum* LMA-436, and *P. camemberti* LMA-435 (□). Inoculation of the fungal strains provided initial counts of approximately 4×10^3 CFU of *G. candidum* LMA-436, 4×10^3 CFU of *P. camemberti* LMA-435, and 1.6×10^2 CFU of *K. lactis* LMA-437 per ml of milk. In both cases, the ripening chamber was held at 90% relative humidity and the ripening was performed at 14°C for 9 days and then at 4°C until day 31.

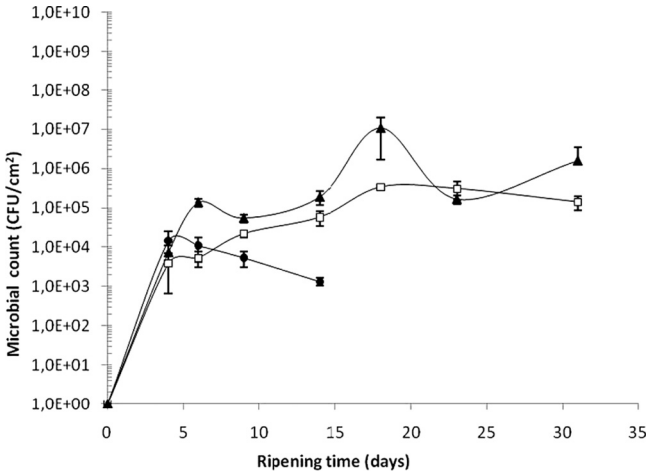

FIG 2 Microbial count on acidified PDA medium (pH 3.5) of the *K. lactis* LMA-437 (●), *G. candidum* LMA-436 (□), and *P. camemberti* (▲) ripening culture.

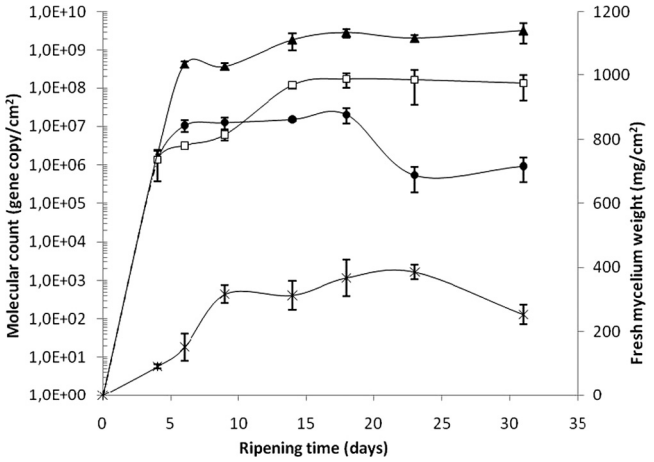

FIG 3 Comparison of the evolution of the mycelium biomass (×) and the surface microbiota quantification of a soft-cheese model curd using a TaqMan real-time qPCR method. The ripening culture contained *K. lactis* LMA-437 (●), *G. candidum* LMA-436 (□), and *P. camemberti* (▲).

strain was considered to be at a concentration close to zero cells per square centimeter of mycelium. When quantified using real-time qPCR, all three microorganisms demonstrated similar growth tendencies. Microbiological methods showed different growth profiles (Fig. 2-3), with the qPCR method generally detecting 10- to 1,000-fold-higher cell counts (17, 43).

We obtained considerable variation in the microbial counts of *P. camemberti*, resulting in a growth curve that was not indicative of the fungal development observed on the cheese surface. Nevertheless, the results show an active growth phase starting at day 4 with *P. camemberti* reaching approximately 1.14×10^7 CFU/cm^2 at day 18 (Fig. 2). The growth profile obtained for *P. camemberti* using qPCR more closely matched the observable mycelium spreading over the cheese surface. The qPCR quantification method revealed that the active growth phase of *P. camemberti* occurred between day 0 and day 6, at which time *P. camemberti* neared its maximum count at 4.31×10^8 gene copy/cm^2. After 18 days, a maximum count of 2.88×10^9 gene copy/cm^2 was attained, and it was maintained up to day 31.

The active growth phase for *G. candidum* LMA-436 occurred from day 4 to day 18. Again, qPCR revealed more rapid growth than what was observed using traditional culture methods, probably because the qPCR technique detected both the mycelial and yeast forms of this organism. Also, the presence of *G. candidum* is detectable at the molecular level in advance of visual detection on a culture plate. Culture methods detected maximal growth at day 18, reaching 3.30×10^5 CFU/cm^2, while the maximum growth detected by qPCR was 1.70×10^8 gene copy/cm^2 (Fig. 2 and 3). In both cases, the stationary phase was stable up to day 31.

The third microorganism of this ecosystem, *K. lactis*, showed only limited growth during the early phase of ripening, between day 4 and day 6, reaching a cell density of 1.08×10^4 CFU/cm^2 (traditional culture method) at day 4 and 1.08×10^7 gene copy/cm^2 (qPCR method) at day 6. After day 14, no *K. lactis* was detected by the microbiological method; however, the qPCR method was able to detect the presence of this microorganism. The culture-dependent methods had clear limitations, since after day 14 it was impossible to enumerate *K. lactis* because of the dominance of the other microorganisms.

Investigation of the global ecosystem grown on SCMC revealed that *P. camemberti* represents nearly 95% of the total population after only 6 days (Fig. 3). This was reflected by the correlation between the growth curve of *P. camemberti* obtained using qPCR and the mycelium weight per square centimeter, except for the weight decrease in the last days of ripening (Fig. 3). Fresh mycelium biomass increased until it reached 386 mg/cm^2 at day 23. As observed elsewhere for Camembert cheese, after this time the mycelia became less dense and less humid and lost height, resulting in a 133 mg/cm^2 weight loss of the rind between day 23 and day 31 (Fig. 3).

Influence of the addition of *D. hansenii* to cheese microbiota in typical Camembert cheese-ripening conditions. The effect of adding the yeast *D. hansenii* LMA-1019 to the previous ecosystem was monitored using the new tools developed in this study. Overall, the alkalinization of the curd surface was slightly faster than what was observed previously (Fig. 1). In this ecosystem, traditional microbiological methods were unable to monitor *K. lactis*, since filamentous species, especially *P. camemberti*, invaded petri dishes and masked the *K. lactis* colonies. Real-time qPCR allowed us to monitor *K. lactis* growth without using culture-dependent methods. Globally, *D. hansenii* appeared to be a good competitor in this ecosystem since this strain negatively affected the cell counts of the other microorganisms. In the presence of *D. hansenii*, the growth kinetics of *G. candidum* and *P. camemberti* were similar to what was previously observed (Fig. 4), but their maximal cell densities were reduced by 346- and 13-fold, respectively (Fig. 5).

The qPCR method showed rapid growth of *D. hansenii* during the first days of ripening, quickly reaching a maximum at day 6. The rapid growth rate of *D. hansenii* between day 0 and day 6 seems to coincide with a lag phase for *G. candidum*. Along with *P. camemberti*, *D. hansenii* colonizes the surface of the cheese in the first 10 days of the ripening period. Moreover, the active growth phase of *G. candidum* coincides, at day 9, with the slowing growth of both *D. hansenii* and *P. camemberti*. *K. lactis* was clearly inhibited by the addition of *D. hansenii*, since about 1×10^4 gene copy/cm^2 was obtained for the entire ripening time without significant

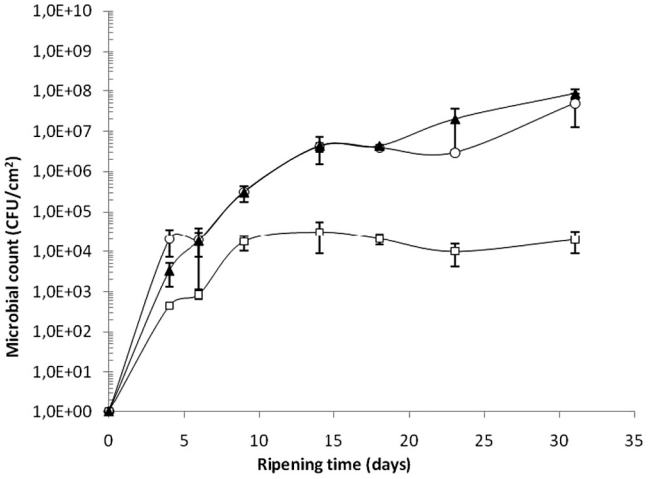

FIG 4 Microbial count on acidified PDA medium (pH 3.5) of the *D. hansenii* LMA-1019 (○), *G. candidum* LMA-436 (□), and *P. camemberti* (▲) ripening culture. *K. lactis* LMA-437 was not detected on acidified PDA medium.

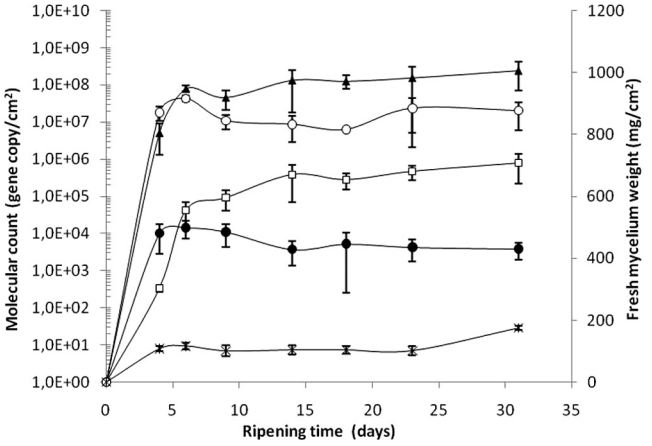

FIG 5 Comparison of the evolution of the mycelium biomass (×) and the surface microbiota quantification of a soft-cheese model curd using a TaqMan real-time qPCR method. The ripening culture contained *K. lactis* LMA-437 (●), *D. hansenii* LMA-1019 (○), *G. candidum* LMA-436 (□), and *P. camemberti* (▲).

growth. Finally, the inhibitory effect of *D. hansenii* on the other microorganisms was reflected in the density of the mycelium biomass measured throughout the ripening, since the mycelium weight did not exceed 175 mg/cm^2, compared to a maximum of 386 mg/cm^2 in the absence of *D. hansenii* (Fig. 5). The cheese rind was evaluated using visual and olfactory assessment (data not shown). The addition of *D. hansenii* to the ripening microbiota of SCMC generated a reduction in mycelium thickness and a more persistent sulfur odor and, finally, modified the white color (beige-pink) and the bloomy aspect of the mycelia.

DISCUSSION

The real-time PCR method proposed in this study overcame several limitations found for traditional microbiological counting. Since few studies have addressed the SYBR green-based real-time PCR quantification of these microorganisms independently, we optimized a TaqMan assay that allowed the use of identical amplification parameters for each strain and eliminated the need for a melting-curve analysis (8, 17, 24). Moreover, qPCR allowed the quantification of *K. lactis* in both ecosystems evaluated in this study, even where standard plating methods failed.

Quantifying mycelium-forming fungi like *P. camemberti* and *G. candidum* using culture-dependent methods resulted in large variations in enumeration, especially in the early ripening stage (before day 10). During this early stage, filamentous fungi produce a large number of hyphae to increase access to the cheese matrix nutrients, especially during their exponential growth phase (32). As noted earlier, culture-dependent methods do not reflect this exponential growth of fungi because cells contained in the hyphal filaments cannot be counted on culture media and because these techniques are highly influenced by the presence of spores (19). Inaccurate evaluation of the exponential growth phase for *P. camemberti* was attributed to the fact that spore formation began at about day 5 (36) and was predominant between day 8 and day 12 of the ripening period (19, 23). Spores can easily be separated from mycelia and form individual colonies on culture medium, while dense mycelia are difficult to spread. This could explain why qPCR detected the growth of *P. camemberti* earlier than culture-dependent methods and why it reflected mycelium development on cheese more accurately. qPCR may lead to overestimation during the stationary and declining growth phases of the microorganisms because the DNA of dead cells may persist and could be quantified (37, 42). This phenomenon was not observed during the latent and active growth phases because the number of living cells is much higher than the number of dead cells. In this study, the qPCR method developed was shown to be a suitable tool because it corresponds to the increase in biomass found in the SCMC.

The SCMC developed in this study appeared to be an excellent model for studying the dynamic of Camembert-type ripening microbiota and for evaluating interactions between strains. Despite slight differences in the strains and ripening parameters used, our results were consistent with the growth dynamics of other Camembert-type ecosystems using simple microbiota (19). Alkalinization observed in SCMC was similar to that reported in other studies and was associated with lactate catabolism and the ammonia produced by ripening cultures (1, 19, 20, 22, 27, 30). *P. camemberti* assimilates lactate mostly for cell growth (as a carbon source for its biosynthesis), while *G. candidum* uses lactate to maintain cell structure (2, 7). It has been suggested that in the case of *G. candidum*, the ammonia produced via amino acid catabolism may be the main factor contributing to the pH increase during cheese ripening (10).

The combination of qPCR and SCMC enabled us to study changes in the fungal ecosystem when a new strain, *D. hansenii*, was added to the consortium. Because of its ability to grow adequately in a cheese matrix and to assimilate lactose, lactate, citrate, and galactose, *D. hansenii* is considered to be a good ripening yeast for the production of some cheese varieties (12, 39, 41). The first impact of adding *D. hansenii*, which is well known to accelerate pH increases through lactate and citrate catabolism, was faster alkalinization of the rind (9, 20, 21, 31, 39). The presence of this strain also affected the growth of *P. camemberti*, *G. candidum*, and *K. lactis*, as reflected by the reduction of the rind density at the surface of the SCMC, which was visually and qualitatively evaluated. The total biomass per square centimeter was also measured and correlated with qPCR detection of reduced fungal gene cop-

ies. However, this trend was difficult to correlate with the culture-dependent methods. Since *D. hansenii* produces sulfur compounds, both the alkalinization profile and the odor of the model cheese may have been modified by the presence of this yeast (6, 14–16, 34). Moreover, *D. hansenii* is known to be unaffected by yeasts or bacterial strains used in red-smear-ripened cheese (31, 32) and, therefore, can establish itself in an ecosystem. Our results support this idea, since the growth of *D. hansenii* was detrimental to all other microorganisms in this ecosystem, especially *K. lactis*, which is known to be a poor competitor in an environment where other microorganisms use the same source of carbon and energy (19). Additional studies are needed to clearly address the behavior of each strain.

This study clearly demonstrates the usefulness of the combination of SCMC and real-time qPCR to understand the behavior of individual strains within the cheese ecosystem. These validated techniques can be used to study fungal behavior in industrial ripened cheeses. In addition, standardized qPCR parameters could facilitate routine analysis for both positive and undesired microbiota, as long as specific oligonucleotides are designed (26).

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