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Abstract

The interactions that occur during the ripening of smear cheeses are not well understood. Yeast-yeast interactions and yeast-bacteria 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 of yeast omissions in the microbial community on ecosystem functioning 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*, and *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-bacteria interactions played a significant role in the establishment of this multi-species ecosystem on the cheese surface. Yeasts were key species in bacterial development, but their influence 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*, their growth relied on a specific yeast, *i.e.*, *G. candidum*. Furthermore, *B. aurantiacum*, *Corynebacterium casei* and *Staphylococcus xylosus* showed a reduced colonization capacity compared with the other bacteria in this model cheese. Bacteria/bacteria interactions could not be clearly identified.

Introduction

Little is known about yeast-bacteria 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, 25, 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 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 of mostly cultivable species, *i.e.*, 10-20 species (12, 27). The microbial diversity of cheese was investigated using both cultivable and non-cultivable approaches such as rep-PCR, FT-IR spectroscopy, 16S rDNA sequencing, cloning and sequencing of 16S rDNA, SSCP, DGGE and TGGE (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 is 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 anti-listerial activity (11, 25). Eppert et al. (11) found single strains of linocin-producing *B. linens* (a bacteriocin-like substance), which reduced *Listeria* spp. populations in cheeses but did not exert an inhibition comparable to that obtained with the ripening consortia from which these strains were isolated. Inversely, none of the 400 isolates from an effective anti-listerial ripening consortium evaluated in the study of Maoz et al. (25) exhibited anti-listerial activity in agar diffusion assays. This implies that the anti-listerial 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 (3) for a review). The multispecies Lotka-Volterra model (22, 36) is a simple model used to measure interactions based on a linear relationship for a given species between growth rate and the populations of each member of the community. Such a model may be a good tool to investigate interactions within a microbial community.

Bonaiti et al. (5), using a three-step dichotomous approach, simplified an ecosystem of 83 strains from Livarot cheese, to four sub-ecosystems composed of nine species based on odor profile. One of these sub-ecosystems showed great similarities with the odor profile of

the 83-strain ecosystem, which had a very similar odor profile to the commercial cheese. This sub-ecosystem 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 model (GLV) 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, color of the cheese surface).

Material and methods

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

The nine microrganisms that composed the model ecosystem were *Arthrobacter arilaitensis* 3M03, *Brevibacterium aurantiacum* 2M23, *Corynebacterium casei* 2M01, *Hafnia alvei* 2E12, *Leucobacter* sp. 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 media. The growth characteristics of the bacteria and yeast as a function of pH and NaCl were tested in a media that contained 0.5 g yeast extract, 1 g casaminoacids, 0.1 g glucose and 1.5 g agar. Salt content was 0, 30, 50, 100 and 150 g l^{-1}, while pH was 5, 5.5, 6, 6.5 and 7. Growth was visually evaluated by checking for the presence of colonies after 2, 4 and 8 days incubation at 12°C.

Growth properties of the microorganisms found in the cheese ecosystem. In this study, two independent experiments were conducted at a five-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 (Exp. I). The cheeses were sampled in duplicate every day for 21 days for microbial enumeration, 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 (Exp. II). All the possible combinations were tested. Cheeses were sampled in triplicate on day 0, 3, 11 and 21 for microbial enumeration, 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 L) was pasteurized full-fat milk, standardized at 29 g/l 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 l 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/l of chymosin at 30 ml/100 l of milk. Coagulation time was 20 min, and 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 standing for 15 min, 70 l of whey were removed prior to molding. Cheeses were shaped in circular polyurethane molds with a diameter of 9 cm and a height of 11 cm. 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, 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 (PDB) or Brain Heart Infusion broth (BHI), respectively, in 50-ml flasks incubated at 25°C for 55 h at 150 rpm. 400 µl of each preculture were then used to inoculate 40 ml of PDB

147 or BHI in 150 ml flasks, which were incubated at 25°C for 66 h at 150 rpm. Five to 10 ml of
148 each preculture were centrifuged at room temperature for 10 min at 4000 rpm. The
149 supernatant was discarded and the cells resuspended in 9 g/l NaCl to obtain a concentration of
150 2 x 10^9 CFU/ml and 2 x 10^7 CFU/ml for the bacteria and the yeast, respectively.
151 Subsequently, 1280 µl of each suspension were mixed and supplemented to make 20 ml with
152 9 g/l NaCl in a volumetric flask. This suspension was used to inoculate the model cheeses.

153
154 *Curd inoculation.* Under sterile conditions, 57 ml of a saline solution containing 92 g/l NaCl
155 were added to 246 g of unsalted curd and mixed three times for 10 s at maximum speed using
156 a Warring blender. 2.4 ml of the ripening culture were then added and mixed, yielding 10^4
157 CFU/g and 10^6 CFU/g of cheese, for the yeast and the bacteria, respectively. Thirty grams
158 were then transferred to sterile crystallizing basins with a diameter of 5.6 cm, and incubated at
159 12°C for 21 days. Two or three cheeses were used at each time point analyzed. Salt content of
160 the cheeses was ~17 g/kg.

161

162 *Analyses.* Surface pH was measured using a surface electrode Blue line 27 (Schott). The pH
163 values were the arithmetic means of three measurements. Surface color was measured using a
164 CM-2002 spectrocolorimeter (Minolta, Carrières sur Seine, France) as described by Mounier
165 et al. (29). The data was processed using the three-dimensional *L* * *a* * *b* response, and logged
166 into the *L* * and *C* * system. *L* * ranges from 0 (black) to 100 (white) and indicates lightness,
167 *a* * and *b* * are the chromaticity coordinates indicating the color directions; + *a* * is the red
168 direction at 0°, - *a* * is the green direction at 180°, + *b* * is the yellow direction at 90° and – *b*
169 * is the blue direction at 270°. Cheese surfaces were photographed using a digital camera.
170 Lactose and lactate content were determined on the whole cheese using HPLC as previously
171 described by Leclercq-Perlat et al. (19). The release of free amino acids was measured on the
172 whole cheese as described by Grunau et al. (14). Ammonia content of the whole cheese was

measured using the Nessler reagent.

Microbiological analyses. Cheese was homogenized using a mortar and pestle, and ~1 g of the cheeses was sampled and transferred into a sterile container. A sterile saline solution (8.5 g/l NaCl) was added to yield a 1:10 dilution, and the mixture was homogenized with an Ultra Turrax® (Labortechnik) at 8000 rpm/min for 1 min. Total bacteria except lactic acid bacteria were enumerated by surface plating in duplicate on BHI agar supplemented with 50 mg/l amphotericin B after five days incubation at 25°C. Yeast population was determined by surface plating in duplicate using Yeast-Glucose-Chloramphenicol agar (YGC) supplemented with 0.01 g/l tetrazolium chloride (TTC) after three days incubation at 25°C. Lactic acid bacteria were enumerated by surface-plating in duplicate on MRS agar after two days incubation at 30°C.

Enumeration of yeast and bacterial species. Each yeast species had a distinct morphotype on YGC supplemented with TTC, which allowed their direct enumeration. For the bacteria, 250 colonies of each cheese sample were removed at random with sterile toothpicks and transferred onto 96-well microtiter plates containing 100 µl of BHI supplemented with 10% (v/v) glycerol and incubated three days at 25°C. The plates were stored at -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/l erythromycin, 1 or 5 mg/l novobiocin, 1 mg/l vancomycin or 1 g/l TTC. After incubation for three 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:

198 $$C_i = \frac{N_i \times C_0}{N_t}$$

199

200 where C_0 is the total bacterial count in CFU/g, N_t is the number of clones replicated, and N_i is

201 the number of clones identified as bacterium *i*.

202

203 *Statistical analysis*

204 The data with repeated measurements (bacterial and yeast population, pH, color, lactate) were

205 compared and statistically assessed using an analysis of variance (ANOVA). When

206 differences were detected by ANOVA, a Student-Newman-Keuls test was used to determine

207 which means were different. Statistical significance was set at $P < 0.05$.

208

209 *Lotka-Volterra modeling*

210 The multispecies Lotka-Volterra model was used in this study. Taking *n* species, the dynamic

211 of the species *i* ($i = 1, \ldots, n$) is the following:

212 $$\frac{dN_i}{dt} = N_i\left(\beta_i + \sum_{j=1}^{n} \alpha_{ij} N_j\right)$$

213 where β_i represents the intrinsic growth rate of the species *I*, and α_{ij} the influence of the

214 species *j* on the growth rate of species *i*. This influence is positive or negative according to the

215 sign of α_{ij}. In this model, the interactions are assumed constant for a given species *j*

216 abundance. To determine the interaction coefficients, the multispecies Lotka-Volterra system

217 can be expressed as a multi-linear regression:

218 $$\frac{d\ln(N_i)}{dt} = \beta_i + \sum_{j=1}^{n} \alpha_{ij} N_j$$

219 The left part of this equation was obtained by deriving the logarithm of the species

220 concentration according to time 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 abundance of the different species was used in the linear model. Inside this simplified system, an interaction coefficient α_{ij} was considered to be significant when $P(\alpha_{ij} \neq 0) > 90\%$.

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 media are compared in Figure S1 (supplementary 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 at pH 5 and 0% NaCl in which *S. xylosus* did not grow. The second group was comprised of *A. arilaitensis*, which grew at a pH equal or greater than 5.5, except in the presence of 0 and 30 g l^{-1} NaCl where it grew at a pH equal 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 or greater than 6, except for *B. aurantiacum*, which grew in the presence of 100 and 150 g l^{-1} NaCl at pH 5.5. In some cases, *C. casei* only grew at a pH equal 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 physico-chemical dynamics during the development of the ecosystem on model cheese

Reproducibility of microbial dynamics. The growth of the three yeasts and six bacteria during cheese ripening are shown in Figures 1A and 1B, respectively. There was a good reproducibility (a difference of less than 0.5 $\log_{10}$ units) between duplicates in the numbers of the yeast and 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 day 12, 16, 17, 18 and 20; *B. aurantiacum* on day 10, 12, 14 and 20 and

C. casei on day 20.

Yeast growth. D. hansenii and *Y. lipolytica* grew during the first days of ripening and had almost similar growth rates (Figure 1A); in contrast, *G. candidum* grew only after two days. A possible explanation for the absence of the 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 down of *D. hansenii* and *Y. lipolytica* growth. Overall, *D. hansenii* dominated the cheese surface until day 5; then, between day 6 and 9, the three yeasts had similar cell numbers, after which *D. hansenii* became progressively subdominant compared with *Y. lipolytica* and *G. candidum*. Indeed, *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.

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 day 0 and 4 (Figure 1B). Growth of all the organisms occurred after day 5-6. *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 bacteria counts decreased slightly from ~10^8 CFU/g on day 0 to 2 x 10^7 CFU/g at the end of ripening (data not shown).

Lactose, lactate and pH dynamics during ripening. Lactose, lactate and pH variations during ripening are shown in Figure 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

279 percent of the lactate was used during growth, which indicates that lactate was not a limiting
280 carbon source. The surface deacidification occurred between day 2 and day 6, with a pH
281 increase from approximately 5.0 to 8.0. This deacidification was highly correlated with the
282 utilization of lactate and the growth of *G. candidum* on the cheese surface (data not shown).
283 *Generalized Lotka-Volterra modeling.* The dendogram of the different species
284 according to their squared correlation coefficient during growth is shown in Figure S2
285 (supplementary material). With a threshold value of 0.75, each yeast was considered to have a
286 specific growth dynamic. In contrast, except for *Leucobacter* sp., the growth dynamics of the
287 bacteria were considered to be correlated. Consequently, GLV modeling was performed on
288 the growth dynamics of five distinct groups that comprised four individual species, *i.e.*, *Y.*
289 *lipolytica*, *G. candidum*, *D. hansenii* and *Leucobacter* sp., and a group of bacteria including *A.*
290 *arilaitensis*, *B. aurantiacum*, C. *casei*, *H. alvei* and *S. xylosus.*
291 The main interactions according to GLV modeling are shown in Figure 3. Yeast-yeast
292 interactions were found to be only negative, while yeast-bacteria interactions were found to be
293 only positive. *G. candidum* interacted negatively with *D. hansenii* and *Y. lipolytica*, while it
294 interacted positively with *Leucobacter sp*. and the group of bacteria. *D. hansenii* was found to
295 have a negative interaction with *Y. lipolytica,* while it had a positive interaction with the group
296 of bacteria. Self-inhibition of *G. candidum* and *D. hansenii* were also found in the model.
297 The model succeeded in representing the growth of the different microbial populations as
298 shown in Figures S3 and S4 (supplementary material), which compare measured and
299 estimated values for the two data sets. Total residual error between estimated and measured
300 values was 0.1 ± 0.4 log CFU/g for both data sets.
301
302 **Effects of single and multiple omissions of yeast in the ecosystem**
303 We aimed at identifying yeast-yeast or yeast-bacteria interactions by comparing the

304 growth of each individual microorganism in the absence or presence of one, two or three
305 yeasts. The utilization of lactose and lactate, the deacidification rate and the color
306 development of the cheese surface were also compared for each inoculum tested.
307 *Reproducibility*. There was good reproducibility between triplicates in terms of lactose and
308 lactate utilization, deacidification and the growth of the microorganisms of the ecosystem as
309 well (data not shown). There was also a good reproducibility between the data of the dynamic
310 study and the omission study in which all the members of the community were inoculated
311 (data not shown).
312
313 *Yeast-yeast interactions*. The viability of *D. hansenii* during the stationary phase was affected
314 in the presence of the other yeasts (see Figure S5 in supplementary material). Populations of
315 *D. hansenii* were significantly lower ($p<0.05$) on day 11 when *D. hansenii* was grown in the
316 presence of *G. candidum* or *G. candidum* and *Y. lipolytica*. Indeed, populations of *D. hansenii*
317 were 0.5 and 0.7 $\log_{10}$ units lower than the *D. hansenii* monoculture in the presence of *G.*
318 *candidum* or *G. candidum* and *Y. lipolytica*, respectively. Moreover, between day 11 and day
319 21, *D. hansenii* populations decreased from 1 to 1.7 $\log_{10}$ units when this organism was co-
320 cultivated with *G. candidum* and/or *Y. lipolytica*, whereas it remained constant in the
321 monoculture. This inhibitory effect was similar regardless of whether *Y. lipolytica* or *G.*
322 *candidum* were present, but was more pronounced in the presence of both species.
323 Populations of *Y. lipolytica* and, to a lesser extent, populations of *G. candidum*, were
324 significantly lower ($p<0.05$) on day 11 when they were grown in the presence of other yeasts
325 (data not shown). Their respective counts were 0.4 and 0.7 $\log_{10}$ units lower than those
326 observed in monoculture. However, there was not any loss in viability of *Y. lipolytica* and *G.*
327 *candidum* during the stationary phase.
328 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 (Figure 4A and 4B), whereas in the presence of *Y. lipolytica*, growth occurred as spaghetti-like structures without formation of pseudohyphae (Figure 4C). This inhibition of mycelial development did not influence cellular growth since only small differences in numbers of *G. candidum* were found (Figure 4D). This phenomenon was also observed in the presence of both *Y. lipolytica* and *D. hansenii*. The idea that an interaction of *Y. lipolytica* on *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 the presence of *Y. lipolytica* compared with the monoculture or in co-culture with *D. hansenii* (Figure 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 co-cultivated with *Y. lipolytica*.

Chemical characteristics of the cheese.

G. candidum showed the highest deacidification rate, followed by *D. hansenii* and *Y. lipolytica*, which had similar deacidification rates (Figure 5A). The pH reached its maximal value, *i.e.*, 8.0, after 11 days when *G. candidum* was present in the ecosystem, whereas pH ranged from 6 to 6.5 for *D. hansenii* and *Y. lipolytica* (Figure 5A) or a combination of both species (data not shown). After 21 days, pH 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 d 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 d, the cheese inoculated with *Y. lipolytica*

had 2-15 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 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 (Figure 6A). After 11 and 21 days, the cheeses that contained *G. candidum* showed significantly higher surface-pH 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 (Figures 6A and 6B). After 11 days, the bacterial count of the cheese inoculated with *G. candidum* by itself was significantly higher ($p<0.05$) than the cheese inoculated with *D. hansenii* or *Y. lipolytica* by itself (Figure 6A). In contrast, with two or three yeasts in the community, total bacterial counts were statistically similar ($p<0.05$) despite the fact that surface pH was significantly lower ($p<0.05$) on the cheese containing *D. hansenii* and *Y. lipolytica* (Figure 6B). After 21 days, total bacterial counts were not statistically different in all cheeses except the cheeses that contained *Y. lipolytica* as the sole yeast, and *D. hansenii* and *Y. lipolytica*, which had counts 1.5 and 1 $\log_{10}$ units lower, respectively (Figures 6A and 6B).

As shown in Figure 7, there were only small differences in the 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.*

379 *hansenii*, the cheeses were dominated by *A. arilaitensis*, which represented between 66 and
380 86% of the total isolates, followed by *H. alvei* (5-25%), *Leucobacter* sp. (2-10%), *S. xylosus*
381 (3-10%), *C. casei* and *B. aurantiacum* (0.4-2%). *H. alvei* (70%), followed by *A. arilaitensis*
382 (26%) and *Leucobacter* sp. (11%), dominated the cheese inoculated with *D. hansenii* and *G.*
383 *candidum*. After 21 d, differences and common patterns were found in the distribution of the
384 bacterial community. *Leucobacter* sp. grew in all the cheeses inoculated with *G. candidum*
385 and represented between 26 and 60%, whereas this species was subdominant (less than 5% of
386 the total isolates) in all cheeses in which *G. candidum* was absent. *A. arilaitensis* dominated in
387 the cheese inoculated with *D. hansenii* or *G. candidum* as the sole yeast (70% of the isolates),
388 while *H. alvei* dominated in cheeses inoculated with *Y. lipolytica* or *Y. lipolytica* and *D.*
389 *hansenii* (70% of the isolates). After 21 days, *S. xylosus*, *B. aurantiacum* and *C. casei*
390 remained subdominant, except in the cheese inoculated with *G. candidum* as the sole yeast in
391 which *B. aurantiacum* represented 10% of the isolates taken in this cheese.
392
393 *Color development of the cheese surface.*
394 There were only small differences in the color development of all the cheeses after 11 days,
395 except the cheese inoculated with no yeast and the cheeses inoculated with *G. candidum* or *G.*
396 *candidum* and *D. hansenii*, which had a lower b* (yellow dimension) probably because *G.*
397 *candidum* formed white mycelia on the surface (data not shown). In contrast, all the cheeses
398 differed considerably in terms of color development after 21 days (Figure 8). The consortium
399 that contained the three yeasts showed the highest a* and b* values, followed by the two other
400 cheeses inoculated with *Y. lipolytica* and *D. hansenii* or *Y. lipolytica* and *G. candidum*. The
401 cheeses inoculated with *G. candidum* by itself and *G. candidum* and *D. hansenii* had high a*
402 but low b* values, while the cheeses inoculated with only *D. hansenii* or *Y. lipolytica* had
403 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 bacteria, on cheese agar (23, 24) or on curd made under aseptic conditions (4, 20, 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 that *G. candidum* grew as spaghetti-like structures instead (Figure 4). Numerous chemical and environmental parameters have been reported to influence the yeast-mycelium formation, such as temperature, glucose levels, pH, nitrogen sources and inoculum size (30). Among these, ammonia and proline, which were produced in greater quantities by *Y. lipolytica* than *D. hansenii*, may be 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 non-dividing yeast cells. Kulkarni and Nickerson (17) showed that proline (10 mM) induced the yeast morphology in

Ceratosystis ulmi in defined liquid media, 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 orientated 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 cell maintenance of *D. hansenii* during its stationary phase.

Yeast-bacteria 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*

454 and developed later in the ripening process. *C. casei* and *B. aurantiacum* were also quite acid-
455 sensitive and did not hold up well under the acidic stress that occurred at the start of ripening,
456 compared to the other members of the bacterial community. This may be responsible for their
457 subdominance in almost all the cheeses.

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

472 *Leucobacter* sp. only grew in the cheeses that contained *G. candidum.* This would imply that
473 *Leucobacter* sp. was highly dependent on *G. candidum* activities either because *G. candidum*
474 rapidly deacidified the surface or because it produced metabolites that enhanced *Leucobacter*
475 sp. growth. Similarly, B. *aurantiacum* represented ~10% of the clones isolated after 21 d in
476 the cheese inoculated with *G. candidum* as the sole yeast, whereas *B. aurantiacum* was
477 subdominant in the other microbial communities. It is possible that *G. candidum* detoxified
478 the environment and released substrates that promoted 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 others, such as *B. linens* or *B. aurantiacum*.

Color development of the cheese surface. The color differentiation that occurred between d 11 and d 21 is probably due to the production of pigments by the bacteria. Interestingly, in some cases, if we compare two cheeses with almost similar bacterial distribution and population, such as the ecosystems that contained only *Y. lipolytica* or *Y. lipolytica* and *D. hansenii*, color differed considerably between the two ecosystems. This would imply that, depending on the yeasts present, species-specific bacterial pigmentation was different in these two cheeses. This is in agreement with 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 effect of *G. candidum* on *Leucobacter* sp. and on the rest of the bacteria that were confirmed in this study. Similarly, the negative interaction between yeasts, such as the inhibition of *G. candidum* on *D. hansenii,* 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 omission study. Inversely, other interactions such as a negative interaction from *Y. lipolytica* on *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 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 orientate 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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Table 1. Selective media used to identify the different bacterial clones.

	Novobiocin		Erythromycin	Vancomycin	TTC‡
	1 mg/l	5 mg/l	20 mg/l	1 mg/l	0.1 g/l
Arthrobacter arilaitensis	-†	-	-	-	+
Brevibacterium aurantiacum	+	-	-	v	v
Corynebacterium casei	+	+	-	-	v
Hafnia alvei	+	+	+	+	+
Leucobacter sp.	-	-	-	+	+
Staphylococcus xylosus	+	+	-	+	+

† -, absence of growth; +, growth; v, variable growth.

‡ TTC: Tetrazolium chloride

Figure legends.

Figure 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.*

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

Figure 3. Main interactions (⟶, positive; ⟶ , negative) according to generalized Lotka-Volterra modeling between the members of the multi-species ecosystem. *Dh*, *Debaryomyces hansenii*; *Yl*, *Yarrowia lipolytica*; *Gc*, *Geotrichum candidum*; *L*s, *Leucobacter* sp; C, group including *Arthrobacter arilaitensis*, *Hafnia alvei***,** *Corynebacterium casei*, *Brevibacterium aurantiacum* and *Staphylococcus xylosus*.

Figure 4. Macromorphology of *Geotrichum candidum* grown as a monoculture (A) or in the presence of *Debaryomyces hansenii* (B) or *Yarrowia lipolytica* (C) and (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* (▲, △).

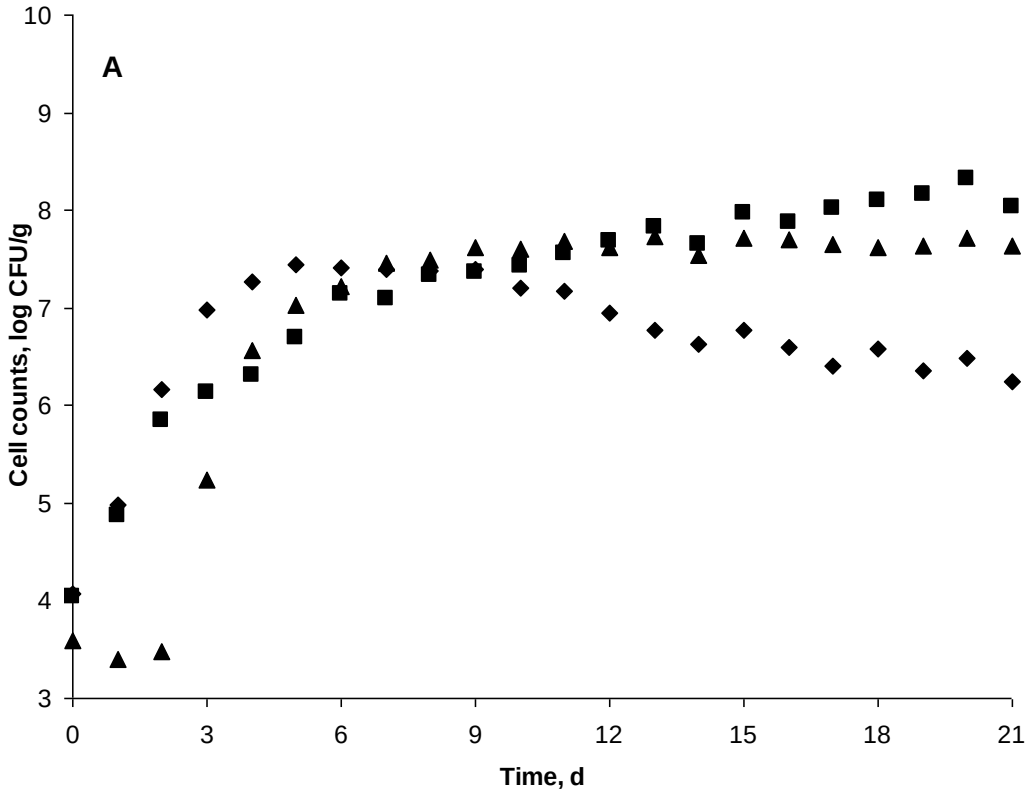
Figure 5. Lactate consumption (closed symbols) and pH increase (open symbols) during ripening in cheeses inoculated with *Debaryomyces hansenii* (▲, △), *Geotrichum candidum* (●, ○) or *Yarrowia lipolytica* (■**,** □).

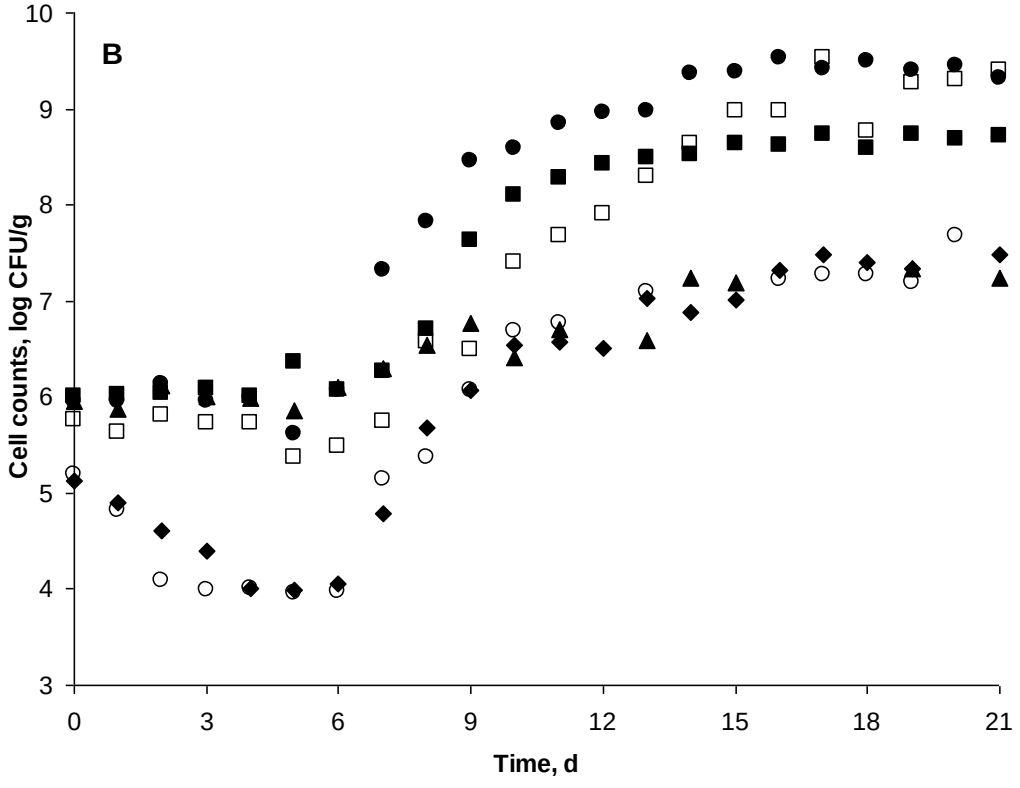
Figure 6. Total bacterial growth and surface pH increase during ripening in cheeses inoculated with (A) no yeast (○), *Debaryomyces hansenii* (■), *Yarrowia lipolytica* (♦), *Geotrichum candidum* (▲) or (B) *D. hansenii* and *Y. lipolytica* (◊), *D. hansenii* and *G. candidum* (△), *Y. lipolytica* and *G. candidum* (□), *D. hansenii, Y. lipolytica,* and *G. candidum* (●).

Figure 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.*

Figure 8. Color of the cheese surface after 21 d 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* (◊), *Y. lipolytica* and *G. candidum* (□) and *D. hansenii, Y. lipolytica,* and *G. candidum* (○).

Figure 1.


A
Cell counts, log CFU/g
Time, d
10
9
8
7
6
5
4
3
0
3
6
9
12
15
18
21


B
Cell counts, log CFU/g
Time, d
10
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706 **Figure 2.**

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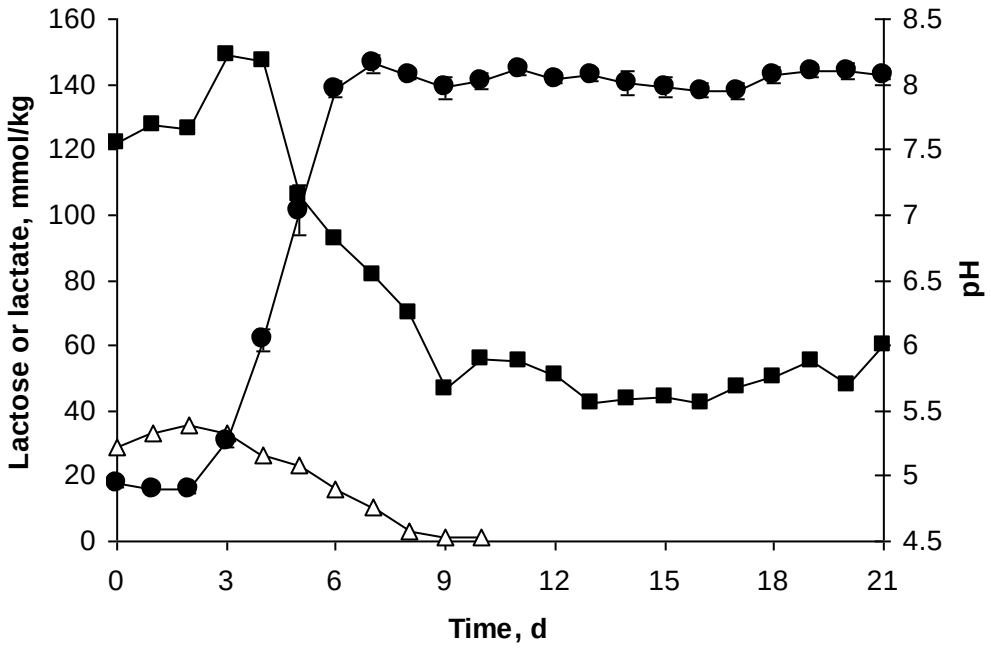

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722 **Figure 3.**

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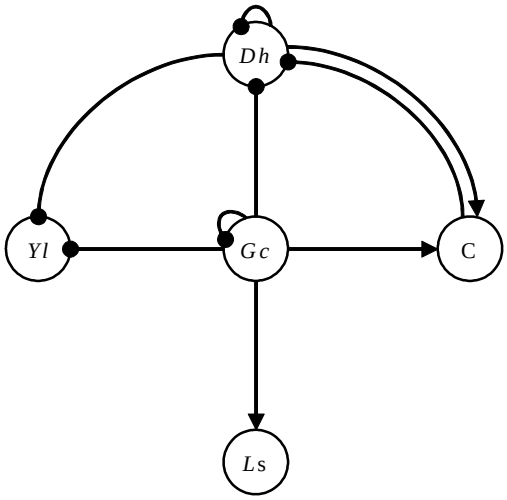

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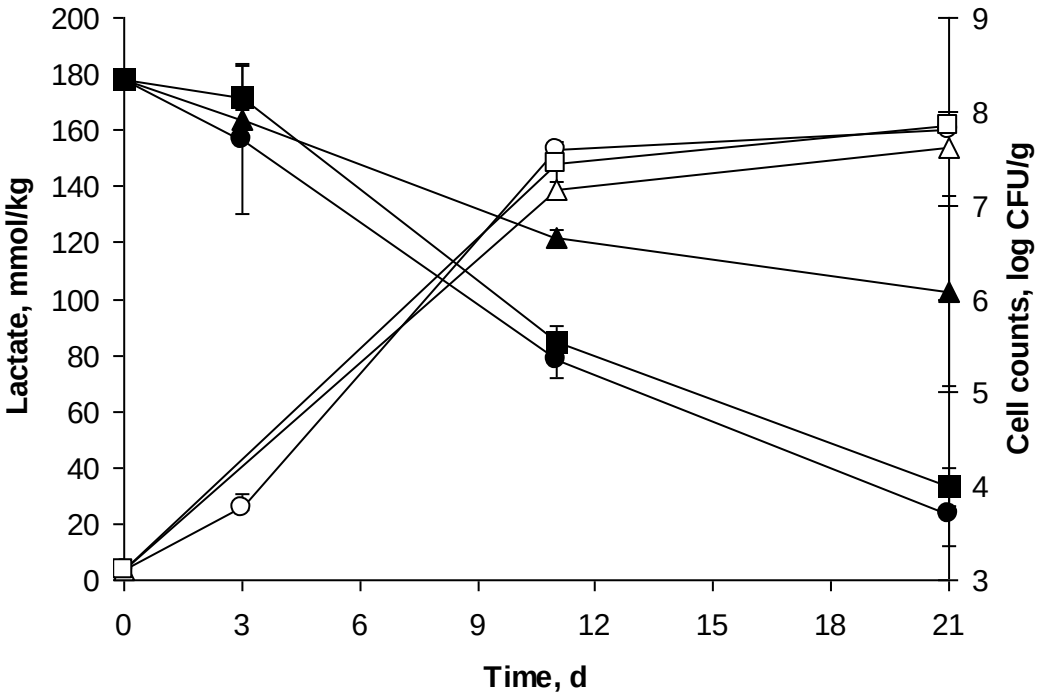
Figure 4.


A
B
C


Lactate, mmol/kg
Cell counts, log CFU/g
Time, d
0
20
40
60
80
100
120
140
160
180
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736 **Figure 5.**

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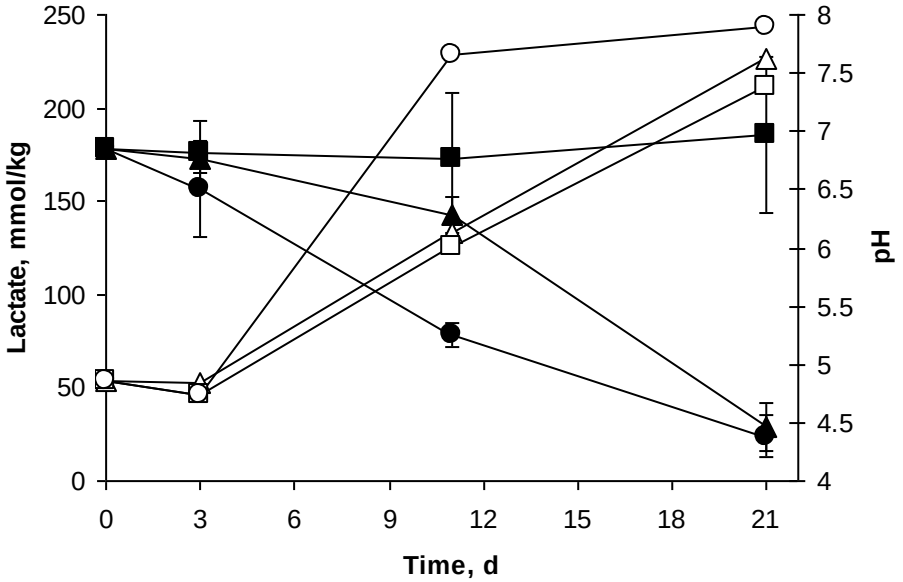

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Figure 6.

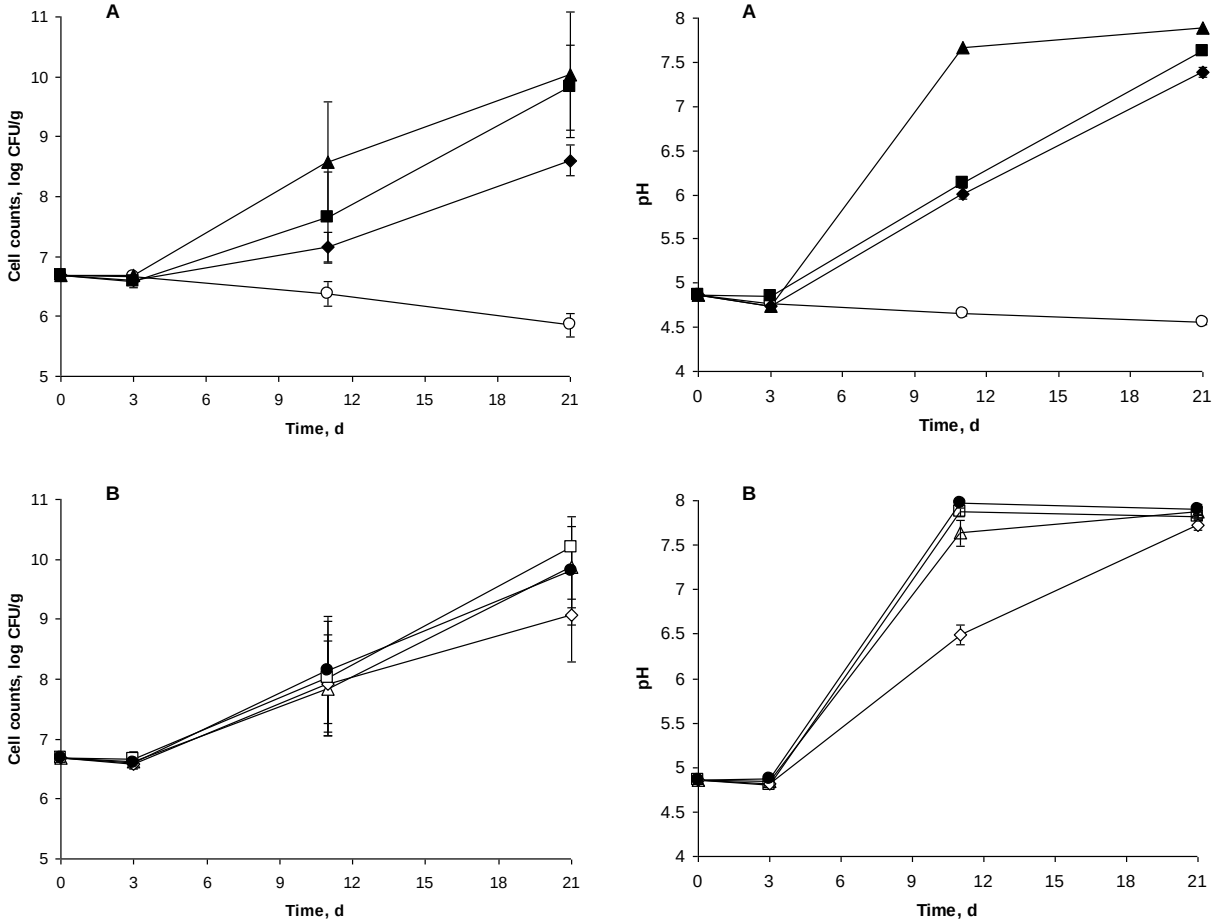
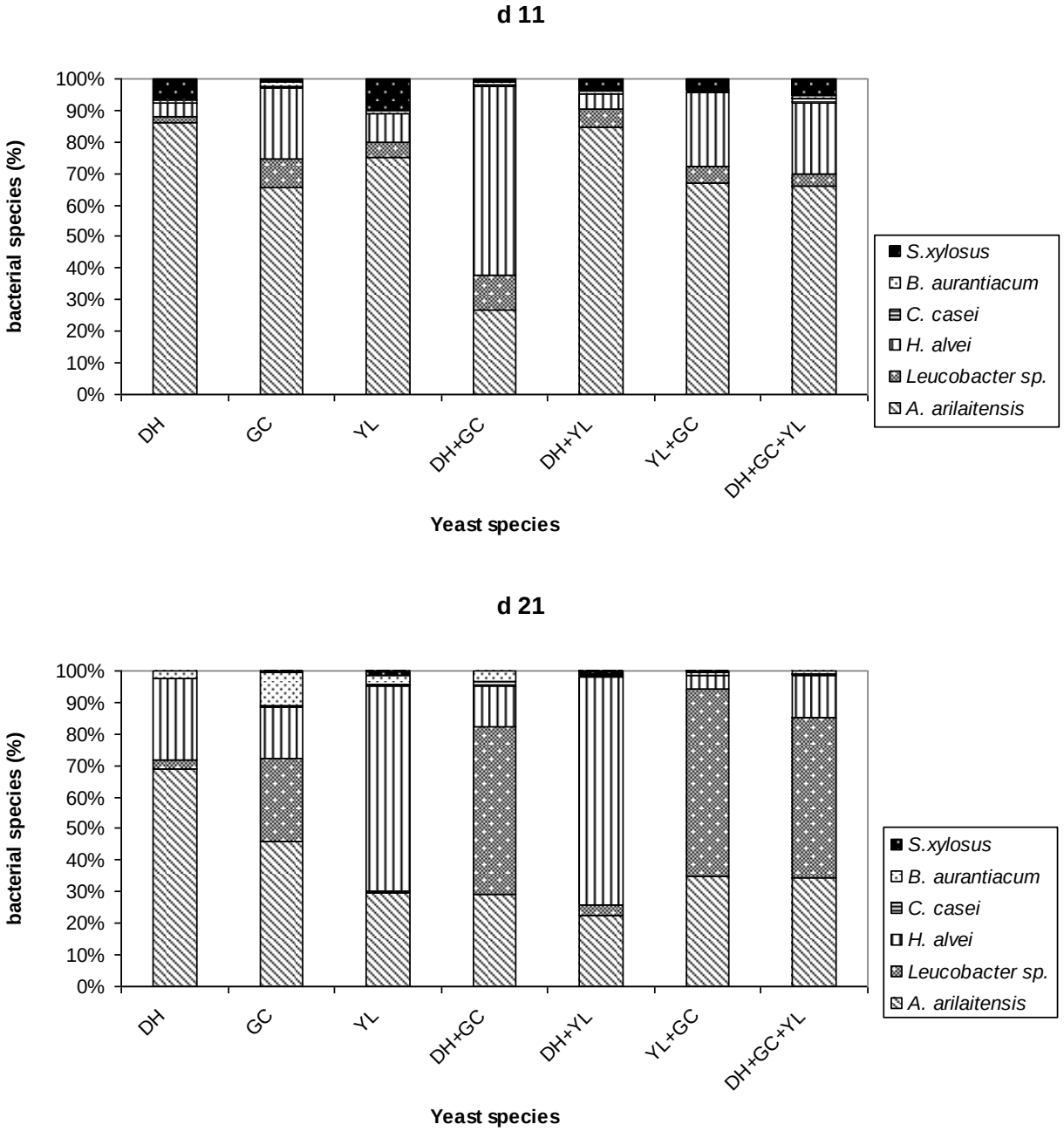
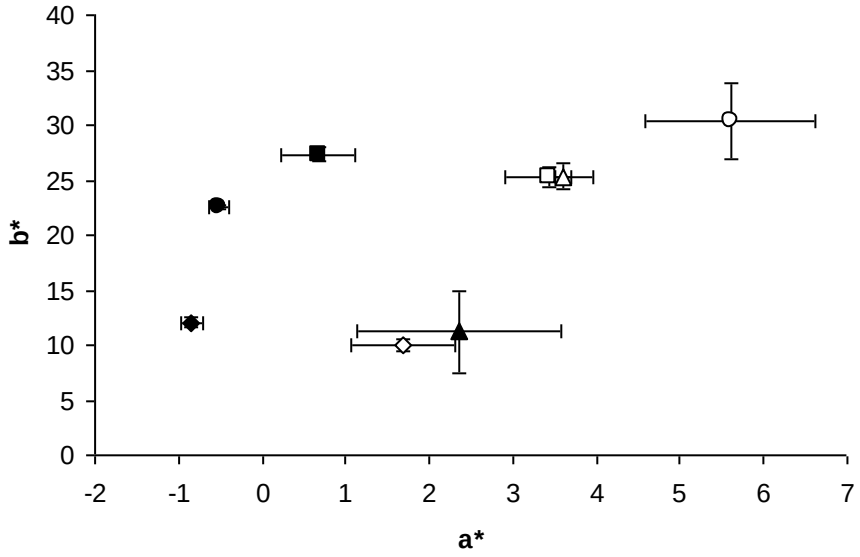

A
Cell counts, log CFU/g
Time, d
A
pH
Time, d
B
Cell counts, log CFU/g
Time, d
B
pH
Time, d

Figure 7.


d 11
bacterial species (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
DH
GC
YL
DH+GC
DH+YL
YL+GC
DH+GC+YL
Yeast species
S.xylosus
B. aurantiacum
C. casei
H. alvei
Leucobacter sp.
A. arilaitensis
d 21
bacterial species (%)
Yeast species
S.xylosus
B. aurantiacum
C. casei
H. alvei
Leucobacter sp.
A. arilaitensis

798 **Figure 8.**

799



800

Supplementary material

Figure legends.

Figure S1. Growth properties of the bacteria of the cheese ecosystem as a function of pH and NaCl content at 12°C. Growth after ■ 2 days, ■ 4 days and □ absence of growth after incubation for 8 days.

Figure S2. Dendrogram of the different species according to their squared correlation coefficient during growth in model cheese used for Lotka-Volterra modelling.

Figure S3. Comparison of experimental populations (○) of experiment I and estimated populations (▬) using Lotka-Volterra modelling of *Debaryomyces hansenii* (Dh), *Yarrowia lipolytica* (Yl), *Geotrichum candidum* (Gc), *Leucobacter* sp. (Le) and a group including *Arthrobacter arilaitensis, Hafnia alvei, Corynebacterium casei, Brevibacterium aurantiacum* and *Staphylococcus xylosus* (C).

Figure S4. Comparison of experimental populations of experiment II (○) and estimated populations (▬) using Lotka-Volterra modelling of *Debaryomyces hansenii* (Dh), *Yarrowia lipolytica* (Yl), *Geotrichum candidum* (Gc), *Leucobacter* sp. (Le) and a group including *Arthrobacter arilaitensis, Hafnia alvei, Corynebacterium casei, Brevibacterium aurantiacum* and *Staphylococcus xylosus* (C).

Figure S5. Growth of *Debaryomyces hansenii* when cultivated as a monoculture (■) or in co-culture with *Yarrowia lipolytica* (▲), *Geotrichum candidum* (○) or *Y. lipolytica* and *G. candidum* (□).

Figure S1.

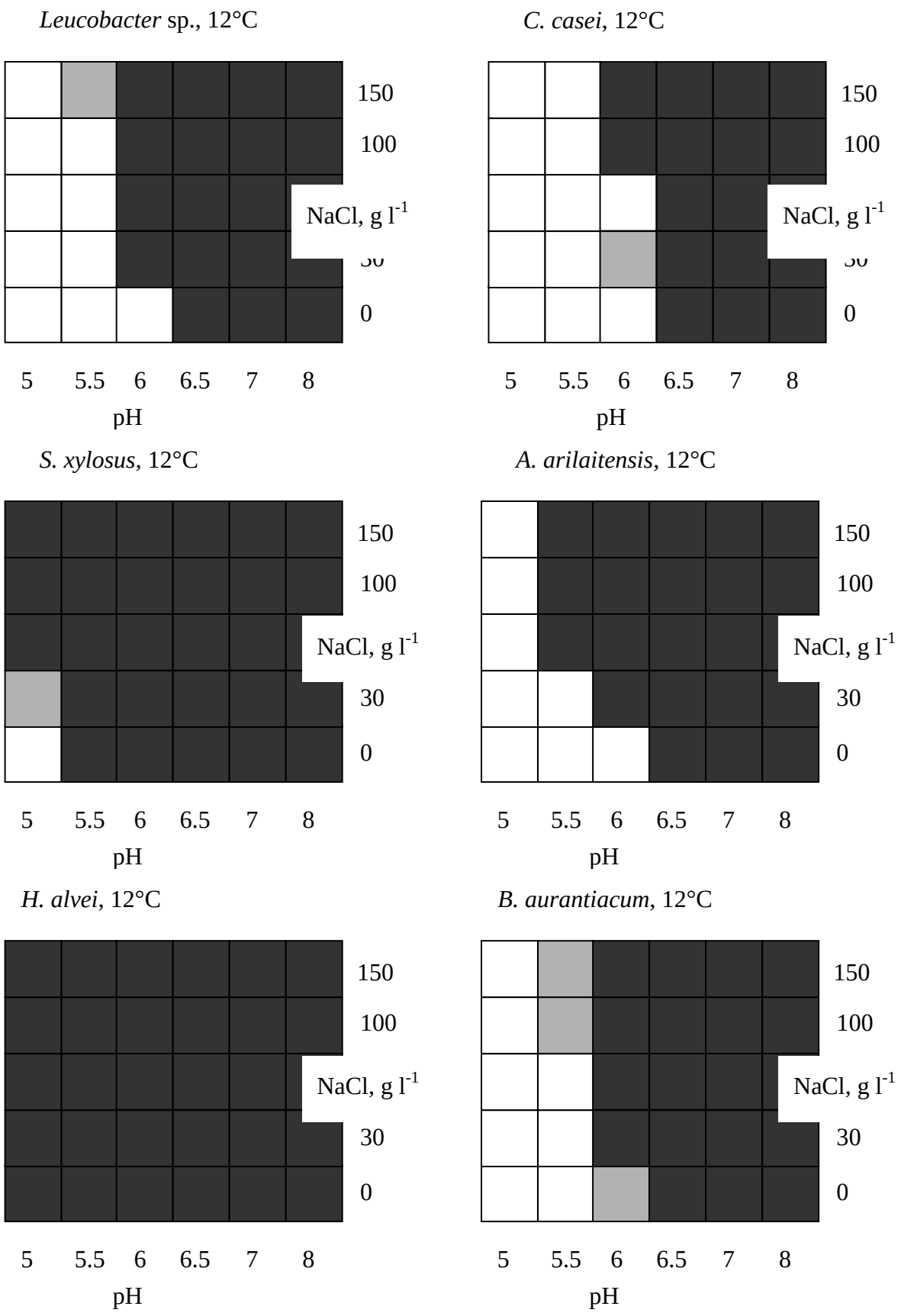
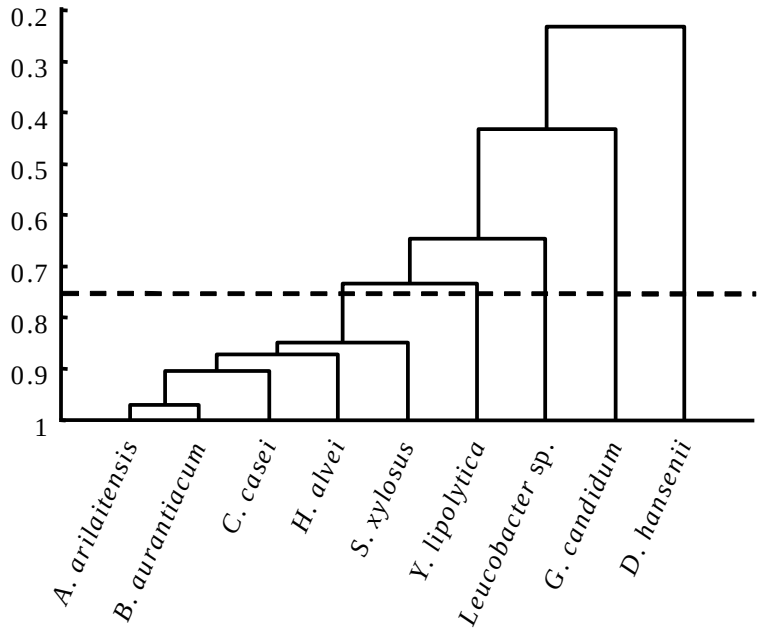

Leucobacter sp., 12°C
C. casei, 12°C
S. xylosus, 12°C
A. arilaitensis, 12°C
H. alvei, 12°C
B. aurantiacum, 12°C
150
100
30
0
NaCl, g l-1
5
5.5
6
6.5
7
8
pH

Figure S2.


0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
A. arilaitensis
B. aurantiacum
C. casei
H. alvei
S. xylosus
Y. lipolytica
Leucobacter sp.
G. candidum
D. hansenii

866

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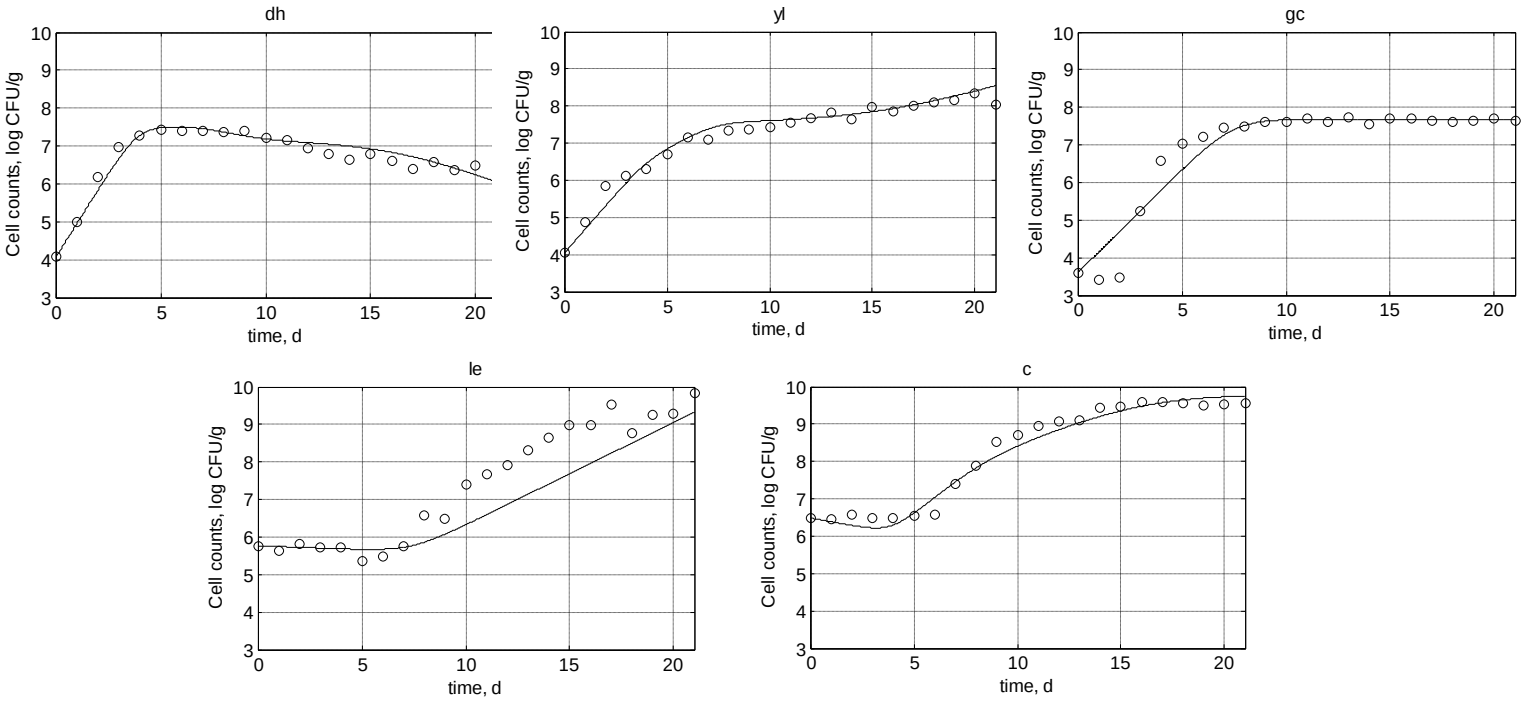
868

869 **Figure S3.**

870



872 **Figure S4.**

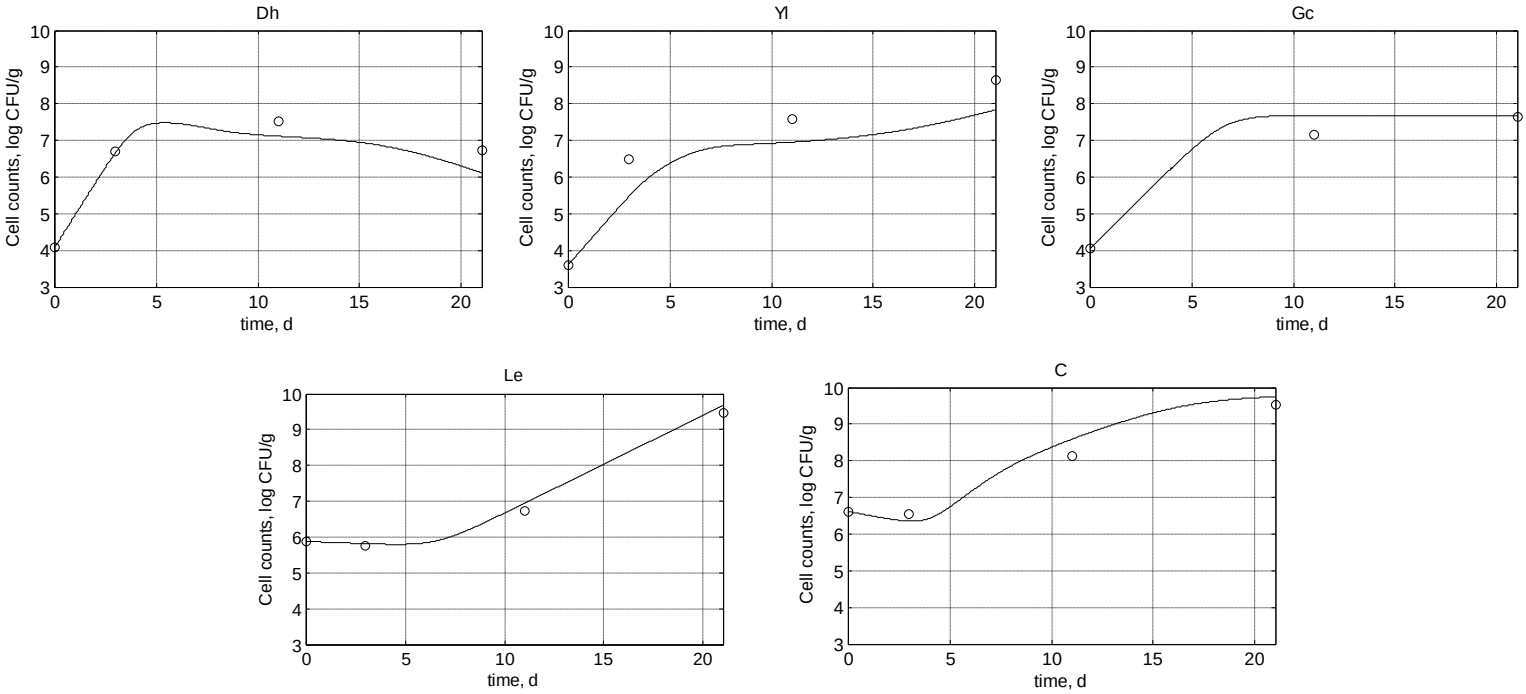


Figure S5.