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Microbial Interactions within a Cheese Microbial Community▽†

Jérôme Mounier,[1] Christophe Monnet,[1] Tatiana Vallaeys,[1] Roger Arditi,[2] Anne-Sophie Sarthou,[1] Arnaud Hélias,[1] and Françoise Irlinger[1]*

UMR782 Génie et Microbiologie des Procédés Alimentaires, INRA, AgroParisTech, 78850 Thiverval Grignon, France,[1] and Ecologie des Populations et Communautés, AgroParisTech, 75000 Paris, France[2]

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The interactions that occur during the ripening of smear cheeses are not well understood. Yeast-yeast interactions and yeast-bacterium interactions were investigated within a microbial community composed of three yeasts and six bacteria found in cheese. The growth dynamics of this community was precisely described during the ripening of a model cheese, and the Lotka-Volterra model was used to evaluate species interactions. Subsequently, the effects on ecosystem functioning of yeast omissions in the microbial community were evaluated. It was found both in the Lotka-Volterra model and in the omission study that negative interactions occurred between yeasts. *Yarrowia lipolytica* inhibited mycelial expansion of *Geotrichum candidum*, whereas *Y. lipolytica* and *G. candidum* inhibited *Debaryomyces hansenii* cell viability during the stationary phase. However, the mechanisms involved in these interactions remain unclear. It was also shown that yeast-bacterium interactions played a significant role in the establishment of this multispecies ecosystem on the cheese surface. Yeasts were key species in bacterial development, but their influences on the bacteria differed. It appeared that the growth of *Arthrobacter arilaitensis* or *Hafnia alvei* relied less on a specific yeast function because these species dominated the bacterial flora, regardless of which yeasts were present in the ecosystem. For other bacteria, such as *Leucobacter* sp. or *Brevibacterium aurantiacum*, growth relied on a specific yeast, i.e., *G. candidum*. Furthermore, *B. aurantiacum*, *Corynebacterium casei*, and *Staphylococcus xylosus* showed reduced colonization capacities in comparison with the other bacteria in this model cheese. Bacterium-bacterium interactions could not be clearly identified.

Little is known about yeast-bacterium interactions, and smear-ripened cheeses offer an interesting model to investigate them. Indeed, the smear cheese microbial community is composed of both yeast and bacteria, is of a known specific composition that constitutes the "inoculum," and shows a reduced diversity and a high stability (12, 13, 23, 27, 34).

The smear is a red-orange, often viscous, microbial mat which is characterized by a succession of microbial communities including both yeast and bacteria. For example, the surface microflora of bacterial smear-ripened cheeses, such as Reblochon, Tilsit, and Limburger, is composed of yeast, mainly *Debaryomyces hansenii* and *Geotrichum candidum*, and of gram-positive catalase-positive organisms, such as coryneform bacteria and staphylococci (2, 9, 10, 35). During the first days of ripening, yeasts colonize the cheese surface and utilize lactate. This utilization progressively leads to the deacidification of the cheese surface, enabling the establishment of a bacterial community that is less acid tolerant (8). These communities are relatively simple compared with other microbial communities, such as soil communities. Indeed, they are composed of a limited number, i.e., 10 to 20 species, of mostly cultivable species (12, 27). The microbial diversity of cheese was investigated using both culture and nonculture approaches, such as repetitive extragenic palindromic sequence-based PCR, Fourier transform infrared spectroscopy, 16S rRNA gene sequencing, cloning and sequencing of 16S rRNA genes, single-strand conformation polymorphism analysis, denaturing gradient gel electrophoresis, and temperature gradient gel electrophoresis (12, 13, 27, 28, 31).

While the succession of yeast and bacteria has been well described, the functional interactions in cheese between yeast and/or bacteria are not yet understood, and only a few interactions have been observed. An early study from Purko et al. (33) on the association between yeasts and *Brevibacterium linens* showed that *B. linens* did not grow on a vitamin-free agar medium. However, when the same medium was inoculated with yeast, it grew around the yeast colonies. Some yeast and bacterial strains have been selected for use by the cheese industry because of their interesting technological properties, such as aroma production or pigmentation. However, it has been shown that these commercial ripening cultures do not necessarily implant on the cheese surface, despite their massive inoculation in the early stages of ripening (7, 12, 27, 28). Mounier et al. (28) showed that the microorganisms that developed on the cheese surface were an adventitious microflora from the cheese environment (brine, ripening shelves, and personnel) which rapidly outnumbered the commercial cultures. Several hypotheses have been advanced to explain these findings. These ripening cultures may be unfit for the cheese habitat, or negative interactions may occur between them and the adventitious microflora. Bacterial and yeast strains have also been selected for their antilisterial activity (11, 23). Eppert et al. (11) found single strains of *B. linens* producing linocin (a bacteriocin-like substance) which reduced *Listeria* populations in cheeses but did not exert an inhibition comparable to that

* Corresponding author. Mailing address: UMR782 Génie et Microbiologie des Procédés Alimentaires, INRA, AgroParisTech, 78850 Thiverval Grignon, France. Phone: 33 (0)1 30 81 54 91. Fax: 33 (0)1 30 81 55 97. E-mail: irlinger@grignon.inra.fr.

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obtained with the ripening consortia from which these strains were isolated. Inversely, none of the 400 isolates from an effective antilisterial ripening consortium evaluated in the study of Maoz et al. (23) exhibited antilisterial activity in agar diffusion assays. This implies that the antilisterial effect is probably not related to the production of inhibitory substances during growth.

In macrosystem ecology, several models that represent intra- and interspecies interactions in food webs have been established (see reference 3 for a review). The multispecies Lotka-Volterra model (22, 26, 36) is a simple model used to measure interactions, based on a linear relationship for a given species, between growth rate and the population of each member of the community. Such a model may be a good tool to investigate interactions within a microbial community.

Bonaïti et al. (5), using a three-step dichotomous approach, simplified an ecosystem of 83 strains from Livarot cheese to four subecosystems composed of nine species based on odor profile. One of these subecosystems showed great similarities with the odor profile of the 83-strain ecosystem, which was very similar to that of the commercial cheese. This subecosystem of nine species was thought to be a good model ecosystem to reproduce cheese surface diversity and to investigate microbial interactions.

The aim of this study was to identify interactions within this ecosystem in model cheeses. In the first part of this study, the growth dynamics of each member of this community were described, and the generalized Lotka-Volterra (GLV) model was used as a preliminary approach to represent inter- and intraspecies interactions. In the second part, specific strains of this community were omitted in order to evaluate the consequences of these omissions on the further development of the rest of the community (species distribution, substrate utilization, and color of the cheese surface).

MATERIALS AND METHODS

Strains. The starters used for the cheese making were frozen Flora Danica cultures (CHN 12 and CHN 15, Chr. Hansen, Arpajon, France). Flora Danica contains a mixture of *Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, citrate-positive strains of lactococci, and *Leuconostoc mesenteroides* subsp. *cremoris*.

The nine microorganisms that composed the model ecosystem were *Arthrobacter arilaitensis* 3M03, *Brevibacterium aurantiacum* 2M23, *Corynebacterium casei* 2M01, *Hafnia alvei* 2E12, *Leucobacter* sp. strain 1L36, and *Staphylococcus xylosus* 1L18 for the bacteria and *Debaryomyces hansenii* 1L25, *Geotrichum candidum* 3E17, and *Yarrowia lipolytica* 1E07 for the yeast. These strains were obtained from the culture collection of the Food Microbiology Laboratory (LMA, Caen, France). They were originally isolated from various batches of Livarot cheese.

Growth properties of the microorganisms of the ecosystem on an agar-based medium. The growth characteristics of the bacteria and yeasts as a function of pH and NaCl were tested in a medium that contained 0.5 g yeast extract, 1 g Casamino Acids, 0.1 g glucose, and 1.5 g agar. The salt content was 0, 30, 50, 100, or 150 g liter^{-1}, while the pH was 5, 5.5, 6, 6.5, or 7. Growth was visually evaluated by checking for the presence of colonies after 2, 4, and 8 days of incubation at 12°C.

Growth properties of the microorganisms found in the cheese ecosystem. In this study, two independent experiments were conducted at a 5-month interval. In the first part of the study, the growth dynamics of the nine species that composed the model ecosystem were investigated on model cheeses. The cheeses were sampled in duplicate every day for 21 days for microbial enumeration and measurement of lactose and lactate content and pH.

In the second part of the study, the effects of single or multiple omissions of the yeast strains that originally composed the ecosystem were evaluated on model cheeses. All the possible combinations were tested. Cheeses were sampled in triplicate on days 0, 3, 11, and 21 for microbial enumeration and measurement of lactose, lactate, ammonia, and free amino acid content, surface pH and color development.

Cheese production. Pilot-scale cheese production (coagulation, cutting, draining, and molding of the curd) according to a process used for Livarot cheese was carried out under aseptic conditions in a sterilized, 2-m^3 chamber as previously described by Leclercq-Perlat et al. (19). The milk used (~100 liters) was pasteurized full-fat milk, standardized at 29 g/liter fat with skim milk. The milk was pasteurized for 2.5 min at 75°C and cooled at 37°C in the chamber. After 1 liter of milk had been pumped into the tank, the milk was inoculated with the starter culture (Flora Danica, Chr. Hansen, Arpajon, France). A filter-sterilized 10% $CaCl_2$ solution (100 ml) was added at the end of pasteurization. It was followed by the addition of the filter-sterilized coagulant containing 520 mg/liter of chymosin at 30 ml/100 liters of milk. The coagulation time was 20 min, and the cutting of the curds took place after 30 min of hardening. The curd was then manually stirred for 5 min at a rate of 10 stirs/min. After the curd had stood for 15 min, 70 liters of whey were removed prior to molding. The cheeses were shaped in circular polyurethane molds with a diameter of 9 cm and a height of 11 cm. The cheeses weighed approximately 350 g. The molds were inverted four times, after 10 min, 2 h, 5 h, and 15.5 h, with a temperature of 20°C in the chamber. After 17 h, the cheeses were demolded, and after another hour, they were transferred to sterile bags and stored at −80°C until use.

Ripening culture. The yeast and bacteria were first precultured in 10 ml of potato dextrose broth or brain heart infusion (BHI) broth, respectively, in 50-ml flasks incubated at 25°C for 55 h at 150 rpm. Amounts of 400 μl of each preculture were then used to inoculate 40 ml of potato dextrose broth or BHI broth in 150-ml flasks, which were incubated at 25°C for 66 h at 150 rpm. Five to 10 ml of each preculture were centrifuged at room temperature for 10 min at 4,000 rpm. The supernatant was discarded, and the cells resuspended in 9 g/liter NaCl to obtain a concentration of 2×10^9 CFU/ml and 2×10^7 CFU/ml for the bacteria and the yeast, respectively. Subsequently, 1,280 μl of each suspension was mixed and supplemented to make 20 ml with 9 g/liter NaCl in a volumetric flask. This suspension was used to inoculate the model cheeses.

Curd inoculation. Under sterile conditions, 57 ml of a saline solution containing 92 g/liter NaCl was added to 246 g of unsalted curd and mixed three times for 10 s at maximum speed using a Warring blender. A 2.4-ml amount of the ripening culture was then added and mixed, yielding 10^4 CFU of yeast/g of cheese and 10^6 CFU of bacteria/g of cheese. Thirty-gram amounts were then transferred to sterile crystallizing basins with a diameter of 5.6 cm and incubated at 12°C for 21 days. Two or three cheeses were used at each time point analyzed. The salt content of the cheeses was ~17 g/kg.

Analyses. The surface pH was measured by using a surface electrode Blue line 27 (Schott). The pH values were the arithmetic means of three measurements. Surface color was measured using a CM-2002 spectrocolorimeter (Minolta, Carrières sur Seine, France) as described by Mounier et al. (29). The data were processed using the three-dimensional L^* a^* b^* response and logged into the L^* and C^* system. L^* ranges from 0 (black) to 100 (white) and indicates lightness and a^* and b^* are the chromaticity coordinates indicating the color directions; $+a^*$ is the red direction at 0°, $-a^*$ is the green direction at 180°, $+b^*$ is the yellow direction at 90°, and $-b^*$ is the blue direction at 270°. The cheese surfaces were photographed using a digital camera. The lactose and lactate contents were determined for the whole cheese by using high-performance liquid chromatography as previously described by Leclercq-Perlat et al. (19). The release of free amino acids was measured for the whole cheese as described by Grunau and Swiader (14). The ammonia content of the whole cheese was measured using Nessler reagent.

Microbiological analyses. The cheese was homogenized by using a mortar and pestle, and ~1 g of each of the cheeses was sampled and transferred into a sterile container. A sterile saline solution (8.5 g/liter NaCl) was added to yield a 1:10 dilution, and the mixture was homogenized with an Ultra Turrax (Labortechnik) at 8,000 rpm/min for 1 min. Total bacteria except for lactic acid bacteria were enumerated by surface plating in duplicate on BHI agar supplemented with 50 mg/liter amphotericin B after 5 days of incubation at 25°C. The yeast population was determined by surface plating in duplicate using yeast-glucose-chloramphenicol agar supplemented with 0.01 g/liter tetrazolium chloride (TTC) after three days of incubation at 25°C. Lactic acid bacteria were enumerated by surface plating in duplicate on MRS agar after two days of incubation at 30°C.

Enumeration of yeast and bacterial species. Each yeast species had a distinct morphotype on yeast-glucose-chloramphenicol agar supplemented with TTC, which allowed their direct enumeration. For the bacteria, 250 colonies of each cheese sample were removed at random with sterile toothpicks, transferred onto 96-well microtiter plates containing 100 μl of BHI broth supplemented with 10% (vol/vol) glycerol, and incubated for 3 days at 25°C. The plates were stored at

TABLE 1. Selective agents used to identify the different bacterial clones[a]

Organism	Novobiocin		Erythromycin (20 mg/liter)	Vancomycin (1 mg/liter)	TTC (0.1 g/liter)
	1 mg/liter	5 mg/liter			
Arthrobacter arilaitensis	−	−	−	−	+
Brevibacterium aurantiacum	+	−	−	v	v
Corynebacterium casei	+	+	−	−	v
Hafnia alvei	+	+	+	+	+
Leucobacter sp.	−	−	−	+	+
Staphylococcus xylosus	+	+	−	+	+

[a] −, absence of growth; +, growth; v, variable growth.

−80°C until use. For bacterial identification, the isolates that grew in microtiter plates were replicated onto five media, i.e., BHI agar containing 20 mg/liter erythromycin, 1 or 5 mg/liter novobiocin, 1 mg/liter vancomycin, or 1 g/liter TTC. After incubation for 3 days at 25°C, the isolates were checked for their ability to grow in the presence of the various selective agents. The combination of the five media was discriminative for each bacterium (Table 1). The counts of each bacterium (C_i) were estimated as follows:

$$C_i(\text{CFU/g}) = \frac{N_i \times C_0}{N_t}$$

where C_0 is the total bacterial count in CFU/g, N_t is the number of clones replicated, and N_i is the number of clones identified as bacterium i.

Statistical analysis. The data with repeated measurements (bacterial and yeast population, pH, color, and lactate content) were compared and statistically assessed using analysis of variance. When differences were detected by analysis of variance, a Student-Newman-Keuls test was used to determine which means were different. Statistical significance was set at a P value of <0.05.

Lotka-Volterra modeling. The multispecies Lotka-Volterra model was used in this study. Taking n species, the dynamic of the species i ($i = 1, \ldots, n$) is the following:

$$\frac{dX_i}{dt} = X_i\left(\beta_i + \sum_{j=1}^{n} \alpha_{ij}X_j\right)$$

where β_i represents the intrinsic growth rate of the species i and α_{ij} the influence of the species j on the growth rate of species i. This influence is positive or negative according to the sign of α_{ij}. In this model, the interactions are assumed constant for the abundance of a given species j. To determine the interaction coefficients, the multispecies Lotka-Volterra system can be expressed as a multilinear regression:

$$\frac{d\log(X_i)}{dt} = \beta_i + \sum_{j=1}^{n} \alpha_{ij}X_j$$

The left part of this equation was obtained by deriving the logarithm of the species concentration according to time by using the cubic spline function without smoothing (Matlab).

In a linear regression model, the correlations between explicative variables have a high impact on parameter identification. The design of experiments makes it possible to avoid the correlations, but this approach is not possible in the present study. Consequently, to avoid too many correlations, the model was not used on each species but on clusters that grouped the different organisms obtained from a squared correlation coefficient with a 0.75 threshold value. For a given cluster, the sum of the abundances of the different species was used in the linear model. Inside this simplified system, the interaction coefficient α_{ij} was considered to be significant when the value of $P(\alpha_{ij} \neq 0)$ was >90%.

RESULTS

Growth properties of the ecosystem microorganisms. The growth characteristics of the bacteria as a function of NaCl content and pH on an agar-based medium are compared in Fig. S1 in the supplemental material. The bacteria could be divided into three groups based on their growth abilities. The first group was comprised of *H. alvei* and *S. xylosus*, which grew under all the conditions tested except pH 5 and 0% NaCl, at which *S. xylosus* did not grow. The second group was comprised of *A. arilaitensis*, which grew at a pH equal to or greater than 5.5 except in the presence of 0 and 30 g liter^{-1} NaCl, where it grew at pHs equal to or greater than 6.5 and 6, respectively. The third group was comprised of *Leucobacter* sp., *B. aurantiacum*, and *C. casei*, which only grew at a pH equal to or greater than 6, except for *B. aurantiacum*, which in the presence of 100 and 150 g liter^{-1} NaCl grew at pH 5.5. In some cases, *C. casei* only grew at a pH equal to or greater than 6.5. The bacteria generally grew better in the presence of increased concentrations of NaCl. Yeast grew under all the conditions tested (data not shown).

Microbial and physicochemical dynamics during the development of the ecosystem on model cheese. (i) Reproducibility of microbial dynamics. The growth rates of the three yeasts and six bacteria during cheese ripening are shown in Fig. 1A and B, respectively. There was a good reproducibility (a difference of less than 0.5 $\log_{10}$ units) between the results of duplicate experiments in the numbers of the yeasts and of the three dominant bacterial species, i.e., *A. arilaitensis*, *Leucobacter* sp., and *H. alvei* (data not shown). The three other bacterial species were only detected occasionally on one or two of the cheeses analyzed because these bacteria had numbers below the detection limit of our method of analysis (approximately 2 $\log_{10}$ units below the total count). *S. xylosus* was not isolated on days 12, 16, 17, 18, and 20; *B. aurantiacum* on days 10, 12, 14, and 20; and *C. casei* on day 20.

(ii) Yeast growth. *D. hansenii* and *Y. lipolytica* grew during the first days of ripening and had very similar growth rates (Fig. 1A); in contrast, *G. candidum* grew only after 2 days. A possible explanation for the absence of an increase in cell numbers of *G. candidum* may be that *G. candidum* had a longer lag phase or formed mycelium at the start of ripening. Indeed, mycelium with hyphae consists of different cells but would give only 1 CFU per agar plate. The growth of *G. candidum* coincided with a slowing of the growth of *D. hansenii* and *Y. lipolytica*. Overall, *D. hansenii* dominated the cheese surface until day 5; then, between days 6 and 9, the three yeasts had similar cell numbers, after which *D. hansenii* became progressively subdominant compared with the numbers of *Y. lipolytica* and *G. candidum*. Indeed, the *G. candidum* and *Y. lipolytica* numbers remained constant or increased slightly, while the *D. hansenii* population decreased by 1.5 $\log_{10}$ units between day 6 and day 21.

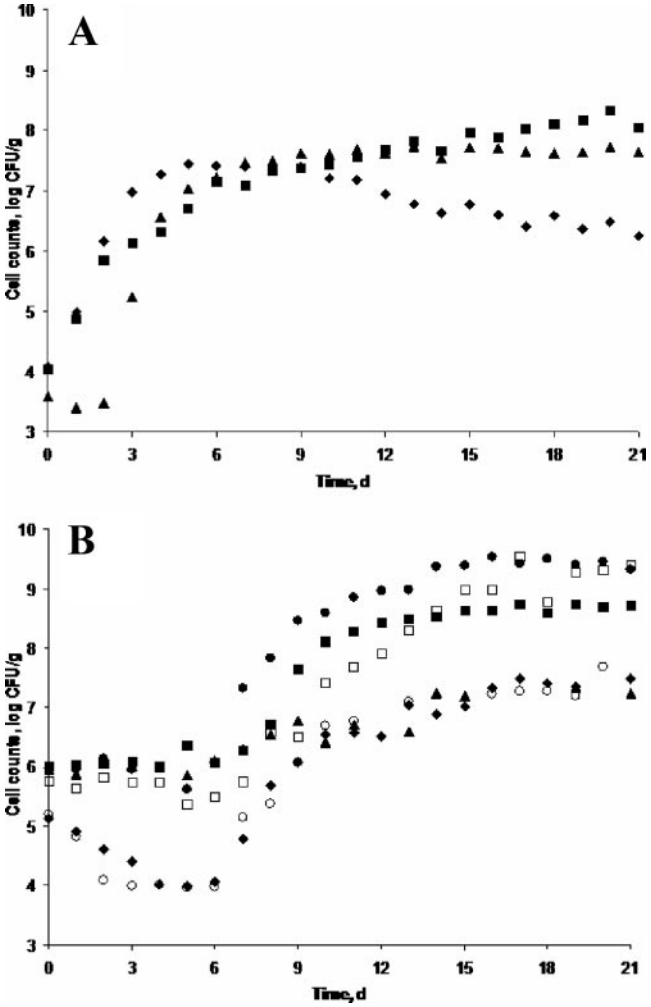

FIG. 1. Yeast (A) and bacterial (B) dynamics of the cheese ecosystem on model cheeses. (A) ◆, *Debaryomyces hansenii*; ■, *Yarrowia lipolytica*; ▲, *Geotrichum candidum*. (B) ●, *Arthrobacter arilaitensis*; ■, *Hafnia alvei*; □, *Leucobacter* sp.; ◆, *Corynebacterium casei*; ○, *Brevibacterium aurantiacum*; ▲, *Staphylococcus xylosus*.

(iii) Bacterial growth. During the first days of ripening, the counts of *H. alvei*, *A. arilaitensis*, *Leucobacter* sp., and *S. xylosus* remained constant, while the populations of *C. casei* and *B. aurantiacum* decreased by approximately 1 log unit between days 0 and 4 (Fig. 1B). Growth of all the organisms occurred after days 5 to 6. *A. arilaitensis*, followed by *H. alvei*, dominated the cheese surface between day 6 and day 9. After day 9, *Leucobacter* sp. counts increased, and this species also became dominant on the cheese surface. *S. xylosus*, *C. casei*, and *B. aurantiacum* remained subdominant throughout the entire ripening period. Lactic acid bacterium counts decreased slightly, from $\sim 10^8$ CFU/g on day 0 to 2×10^7 CFU/g at the end of ripening (data not shown).

(iv) Lactose, lactate, and pH dynamics during ripening. Lactose, lactate, and pH variations during ripening are shown in Fig. 2. Lactose was used first and was totally depleted on day 8. After an increase during the first days of ripening, probably due to a slight acidification by the lactic acid bacteria, lactate was consumed from day 5 to day 9, but was not depleted. Sixty

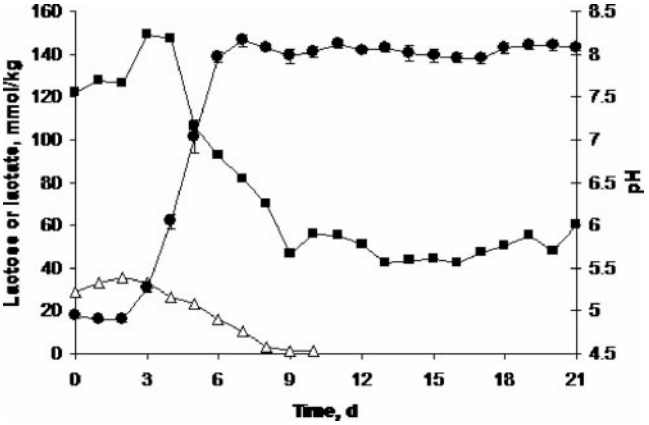

FIG. 2. Lactose (△), lactate (■), and pH (●) variations during the growth of the cheese ecosystem on model cheese.

percent of the lactate was used during growth, which indicates that lactate was not a limiting carbon source. The surface deacidification occurred between day 2 and day 6, with a pH increase from approximately 5.0 to 8.0. This deacidification was highly correlated with the utilization of lactate and the growth of *G. candidum* on the cheese surface (data not shown).

(v) GLV modeling. A dendrogram of the different species according to their squared correlation coefficients during growth is shown in Fig. S2 in the supplemental material. With a threshold value of 0.75, each yeast was considered to have a specific growth dynamic. In contrast, except for *Leucobacter* sp., the growth dynamics of the bacteria were considered to be correlated. Consequently, GLV modeling was performed on the growth dynamics of five distinct groups that comprised four individual species, i.e., *Y. lipolytica*, *G. candidum*, *D. hansenii*, and *Leucobacter* sp., and a group of bacteria including *A. arilaitensis*, *B. aurantiacum*, *C. casei*, *H. alvei*, and *S. xylosus*.

The main interactions according to GLV modeling are shown in Fig. 3. Yeast-yeast interactions were found to be only

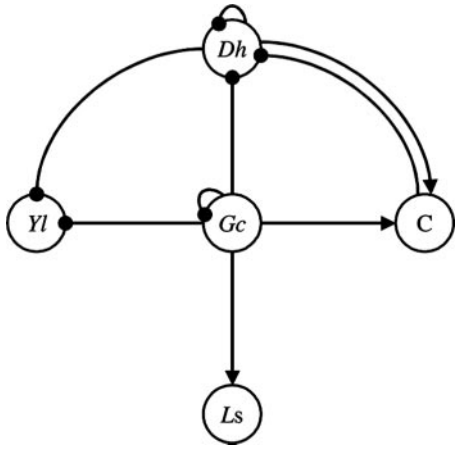

FIG. 3. Main interactions (→, positive; —●, negative) according to GLV modeling between the members of the multispecies ecosystem. *Dh*, *Debaryomyces hansenii*; *Yl*, *Yarrowia lipolytica*; *Gc*, *Geotrichum candidum*; Ls, *Leucobacter* sp.; C, group including *Arthrobacter arilaitensis*, *Hafnia alvei*, *Corynebacterium casei*, *Brevibacterium aurantiacum*, and *Staphylococcus xylosus*.

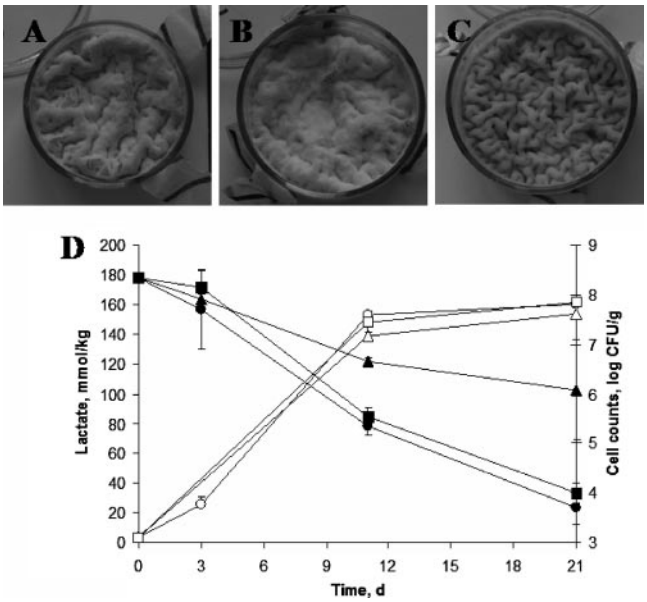

FIG. 4. Macromorphology of *Geotrichum candidum* grown as a monoculture (A) or in the presence of *Debaryomyces hansenii* (B) or *Yarrowia lipolytica* (C). (D) Lactate utilization (closed symbols) and *G. candidum* counts (open symbols) in model cheeses containing *G. candidum* (●, ○), *G. candidum* and *D. hansenii* (■, □) or *G. candidum* and *Y. lipolytica* (▲, △). Error bars show standard deviations.

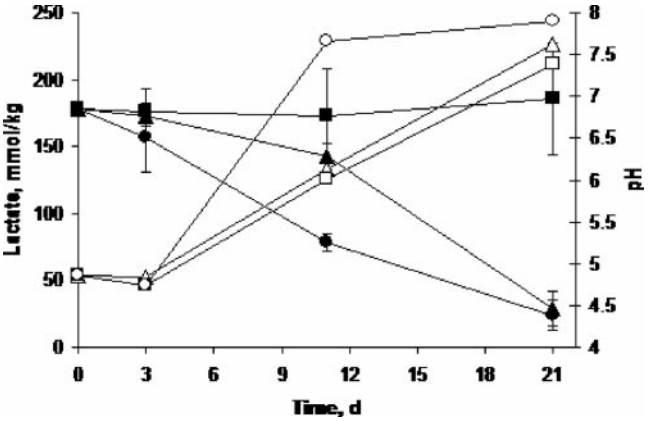

FIG. 5. Lactate consumption (filled symbols) and pH increase (open symbols) during ripening in cheeses inoculated with *Debaryomyces hansenii* (▲, △), *Geotrichum candidum* (●, ○), or *Yarrowia lipolytica* (■, □). Error bars show standard deviations.

negative, while yeast-bacterium interactions were found to be only positive. *G. candidum* interacted negatively with *D. hansenii* and *Y. lipolytica*, while it interacted positively with *Leucobacter* sp. and the group of bacteria. *D. hansenii* was found to have a negative interaction with *Y. lipolytica*, while it had a positive interaction with the group of bacteria. Self-inhibition of *G. candidum* and *D. hansenii* was also found in the model.

The model succeeded in representing the growth of the different microbial populations, as shown in Fig. S3 and S4 in the supplemental material which compare the measured and estimated values for the two data sets. The total residual error between the estimated and measured values was 0.1 log CFU/g ± 0.4 log CFU/g (mean ± standard deviation) for both data sets.

Effects of single and multiple omissions of yeast in the ecosystem. We aimed at identifying yeast-yeast or yeast-bacterium interactions by comparing the growth of each individual microorganism in the absence or presence of one, two, or three yeasts. The utilization of lactose and lactate, the deacidification rate, and the color development of the cheese surface were also compared for each inoculum tested.

Reproducibility. There was good reproducibility between the results of triplicate experiments in terms of lactose and lactate utilization and deacidification, as well as for the growth of the microorganisms of the ecosystem (data not shown). There was also good reproducibility between the data from the dynamic study and the omission study in which all the members of the community were inoculated (data not shown).

Yeast-yeast interactions. The viability of *D. hansenii* during the stationary phase was affected in the presence of the other yeasts (see Fig. S5 in the supplemental material). Populations of *D. hansenii* were significantly lower ($P < 0.05$) on day 11 when *D. hansenii* was grown in the presence of *G. candidum* or *G. candidum* and *Y. lipolytica*. Indeed, populations of *D. hansenii* were 0.5 and 0.7 $\log_{10}$ units lower in the presence of *G. candidum* or *G. candidum* and *Y. lipolytica*, respectively, than in the *D. hansenii* monoculture. Moreover, between day 11 and day 21, *D. hansenii* populations decreased by 1 to 1.7 $\log_{10}$ units when this organism was cocultivated with *G. candidum* and/or *Y. lipolytica*, whereas it remained constant in the monoculture. This inhibitory effect was similar regardless of whether *Y. lipolytica* or *G. candidum* was present but was more pronounced in the presence of both species. Populations of *Y. lipolytica* and, to a lesser extent, populations of *G. candidum*, were significantly lower ($P < 0.05$) on day 11 when they were grown in the presence of other yeasts (data not shown). Their respective counts were 0.4 and 0.7 $\log_{10}$ units lower than those observed in monoculture. However, there was not any loss in viability of *Y. lipolytica* and *G. candidum* during the stationary phase.

Interestingly, *Y. lipolytica*, but not *D. hansenii*, greatly influenced the mycelium formation of *G. candidum*. In the monoculture or in the sole presence of *D. hansenii*, *G. candidum* grew in the form of white mycelium which covered the surface of the model cheeses (Fig. 4A and B), whereas in the presence of *Y. lipolytica*, growth occurred as spaghetti-like structures without the formation of pseudohyphae (Fig. 4C). This inhibition of mycelial development did not influence cellular growth since only small differences in numbers of *G. candidum* were found (Fig. 4D). This phenomenon was also observed in the presence of both *Y. lipolytica* and *D. hansenii*. The idea that an interaction of *Y. lipolytica* with *G. candidum* occurred was also reinforced because the rate of utilization of lactate in the cheese containing *G. candidum* and *Y. lipolytica* was decreased in comparison with that in the monoculture or in coculture with *D. hansenii* (Fig. 4D). Ninety percent of the lactate was used after 21 days when *G. candidum* grew as the sole yeast or in the presence of *D. hansenii*, while only 44% was used when this organism was cocultivated with *Y. lipolytica*.

Chemical characteristics of the cheeses. *G. candidum* showed the highest deacidification rate, followed by *D. hansenii* and *Y. lipolytica*, which had similar deacidification rates (Fig. 5). The pH reached its maximal value, i.e., 8.0, after 11

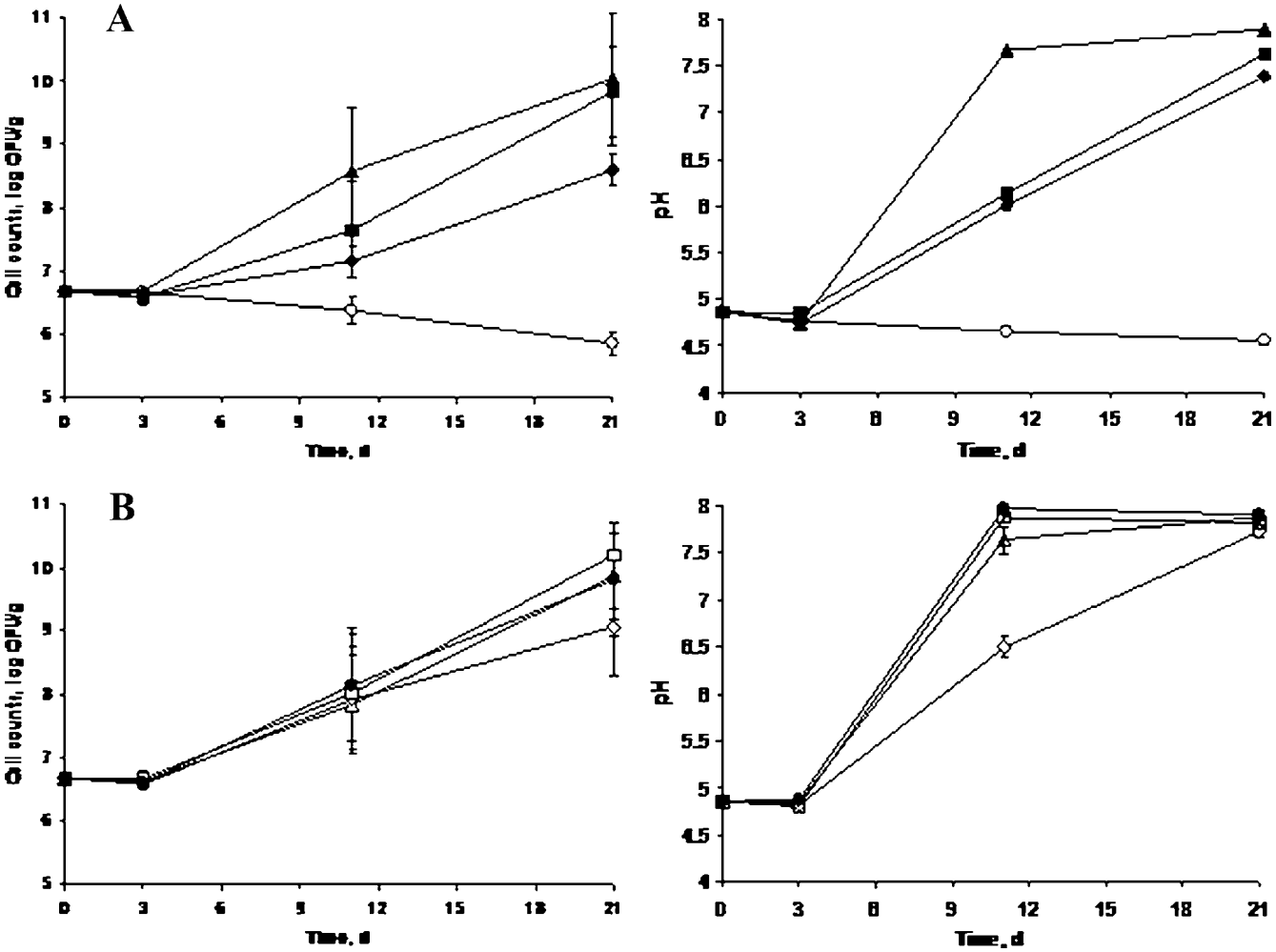

FIG. 6. Total bacterial growth and surface pH increase during ripening in cheeses inoculated with (A) no yeast (○), *Debaryomyces hansenii* (■), *Yarrowia lipolytica* (◆), or *Geotrichum candidum* (▲) or (B) *D. hansenii* and *Y. lipolytica* (◇), *D. hansenii* and *G. candidum* (△), *Y. lipolytica* and *G. candidum* (□), or *D. hansenii*, *Y. lipolytica*, and *G. candidum* (●). Error bars show standard deviations.

days when *G. candidum* was present in the ecosystem, whereas the pH values ranged from 6 to 6.5 for *D. hansenii* and *Y. lipolytica* (Fig. 5) or a combination of both species (data not shown). After 21 days, the pH values ranged from 7.4 to 8.0. The higher pH of cheese containing *G. candidum* may be attributable to the fact that *G. candidum* utilized more lactate than *D. hansenii* between days 0 and 11. *D. hansenii* produced a small amount of NH_3 (data not shown). *Y. lipolytica* did not utilize lactate but produced large amounts of NH_3 (data not shown). Amino acids and compounds such as ornithine and γ-amino-*n*-butyric acid (GABA) differed between cheeses (data not shown). After 21 days, the cheese inoculated with *Y. lipolytica* had 2 to 15 times more free amino acids, depending on the amino acid considered, than the cheeses inoculated with *D. hansenii* or *G. candidum* and the cheese with no yeast. Except for asparagine, cysteine, ornithine, and GABA, all amino acids were produced in large quantities in the cheese inoculated with *Y. lipolytica* compared with the quantities in cheeses inoculated with the two other yeasts (data not shown).

Development of the bacterial community. The growth of the bacteria in the cheese model was considerably influenced by the yeasts that were either present or not in the initial inocula. Growth of the bacteria did not occur when yeasts were not inoculated (Fig. 6A). After 11 and 21 days, the cheeses that contained *G. candidum* showed significantly higher surface pH values than the cheeses inoculated with *D. hansenii* and/or *Y. lipolytica*. The differences in surface pH between cheeses inoculated with *D. hansenii* and/or *Y. lipolytica* were much lower when *D. hansenii* and *Y. lipolytica* were combined than when they were the sole yeasts inoculated (Fig. 6A and B). After 11 days, the bacterial count of the cheese inoculated with *G. candidum* by itself was significantly higher ($P < 0.05$) than that of the cheese inoculated with *D. hansenii* or *Y. lipolytica* alone (Fig. 6A). In contrast, with two or three yeasts in the community, total bacterial counts were statistically similar ($P < 0.05$) despite the fact that the surface pH was significantly lower ($P < 0.05$) on the cheese containing *D. hansenii* and *Y. lipolytica* (Fig. 6B). After 21 days, total bacterial counts were not statistically different in all cheeses except for the cheese that contained *Y. lipolytica* as the sole yeast and the cheese that contained *D. hansenii* and *Y. lipolytica*, which had counts that were 1.5 and 1 $\log_{10}$ units lower, respectively (Fig. 6A and B).

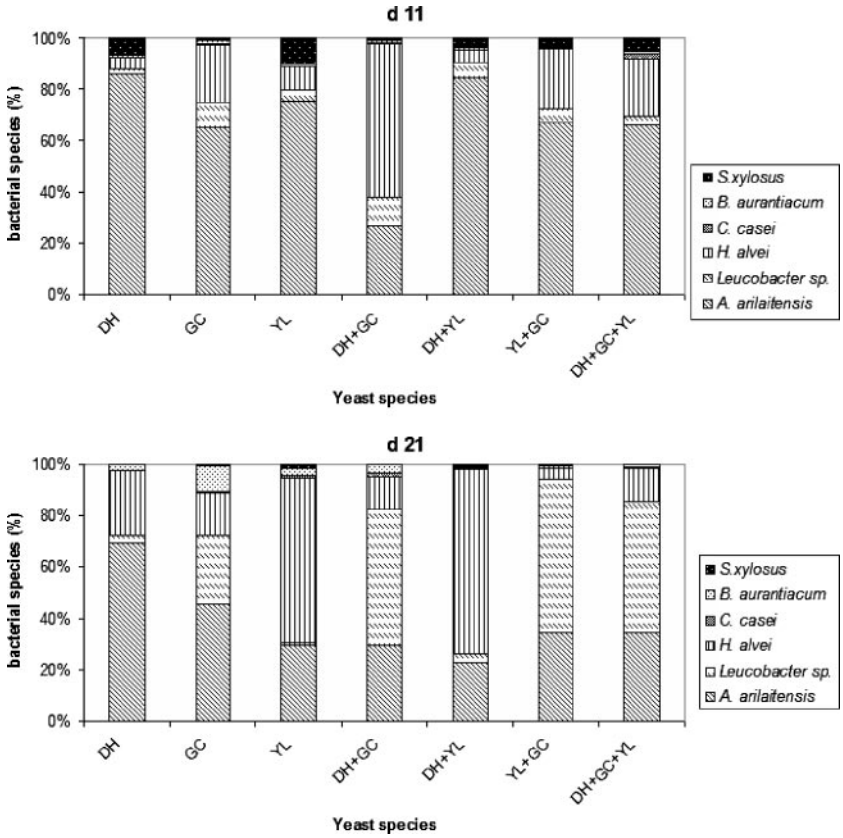

FIG. 7. Distribution of the bacterial species in the model cheese after 11 and 21 days as a function of the yeast inoculated. DH, *Debaryomyces hansenii*; YL, *Yarrowia lipolytica*; GC, *Geotrichum candidum*.

As shown in Fig. 7, there were only small differences in distribution of the bacterial species on the different cheeses after 11 days, except for the cheese inoculated with *D. hansenii* and *G. candidum*. Except in the cheese inoculated with *G. candidum* and *D. hansenii*, the cheeses were dominated by *A. arilaitensis*, which represented between 66 and 86% of the total isolates, followed by *H. alvei* (5 to 25%), *Leucobacter* sp. (2 to 10%), *S. xylosus* (3 to 10%), and *C. casei* and *B. aurantiacum* (0.4 to 2%). *H. alvei* (70%), followed by *A. arilaitensis* (26%) and *Leucobacter* sp. (11%), dominated the cheese inoculated with *D. hansenii* and *G. candidum*. After 21 days, differences and common patterns were found in the distribution of the bacterial community. *Leucobacter* sp. grew in all the cheeses inoculated with *G. candidum* and represented between 26 and 60% of the total isolates, whereas this species was subdominant (less than 5% of the total isolates) in all cheeses in which *G. candidum* was absent. *A. arilaitensis* dominated in the cheese inoculated with *D. hansenii* or *G. candidum* as the sole yeast (70% of the isolates), while *H. alvei* dominated in cheeses inoculated with *Y. lipolytica* or *Y. lipolytica* and *D. hansenii* (70% of the isolates). After 21 days, *S. xylosus*, *B. aurantiacum* and *C. casei* remained subdominant except in the cheese inoculated with *G. candidum* as the sole yeast, in which *B. aurantiacum* represented 10% of the isolates taken in this cheese.

Color development of the cheese surface. There were only small differences in color development of all the cheeses after 11 days except for the cheese inoculated with no yeast and the cheeses inoculated with *G. candidum* or *G. candidum* and *D. hansenii*, which had a lower b^* (yellow dimension), probably because *G. candidum* formed white mycelia on the surface (data not shown). In contrast, all the cheeses differed considerably in terms of color development after 21 days (Fig. 8). The consortium that contained the three yeasts showed the highest

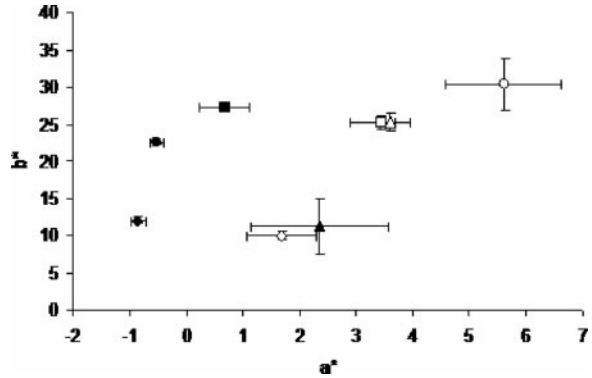

FIG. 8. Color of the cheese surface after 21 days as a function of the chromaticity coordinate a^* (red dimension) and b^* (yellow dimension) values. Cheeses were inoculated with no yeast (◆); *Debaryomyces hansenii* (■); *Geotrichum candidum* (▲); *Yarrowia lipolytica* (●); *D. hansenii* and *Y. lipolytica* (△); *D. hansenil* and *G. candidum*, (◇); *Y. lipolytica* and *G. candidum* (□); and *D. hansenii*, *Y. lipolytica*, and *G. candidum* (○). Error bars show standard deviations.

a^* and b^* values, followed by the two other cheeses inoculated with *Y. lipolytica* and *D. hansenii* or *Y. lipolytica* and *G. candidum*. The cheeses inoculated with *G. candidum* by itself and *G. candidum* and *D. hansenii* had high a^* but low b^* values, while the cheeses inoculated with only *D. hansenii* or *Y. lipolytica* had high b^* but low a^* values.

DISCUSSION

In this study, the dynamics of a nine-species cheese ecosystem and the effects of the omission of one, two, or three yeasts on the growth of this community were investigated in model cheeses. To our knowledge, all the studies about the growth behavior of microorganisms isolated from cheese have been done on mixed cultures with only two microorganisms, generally a yeast and a bacterium, on cheese agar (24, 25) or on curd made under aseptic conditions (4, 20, 21, 29). Despite the fact that such studies provide interesting information on the individual growth characteristics of these organisms and their contribution to ripening, they do not take account of the fact that the cheese microflora is much more diverse and that interactions may exist between the members of these communities. These interactions may strongly influence their implantation and colonizing capacity in cheese, as shown in this study.

Yeast-yeast interactions. *G. candidum* was isolated from nearly all smear-ripened cheeses. This organism imparts a uniform, white velvety coat on the surface of some cheeses, such as St. Marcellin, while on others, such as Livarot, it is not the case (6). In this study, it was found that when *Y. lipolytica* was grown in association with *G. candidum*, hyphal formation was inhibited and *G. candidum* grew as spaghetti-like structures instead (Fig. 4). Numerous chemical and environmental parameters, such as temperature, glucose levels, pH, nitrogen sources, and inoculum size (30), have been reported to influence yeast mycelium formation. Among these, ammonia and proline, which were produced in greater quantities by *Y. lipolytica* than *D. hansenii*, may provide an explanation for this observation. Palkova and Forstova (32) showed that, between different yeast taxa, ammonia induction triggered changes in colony morphology in which pseudohyphae decomposed into nondividing yeast cells. Kulkarni and Nickerson (17) showed that proline (10 mM) induced the yeast morphology in *Ceratocystis ulmi* in defined liquid medium and that budding yeasts were only formed above 10^6 blastidiospores per ml. However, in our study, other factors may be involved, and further investigations are being pursued to understand this interaction. *G. candidum* was also less metabolically active or its metabolism was differently oriented in the presence of *Y. lipolytica* because *G. candidum* was less effective in utilizing lactate in spaghetti-like structures than in mold-like structures. Indeed, mycelium-like structures may provide a better access to substrates in the cheese matrix.

The presence of other yeasts in the cheese had only a small effect on the growth of each individual yeast. This may be explained by the fact that each yeast utilized different energy sources for growth. Barnett et al. (1) showed that *D. hansenii* assimilates lactose and lactate while *G. candidum* and *Y. lipolytica* only assimilate lactate. In this study, *Y. lipolytica* did not utilize lactate. The energy source of *Y. lipolytica* remained unclear, but nitrogen compounds are likely to be its main energy source. *D. hansenii* populations were found to significantly decrease in the presence of other yeasts. This indicates that competition for nutrients or negative interactions (inhibition) occurred between yeasts, which affected the cell maintenance of *D. hansenii* during its stationary phase.

Yeast-bacterium interactions. In this study, it was demonstrated that the bacterial development and distribution of the different species were modified depending on the yeast present in the ecosystem. It is obvious, because of the different levels of acid sensitivity of the bacteria, that the deacidification rate of the yeasts influenced the bacterial development on the cheese surface. Indeed, in most cases, the bacteria reached higher population levels when the deacidification was more rapid. The growth characteristics of each bacterial strain as a function of pH determined in agar-based media gave us an insight into the growth ability of each bacterium. For example, *Leucobacter* sp. was much more acid-sensitive than *H. alvei* and developed later in the ripening process. *C. casei* and *B. aurantiacum* were also quite acid sensitive and did not hold up well in comparison to the other members of the bacterial community under the acidic stress that occurred at the start of ripening. This may be responsible for their subdominance in almost all the cheeses.

Surface pH was not the only factor that influenced bacterial development. For example, *S. xylosus*, which is able to grow at relatively low pH on agar, did not well colonize the cheese surface in comparison to the colonization of *A. arilaitensis*, *H. alvei*, or *Leucobacter* sp. This also indicates that growth abilities obtained in pure culture on agar-based media cannot be extrapolated to more-complex media and multispecies ecosystems. *S. xylosus* may have a limited colonization capacity in cheese because the nutrients available may not have been sufficient to support growth, or competition may have occurred between this strain and the different yeasts and bacteria. In biodiversity studies, it has been reported that *Staphylococcus* spp. were often predominant in the early stages of ripening but were rapidly outnumbered by other bacteria at the later stages of ripening (15, 28, 34). However, in coculture studies, *Staphylococcus saprophyticus* was able to reach high numbers, i.e., 10^{10} CFU/g with *D. hansenii*, in model cheese curd (29), while it did not reach such numbers in cheese (28). Therefore, *Staphylococcus* strains may have a limited colonization capacity in this type of cheese, especially when the microflora is much more complex.

Leucobacter sp. grew only in the cheeses that contained *G. candidum*. The growth of *Leucobacter* sp. only in cheeses containing *G. candidum* would imply that *Leucobacter* sp. was highly dependent on *G. candidum* activities, either because *G. candidum* rapidly deacidified the surface or because it produced metabolites that enhanced *Leucobacter* sp. growth. Similarly, *B. aurantiacum* represented ~10% of the clones isolated after 21 days in the cheese inoculated with *G. candidum* as the sole yeast, whereas *B. aurantiacum* was subdominant in the other microbial communities. It is possible that *G. candidum* detoxified the environment and released substrates that promoted the growth of *B. aurantiacum* under these conditions.

A. arilaitensis and, in most cases, *H. alvei* were found to represent a large part of the bacteria under all the conditions tested. Therefore, these species may not be highly dependent on a specific yeast interaction, with the exception of surface deacidification. *A. arilaitensis* has been found to dominate the

microflora of many European cheeses (12, 13, 16). This shows the high colonization capacity of this species compared with the colonization capacities of others, such as *B. linens* or *B. aurantiacum*.

Color development of the cheese surface. The color differentiation that occurred between day 11 and day 21 is probably due to the production of pigments by the bacteria. Interestingly, in some cases, if we compare two cheeses with very similar bacterial distributions and populations, such as the ecosystems that contained only *Y. lipolytica* or *Y. lipolytica* and *D. hansenii*, the colors differed considerably between the two ecosystems. This would imply that, depending on the yeasts present, species-specific bacterial pigmentations were different in these two cheeses. This is in agreement with the results of a previous study of Leclercq-Perlat et al. (18) in which it was shown that *B. linens* pigmentation differed depending on the yeast used for deacidification. The ecosystem that contained the three yeasts yielded the strongest color development. This suggests that each yeast would have different ecosystem functions in terms of color development and that the combination of the three yeasts led to the highest pigment production by the bacteria.

Lotka-Volterra modeling. In this study, Lotka-Volterra modeling was used for the first time on a microbial ecosystem as a preliminary approach to represent inter- and intraspecies interactions. This approach made it possible to identify the positive interactions between the bacteria and the yeast during ripening, i.e., the positive effects of *G. candidum* on *Leucobacter* sp. and on the rest of the bacteria that were confirmed in this study. Similarly, negative interaction between yeasts, such as the inhibition of *D. hansenii* by *G. candidum*, was also found in the Lotka-Volterra model. However, this model showed interactions, such as a negative interaction of the bacteria on *D. hansenii*, which could not be explained by the results of the omission study. Inversely, other interactions, such as a negative interaction of *Y. lipolytica* with *D. hansenii*, were not significant in the model but observed in situ. Further data would be necessary to confirm or invalidate the interactions observed in the model. Because the growth of most of the bacteria was highly correlated, we could not measure interactions between each individual bacterial species and the yeasts. Despite the limits of this approach, the use of the GLV model on only one set of data provided us with an insight into the main interactions. Therefore, GLV modeling may be useful as a preliminary step to orient interaction studies.

The smear-cheese microbial community is a beneficial biofilm because it is responsible for the flavor and appearance of this type of cheese. For a better understanding of the interactions that occur, it would be interesting to investigate the spatial distribution of these microorganisms on the cheese surface, using fluorescence in situ hybridization, for example.

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