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Original article

Contribution of several cheese-ripening microbial associations to aroma compound production

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Abstract – The aromatic potential of various cocultures of yeasts, *Brevibacterium linens* and lactic acid bacteria (LAB) was studied in cheese-based medium. Three yeasts (*Debaryomyces hansenii*, *Geotrichum candidum* and *Kluyveromyces lactis*) were cultivated in association with *B. linens*, in the presence or in the absence of LAB – added as the commercial lactic acid starter *Flora Danica*®. Various parameters were analysed such as aroma compound production, the growth of each microorganism and lactose/lactate degradation. All tested yeasts could grow in all the associations regardless of the presence or the absence of LAB. LAB enhanced the growth of *B. linens* in *D. hansenii* associations, but they reduced *B. linens'* growth when associated with *K. lactis*. When cultivated alone, LAB produced very few aroma compounds and in lesser amount than the yeast-*B. linens* associations. In pure cultures of LAB, ethanol was the major volatile compound, and only scanty amounts of other volatile compounds were produced. The *K. lactis-B. linens* association exhibited the most diversified aroma compound profile with high quantities of *S*-methyl thioacetate and ethyl acetate. LAB promoted the synthesis of volatile sulphur compounds in this association.

Ripening / interaction / microbial association / aroma compound

Résumé – Contribution de plusieurs associations microbiennes à l'affinage d'un milieu fromager. Le potentiel aromatisant de plusieurs co-cultures constituées de levures, de *Brevibacterium linens* et de bactéries lactiques (BL), a été étudié dans un milieu fromager. Trois levures (*Debaryomyces hansenii*, *Geotrichum candidum* et *Kluyveromyces lactis*) ont été cultivées en association avec *B. linens*, avec ou sans BL – ajoutées sous forme du levain lactique *Flora Danica*®. Plusieurs paramètres ont été analysés parmi lesquels la production de composés d'arôme, la croissance de chaque micro-organisme et la dégradation du lactose/lactate. Toutes les levures étudiées se sont développées que ce soit en présence ou en l'absence de BL. La présence de BL a favorisé la croissance de *B. linens* dans les associations avec *D. hansenii*, alors que la croissance de *B. linens* s'est trouvée réduite dans les associations avec *K. lactis*. Lorsqu'elles ont été cultivées seules, les BL ont produit qualitativement et quantitativement moins de composés d'arôme que les associations levures-*B. linens*. Les cultures pures de BL ont produit majoritairement de l'éthanol et seulement de très faibles quantités d'autres composés volatils. L'association *K. lactis-B. linens* a permis d'obtenir la plus grande diversité de composés d'arôme, avec des quantités importantes de *S*-methylthioacétate et d'acétate d'éthyle. Les BL ont permis d'augmenter les quantités de composés soufrés volatils produites dans cette association.

Affinage / interaction / association microbienne / composé aromatique

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1. INTRODUCTION

The role played by mixed microbial populations in the quality and the typical flavour of several fermented foodstuffs is of great interest. Microbial consortia are able to develop valuable properties which are often the result of the activities of a group of microorganisms rather than a single one. This is the case for cheese ripening, which is made possible by a complex ecosystem in which bacteria, yeasts and filamentous fungi are involved.

While some cheeses such as Cheddar are made using solely LAB [32], others such as Camembert require the presence of four groups of microflora i.e. LAB, surface bacteria, yeasts and moulds [19]. A wide range of yeasts and surface bacteria have been isolated from traditional cheese, but little is known about their behaviour when cultivated in association. However, it is clear that the microbial community of this ecosystem strongly contributes to the synthesis of volatile aroma compounds: among others, volatile sulphur compounds (VSC), esters, alcohols, aldehydes and ketones, which were commonly found in ripened cheeses such as Cheddar, Camembert or Emmental [37]. These compounds impart a basic "cheesy" flavour; however, analysis of Cheddar, Limburger, Camembert and other mould-ripened varieties indicated that they can also generate specific notes ("cabbage", "garlic" or "fruity") [29, 33].The organoleptic properties and the identity of these aroma compounds – which are especially pronounced at very low concentrations due to their low perception thresholds – participate in the overall quality of the final product [21].

It is generally admitted that the cheese microflora act in a sequential manner and that interactions between microorganisms could play a major role in the ripening process. So far, possible interactions between cheese-ripening yeasts, moulds and surface bacteria in the cheese ecosystem have been essentially assessed in terms of growth of the different populations [1, 34], but the development of particular functionalities, such as aroma compound synthesis, has been investigated infrequently in microbial associations cultured in a cheese-based medium [27].

Information is already available on the ability of pure cultures of several cheese-ripening yeasts and bacteria [3, 8, 9] or lactic acid bacteria (LAB) [37] to produce aroma compounds, and various metabolic pathways leading to the generation of these compounds have been suggested [26]. However, the precise contribution of each microorganism to cheese aroma compound production in association with other microorganisms of the cheese ecosystem still remains unclear. Yeasts, such as *Geotrichum candidum,* have a strong potential for producing VSC through the degradation of L-methionine when cultivated as pure culture, while others, such as *Kluyveromyces lactis* were found to be more prone to producing esters [3]. It is expected that these yeasts could contribute to different specific flavour notes during ripening. Surface cheese-ripening bacteria, especially *Brevibacterium linens*, are known for their ability to produce VSC through the synthesis of a common precursor from L-methionine, methanethiol (MTL) [18]. Since these bacteria are salt-tolerant but also acid-sensitive [7], they generally develop in the late stage of ripening, after yeasts have deacidified the curd [24]. In LAB, the catabolism of amino acids – in relation to aroma compound synthesis – was thoroughly investigated and was revealed to be highly strain-dependent [37]. However, it is not clear what the actual impact could be of cultivating LAB in association with cheese-ripening yeasts and bacteria on the synthesis of aroma compounds. Although pure culture approaches provided useful information on metabolic pathways leading to aroma compounds, the in situ behaviour of microflora and interaction phenomena between cheese microorganisms were not fully investigated.

Different yeast(s)-bacteria associations were studied in a cheese-based medium. Three yeasts – *Debaryomyces hansenii, G. candidum* and *K. lactis* – were co-cultured

with the cheese-ripening bacterium *B. linens* and, in some cases, associated with LAB. Not only the influence of the yeast used, but also the possible influence of LAB on aroma compound production were investigated for each microbial association.

2. MATERIALS AND METHODS

2.1. Cheese curd production

Camembert-type experimental cheeses were prepared under aseptic conditions, in a sterilised 2-m^3 pilot-scale cheese-making chamber as previously described [25].

The chamber temperature was maintained at 28 ± 1 °C. Coagulation, cutting, draining and shaping of the curds were performed inside this chamber. In each cheese-making trial, 50 cheeses of 300 g average wet weight were produced from 100 L of reconstituted milk. Medium-heat skim milk powder (total nitrogen > 35% m/m, total fat < 1.5% m/m) was purchased (Armor Protéines, Saint-Brice-en-Coglès, France). The powder was suspended in sterile distilled water at a concentration of 120 g·L^{-1}. Commercial cream (Elle & Vire®, Condé-sur-Vire, France) was added to the milk to obtain a final concentration of 29 g·L^{-1} of total fat. After homogenisation, the milk was allowed to rest at 4 °C overnight.

Prior to entrance into the clotting vat, the milk was pasteurised for 30 sec at 87 ± 1 °C, and cooled to 34 ± 1 °C. Milk pH varied between 6.5 and 6.6. Inoculation with the lactic acid starter (1.5% v/v) was performed when the first litre of milk reached the clotting tank. The mesophilic lactic acid starter, which served as inoculum, was prepared using a *Flora Danica*® lyophilisat (CHN11, Chr Hansen, Arpajon, France). One gram of the lyophilisat was dissolved in 50 mL sterile skim milk (reconstituted at 100 g·L^{-1}) containing glycerol (5% v/v) for cryoprotection. This mixture was then transferred to 10-mL tubes and stored at –80 °C. Before each cheese-making trial one aliquot of the starter was cultured at 30 °C in 1.5 L sterile skim milk for 16 h.

Eighty to 100 min after the addition of the lactic acid starter, milk pH reached 6.3. Rennet containing 520 mg·L^{-1} of chymosin (Degussa, Beaune, France) was then added at a concentration of 0.2 mL·L^{-1}. Coagulation occurred after 15 min followed by 40 min of hardening. The curd was subsequently cut into cubes (20 × 20 mm). At the end of the draining step, around 35 L of whey were expelled to obtain an average cheese dry matter ranging between 400 and 420 g·kg^{-1}. The resulting curd was then shaped using polyurethane moulds (diameter 110 mm, height 107 mm). The cheese-containing moulds were inverted twice after 30 min and 5 h. Three hours after moulding, the temperature of the cheese-making chamber was reduced to 20–22 °C. The cheeses were withdrawn 24 h after the moulding, packed in sterile polyethylene bags and kept at –80 °C until they were transformed into cheese slurries.

2.2. Preparation of the cheese slurry

Cheese curds were thawed (4 °C overnight) and mixed (60% wet weight) with 40% sterile brine (42.5 g·L^{-1} of NaCl) using a laboratory blender (Waring, Fisher Labosi, Elancourt, France). The cheese slurry obtained was then heated at 100 °C for 30 min to destroy the lactic acid bacteria and other possible contaminants. After cooling (25 °C), the cheese slurry was homogenised: 25 000 rpm, 2 min (Ultra-Turrax®, VWR International, Fontenay-sous-Bois, France), and 300 g of this cheese slurry were transferred into sterile 500-mL flasks (300 g/flask). Samples were taken before repartition to check the absence of residual microorganisms. Aerobic plate counts were achieved as described below.

2.3. Microorganisms and culture conditions

In this study, yeasts were cultivated in association with *B. linens* in model cheese

Table I. Codes attributed to the various microbial associations tested in this study.

Association		LAB#	*B. linens*	*D. hansenii*	*G. candidum*	*K. lactis*
Dh	1	-	+	+	-	-
	2	+	+	+	-	-
Gc	1	-	+	-	+	-
	2	+	+	-	+	-
Kl	1	-	+	-	-	+
	2	+	+	-	-	+
KlDh	1	-	+	+	-	+
	2	+	+	+	-	+
LAB		+	-	-	-	-

Lactic acid bacteria.

medium at 14 °C. Each association (yeast(s)-*B. linens*) was cultivated in the presence or in the absence of LAB.

Three cheese-ripening yeasts, one cheese-ripening bacterium and lactic acid bacteria were used. Among the yeasts, two were from our laboratory collection (UMR GMPA, Inra de Grignon, Thiverval-Grignon, France) and originally isolated from French cheeses, namely *Kluyveromyces lactis* 44_8 and *Debaryomyces hansenii* 304, and one is a commercial strain, *G. candidum* GcG (Degussa, La-Ferté-sous-Jouarre, France). All the yeasts were cultivated in association with one surface bacterium, *B. linens* CNRZ 918. In some associations, LAB were added as the commercial lactic acid starter *Flora Danica*® lyophilisat (CHN11, Chr Hansen, Arpajon, France). The different associations tested are described in Table I.

The microorganisms were stored at –20 °C in non-fat dry milk containing 5% v/v glycerol until ready-to-use aliquots were prepared. High-density microbial suspensions were obtained according to the procedure described earlier [24].

Cultures of microorganisms were conducted in the cheese slurry prepared as described above. Each Erlenmeyer flask containing 300 g of cheese slurry was inoculated with the appropriate microbial association. Cheese slurries were inoculated to obtain concentrations of 5×10^5, 10^5, 5×10^4, and 3×10^6 cfu·g^{-1} of slurry of *K. lactis*, *G. candidum*, *D. hansenii* and *B. linens*, respectively. LAB concentration was 5×10^5 to 10^6 cfu·g^{-1} of slurry. For each association, all experiments were carried out in triplicate and results were reported as means ± standard deviations. Cultures were incubated at 14 °C under agitation (140 rpm) for 41 d. Samples were taken at regular time intervals for biochemical and microbiological analyses.

2.4. Viable cell counts

Viable cell counts of yeasts were determined following a standard aerobic plate count procedure on Yeast Extract Glucose Chloramphenicol Agar (Biokar, Paris, France). The dishes were incubated at 25 °C for 2 to 3 d. Likewise, aerobic plate counts were performed on Brain Heart Infusion medium (Biokar) containing the fungicide amphotericin B (50 mg·L^{-1}) to compute the viable cells of *B. linens*. The dishes were incubated at 25 °C for 7 d. Viable cell counts of LAB were performed on MRS (Man, Rogosa, Sharpe) medium (Biokar) containing amphotericin B (50 mg·L^{-1}). Dishes were incubated at 25 °C for 3 d minimum. The results are expressed as cfu·g^{-1} of cheese slurry.

2.5. Lactate and sugar analysis by HPLC

For each sampling time, samples (15 g) were taken for high pressure liquid chromatography (HPLC) analysis and stored at –20 °C. Prior to analysis, each culture suspension was thawed and mixed with 20 mL of distilled water. The mixture was homogenised (Ultra-Turrax®, 25 000 rpm, 30 sec) after incubation at 50 °C for 1 h. The resulting homogenates were subsequently treated for either lactate or sugar measurements by HPLC as previously described [23].

2.6. Volatile compound analysis by dynamic headspace GC-MS

Two grams of culture were taken and stored at –80 °C until gas chromatography analysis was performed. Before the analysis, samples were thawed at 4 °C, homogenised vigorously and diluted to 10^{-1} with water. NaCl 10% (w/w) was added to the diluted samples in order to enhance the extraction by salting-out. Five millilitres of the mixture were analysed using a headspace analyser (HP 7695A purge and trap concentrator; Hewlett Packard, Palo Alto, CA, USA) coupled to a gas chromatograph (HP 6890; Hewlett Packard) and a mass spectrometer detector (HP 6890A quadrupole mass spectrometer; Hewlett Packard) as previously described [27]. Volatile compounds were identified according to their total ionic current and quantified using external standards' calibration curves. All sulfides were quantified using dimethyl disulfide (Acros, Noisy-Le-Grand, France) standard calibration curve except dimethyl trisulfide for which the corresponding standard (Acros) was used. Methyl thiobutyrate's (Sigma-Aldrich, St-Quentin-Fallavier, France) calibration curve was used for quantifying the thioesters. All esters and aldehydes were quantified using pure ethyl acetate (Prolabo, Fontenay-sous-Bois, France) as external standard; and 3-methyl butanol's (Sigma-Aldrich) standard curve was used to quantify the alcohols present in the samples analysed. The results are expressed as microgram of compound per kilogram of the cheese slurry.

2.7. Data analysis

Data were analysed using Statgraphics Plus software (Sigma Plus, Toulouse, France). Values presented throughout the results section are means of three replicates. A one-way analysis of variance was performed. The Scheffe's multiple range test ($\alpha \leq 0.05$) was applied to the individual variables to compare means and to assess their difference significance. Asterisks were assigned to significantly different variables.

3. RESULTS

3.1. Growth of microorganisms

The growth of all microorganisms is presented in Figure 1. Regardless of the association, the growth of LAB exhibited a relatively similar pattern. During the first 12 d, the total LAB population increased from an initial population of around 10^6 cfu·g^{-1} to 5×10^8 cfu·g^{-1} followed by a plateau. When LAB were cultivated alone, a dramatic decrease of more than 2 log units in the LAB population was noticed after d 26.

B. linens showed noticeable development in the associations Dh, Kl and KlDh, reaching concentrations as high as 7×10^9 cfu·g^{-1} (association KlDh1 at d34) while no growth was observed when associated with *G. candidum* (Gc-type associations). When cultivated with *D. hansenii* (Dh-type associations), the presence of LAB promoted the growth of *B. linens* particularly between d 12 and d 26. Conversely, a negative effect on the growth of *B. linens* was observed in the presence of LAB when associated with only *K. lactis* (association type Kl). The difference in *B. linens* populations in the presence or in the absence of LAB was less pronounced in the association type KlDh though it remained significant ($P < 0.05$). Therefore, it seems that the negative effect of LAB on

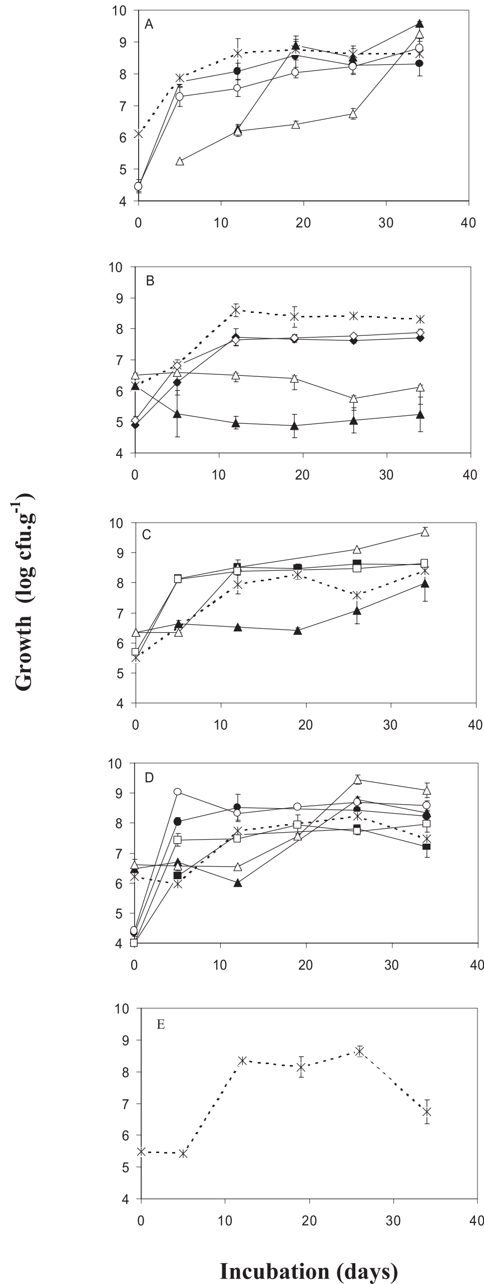

Figure 1. Changes in viable cell counts of microorganisms cultivated in association in model cheese medium. (A) Association type Dh; (B) association type Gc; (C) association type Kl; (D) association type KlDh; and (E) association type LAB. Open symbols: in the absence of LAB; closed symbols: in the presence of LAB; dotted lines: LAB. ● *D. hansenii,* ◆ *G. candidum,* ■ *K. lactis,* ▲ *B. linens*.

B. linens growth observed in the Kl association could be counterbalanced to a certain extent by the presence of *D. hansenii*, in the KlDh association.

Elevated yeast populations over 10^8 cfu·g^{-1} were generally reached by the end of the culture. Yeasts showed a rapid growth in the first few days of culture, and reached a plateau after 5 d of incubation. Maximum populations of around 10^8 cfu·g^{-1} for *K. lactis* and 10^9 cfu·g^{-1} for *D. hansenii* were obtained. The stationary growth phase was reached after 12 d for *G. candidum* (6×10^7 cfu·g^{-1}).

3.2. Lactose and lactate consumption and pH evolution

The concentrations of lactose and lactate and pH changes throughout the cultures are presented in Figure 2. None of the glucose, galactose or glycerol was detected in the cheese slurries. The initial concentration of lactate was 10 ± 1 g·kg^{-1} and the only sugar present initially was lactose (12 ± 1 g·kg^{-1}). When LAB were cultivated alone (Fig. 2E), an increase of 1.3 times in lactate concentration was observed between d 5 and d 19 concomitantly to the degradation of about 60% of the lactose present in the medium. The concentrations of these substrates stabilised thereafter until the end of the culture. A slight, but significant ($P < 0.05$) decrease in pH (0.4 pH units) was noticed between d 0 and d 34 (Fig. 2E). This clearly indicates that LAB were fully active at least during the first 20 d of incubation. When *G. candidum* was cultivated in association with *B. linens* (Gc1), lactose was not consumed throughout the culture while 77% of the initial lactate was depleted by d 41. The presence of LAB enabled total exhaustion of lactose in Gc-type associations; it was totally depleted between d 5 and d 19 in Gc2. Conversely, in the same cultures, lactate consumption was hardly influenced by the presence of LAB. 57% of this substrate was consumed from d 5 to d 26 in Gc2 versus 66% in Gc1. In Gc-type associations, pH modestly but steadily increased from an initial value of 4.6 to reach 5.2 after 34 d. In the association Dh1, lactose was totally depleted after 26 d, while only 5 d were necessary for its depletion in the presence of LAB (in the association Dh2) (Fig. 2A). The increase in pH followed the same pattern in both the Dh1 and Dh2 associations, the final pH being slightly higher than 7. Lactose catabolism was rapid in Kl-type associations. Indeed, all lactose initially present was degraded within 5 d of culture; however, lactate degradation was more gradual and was complete after 19–26 d of incubation. pH increased concomitantly to lactate consumption and reached 6.3 at the end of the culture (Fig. 2C). In KlDh-type associations, lactate was totally exhausted after 12 d of incubation. It followed the same pattern in the presence or in the absence of LAB. Lactose degradation was more rapid in the association KlDh1, than in KlDh2, during the first few days of culture. After 5 d of culture, 52% of the lactose was degraded in KlDh1 versus only 7% in KlDh2, which corresponds to a simultaneous increase in both *K. lactis* and *D. hansenii* populations in KlDh1. After 12 d, lactose was totally depleted in both KlDh1 and KlDh2. While the pH remained stable after 12 d in KlDh1, it kept increasing in KlDh2 and reached 7.1 at the end of the culture.

3.3. Volatile sulphur compounds

The results of GC-MS analyses of culture samples revealed that VSC were maximally produced at the end of the culture (d 41) for all associations, except for associations of type Kl in which it occurred at d 34 (Tab. II). Five major sulphur compounds were generally detected in cultures, namely methanethiol (MTL), dimethyl sulfide (DMS), dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS) and *S*-methyl thioacetate (MTA). In addition, scant quantities of S-methyl thiobutyrate (MTB) were also detected in some cultures. At d 0, only trace amounts of DMDS were found in the samples (data not shown). No sulphur aroma compounds were produced in Gc-type associations (where *B. linens* did not develop) apart from

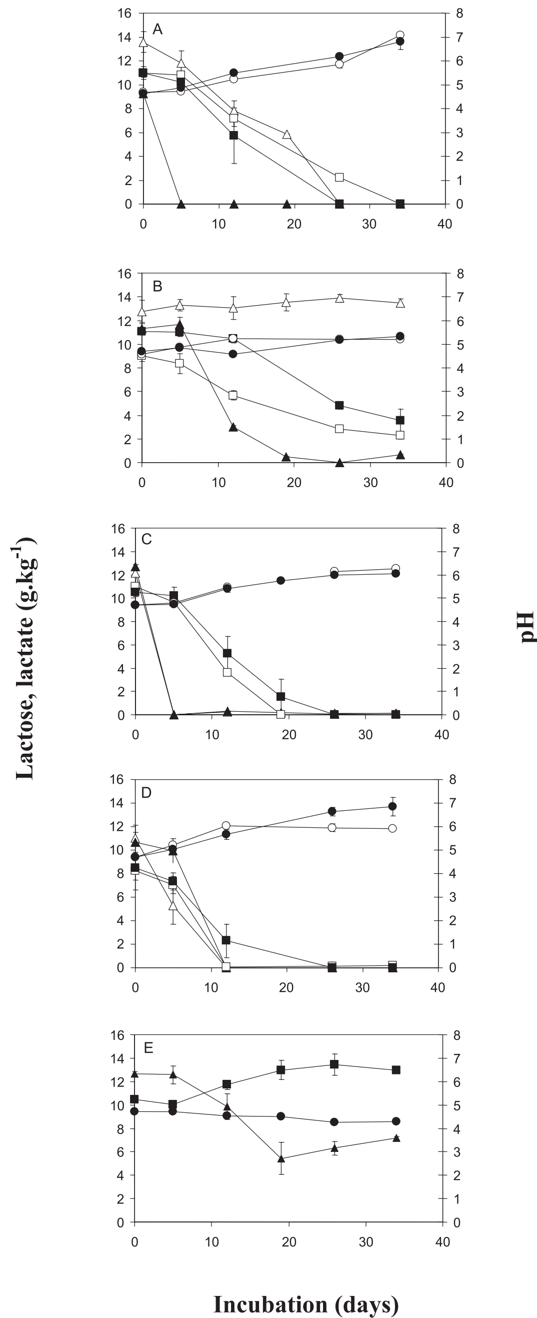

Figure 2. Evolution of pH, and residual concentrations of lactate and lactose during incubation of microorganisms in association in model cheese medium. (A) Association type Dh; (B) association type Gc; (C) association type Kl; (D) association type KlDh; and (E) association type LAB. Open symbols: in the absence of LAB; closed symbols: in the presence of LAB. ■ lactate, ▲ lactose, ● pH.

Table II. Maximal[#] concentrations ($\mu g \cdot kg^{-1}$) of volatile sulphur compounds produced by microbial associations[¥] grown on model cheese medium.

Compounds	MTL	DMS	MTA	DMDS	DMTS	MTB
Dh1	1522 ± 393[‡]	508 ± 78	tr*	2940 ± 269*	113 ± 7*	tr*
Dh2	2207 ± 396	501 ± 90	32 ± 12*	1619 ± 3*	81 ± 1*	13 ± 1*
Kl1	14 ± 2*	12 ± 2*	277 ± 47*	156 ± 10*	19 ± 3*	6 ± 0*
Kl2	nd*	6 ± 1*	18 ± 8*	6 ± 2*	tr*	nd*
KlDh1	nd*	nd*	nd	232 ± 59*	nd*	nd
KlDh2	3571 ± 3081*	353 ± 212*	nd	598 ± 181*	17 ± 7*	tr

MTL: methanethiol; **DMS**: dimethyl disulfide; **MTA**: *S*-methyl thioacetate; **DMDS**: dimethyl disulfide; **DMTS**: dimethyl trisulfide; **MTB**: *S*-methyl thiobutyrate. [#] Obtained after 41 and 34 d of culture for associations (Dh, and KlDh) and KL, respectively. [¥] Associations Gc and LAB, which produced low amounts of sulphur compounds, were not presented in this table. [‡] Results are means of three replicates. Within the same column and the same association group (Dh, Kl or KlDh), means bearing an asterisk are significantly different according to the Scheffe's multiple range test ($P \leq 0.05$) described in the Materials and Methods section. Tr: traces (< 5 ppb). nd: not detected.

small amounts of DMDS (10 ± 1 $\mu g \cdot kg^{-1}$ in Gc1). Significant amounts of DMDS and DMTS were found in the association Dh1, reaching 2940 ± 269 $g \cdot kg^{-1}$ and 113 ± 7 $g \cdot kg^{-1}$, respectively. The presence of LAB caused the amounts of DMDS and DMTS to drop significantly (< 0.05) in Dh-type associations, but no effect was observed for MTL and DMS. The opposite effect was observed for MTA and MTB, although both compounds were produced in limited amounts. For Kl-type associations, MTA and DMDS were the most abundant sulphur compounds produced. Kl1 is the association for which the highest amounts of MTA (Tab. II) were produced. MTA and DMDS concentrations were, respectively, 15 and 26 times lower in the presence of LAB in Kl-type associations. Likewise, for all the minor sulphur compounds (MTL, DMS, DMTS and MTB), the presence of LAB had a negative effect on VSC production in Kl-type cultures. Interestingly, the comparison between the Dh1, Kl1 and KlDh1 associations showed that the capacities of the microorganisms to produce VSC were not cumulative since a significant drop in DMDS concentrations and a nearly complete inhibition in other VSC production was noticed when and were associated (KlDh1 association) compared with when they were cultivated separately (Kl1 or Dh1 associations). However, the presence of LAB in the KlDh2 association partially restored the capacity to produce VSC, with the exception of -methyl thioesters.

3.4. Non-sulphur volatile compounds

Non-sulphur aroma compounds were also detected in cultures. These were mainly alcohols i.e. ethanol and 3-methyl butanol and their corresponding aldehydes, respectively, acetaldehyde and 3-methyl butanal (Tab. III). These compounds were generally produced during the early stages of culture. Their maximal concentration was reached after 12 d of incubation. However, for 3-methyl butanal, production was found to occur later in most cultures (d 34 for Kl and LAB; d 41 for Dh and KlDh).

Considerable amounts of ethyl acetate were also detected in Kl-type associations; its concentration reached its maximum level at d 12. Other esters were detected in such cultures, namely ethyl butyrate, ethyl propionate,

Table III. Maximal# concentrations ($\mu g \cdot kg^{-1}$) of the most frequently detected non-sulphur volatile compounds in the cultures of microbial associations grown on model cheese medium.

Compounds	Ethanol	3-Methyl butanol	Acetaldehyde	3-Methyl butanal	Ethyl acetate
Dh1	22452 ± 5667*‡	6286 ± 124*	888 ± 338*	118 ± 41	nd
Dh2	920 ± 439*	2978 ± 434*	97 ± 37*	99 ± 53	nd
Gc1	755 ± 166*	198 ± 93*	163 ± 48	288 ± 60*	nd
Gc2	4786 ± 2536*	992 ± 38*	218 ± 66	70 ± 0.3*	nd
Kl1	37418 ± 2896	10204 ± 533	574 ± 50	220 ± 89*	6478 ± 1095
Kl2	44357 ± 7822	12724 ± 1719	633 ± 47	16 ± 3*	5495 ± 322
KlDh1	7627 ± 1101*	16148 ± 7650	307 ± 2*	81 ± 38	nd
KlDh2	68854 ± 12570*	20863 ± 2552	nd*	44 ± 13	nd
LAB	1860 ± 369	nd	18 ± 6	13 ± 3	48 ± 6

Obtained after 12 d for all cultures and compounds except 3-methyl butanal for which the maximum level was measured after 12, 34 and 41 d of culture for the associations Gc, (Kl and LAB) and (Dh, and KlDh), respectively. ‡ Results are means of three replicates. Within the same column and the same association group (Dh, Gc, Kl or KlDh), means bearing an asterisk are significantly different according to the Scheffe's multiple range test ($P \leq 0.05$) described in the Materials and Methods section. nd: not detected.

3-methylbutyl acetate and propyl acetate. Their concentrations were not significantly affected by the presence of LAB. Maximal concentrations for these compounds, 562 ± 12 $\mu g \cdot kg^{-1}$ for isoamyl acetate, 636 ± 84 $\mu g \cdot kg^{-1}$ for ethyl propionate, 9 ± 1 $\mu g \cdot kg^{-1}$ for propyl acetate and 106 ± 2 $\mu g \cdot kg^{-1}$ for ethyl butyrate, were reached at d 12. Alcohols corresponding to the esters detected in Kl associations were also detected in high amounts in these cultures.

Again, the poorest aromatic compound-producing ability was obtained in LAB cultures. In these cultures, ethanol was by far the most abundant aroma compound. However, there is no clear-cut effect of adding LAB to yeast(s)-*B. linens* associations on non-sulphur aroma compound production. The effect was rather dependent on the yeast, or the yeast combination considered. The results of the statistical analysis showed that in the absence of LAB, no gain in non-sulphur aroma compounds was obtained by associating *K. lactis* and *D. hansenii* compared with when they were cultivated separately. For instance, significantly less ethanol was yielded in KlDh1 than in Dh1 and Kl1. For the KlDh2 association, the effect of LAB on the level of aroma compounds, when compared with KlDh1, differed from one compound to another.

4. DISCUSSION

All yeasts exhibited rapid growth in the various associations tested. This fast growth in the first 10–15 d of incubation in our cheese-based medium corresponds to the development pattern usually reported for *K. lactis* [24] and *D. hansenii* [25] in soft cheese. Conversely, *B. linens* growth occurred later – generally after 5 d of culture – and reached a plateau after d 10 or more, depending on the yeast it was associated With [24, 25]. Lactose and lactate also exhibited similar degradation patterns to those observed during cheese ripening [23–25]. The starter used in this experiment – which is extensively used in soft cheese-making – is mostly composed of homofermenters, which explains the high quantity of lactate detected [12, 13].

Our data gave evidence of possible interactions between microorganisms with respect

to growth and/or aroma compound production. For instance, it was found that the LAB population could be maintained for a longer period of time by the presence of yeasts and *B. linens.* Yeasts have been found to support the growth of LAB during cheese ripening by providing growth factors such as amino acids and vitamins [35]. We also found that the presence of LAB promoted the growth of *B. linens* when cultivated with *D. hansenii*. A growth-promoting effect of yeasts on *B. linens* has been reported in the literature. It occurs through the synthesis of pantothenic acid and other vitamins [31]. The positive effect of associating LAB with *D. hansenii* on the growth of *B. linens* could therefore be attributable to a cooperative production of such vitamins by these two microorganisms.

In Gc-type associations, pH remained too low for *B. linens* – which is acid-sensitive – to develop. Previous studies on *B. linens* have shown that this bacterium could not develop at a pH below 5.8 [10]. The low pH prevailing in post-manufacture curd, while tolerated by yeasts and LAB, is detrimental to the survival and/or growth of acid-sensitive species such as cheese surface bacteria [7].

Our results show that there is a substantial gain in VSC-producing abilities in yeast(s)-*B. linens* associations (with or without LAB) as compared with LAB cultivated alone. It was also found that, when produced, VSC synthesis coincided with maximal development of *B. linens*. Gc-type associations only produced trace amounts of VSC, although *G. candidum* was found to produce high concentrations of VSC when cultivated on synthetic medium with glycerol as carbon source [3]. It was also able to synthesise thioesters on cheese-based medium [6]. However, our experimental conditions are distinct from those used by Berger et al. [6] with regard to moisture content and aeration of the cultures. Moreover, in a previous study, we demonstrated that thioester formation from L-methionine by *G. candidum* is impaired when lactic acid is provided as the major carbon source [4]. Only low quantities of VSC were produced by LAB when cultivated alone. LAB and mainly *L. lactis*, the major species in our starter mixture, was found to possess two enzymes, cystathionine β-lyase and cystathionine γ-lyase, which were able to convert L-methionine to methanethiol [2, 16]. However, these enzymes exhibit low specificity towards L-methionine. Moreover, their activities were found to be inhibited by L-methionine and L-cysteine present in cheese [15], which could explain the low production of sulphur compounds by LAB. In Dh- and Kl-type associations, the presence of LAB caused the amounts of VSC – namely DMDS and DMTS – to drop. This indicated that LAB did not impair the degradation of L-methionine to MTL, but rather the auto-oxidation of this latter to DMDS and DMTS. In Dh-type associations, thioester production was increased in the presence of LAB. *S*-methyl thioester production by microorganisms is achieved via the acylation of MTL [20, 36]. This suggests that in the presence of LAB, thioesters formation from MTL and acetyl CoA or butyryl CoA is favoured, which reduces the availability of this thiol for oxidation to DMDS and DMTS. The involvement of LAB in the enhancement of MTA and MTB may be by indirectly supporting the growth of *B. linens* in Dh associations. Indeed, *B. linens* was found to be able to produce various *S*-methyl thioesters, among which were MTA and MTB [22]. When associated, *B. linens* and *K. lactis* produced the highest amounts of the thioester MTA. A previous study demonstrated that *K. lactis* required a MTL donor for MTA synthesis [3]. In Kl-type associations, *B. linens* probably acts as a MTL donor, which resulted in MTA synthesis. This gives an example of metabolic interactions between two microorganisms, one *K. lactis*, providing the acyl moiety, the other *B. linens*, providing MTL.

In all cultures, the overall concentration of alcohols was higher than that of aldehydes. Indeed, in most cheese varieties, aldehydes are transitory compounds; they do not accumulate since they are subsequently

reduced to alcohols [14]. Aldehydes are transformed to alcohols via the action of alcohol dehydrogenases or aldo-keto reductases. These enzymes are widely distributed in microorganisms, among which are *K. lactis* and *L. lactis* [5, 11, 17].

3-methyl butanal most probably resulted from the catabolism of the branched-chain amino acid L-leucine through a transamination, giving rise to an α-ketoacid followed by a decarboxylation [37] that leads to the corresponding aldehydes. Such a pathway, already reported in yeasts [37], is quite active in Dh, Kl and KlDh associations as indicated by the presence of significant amounts of 3-methyl butanol.

Significant amounts and a wide variety of esters are produced by Kl-type associations. The ability of *K. lactis* to produce esters – as compared with *D. hansenii* and *G. candidum* – has already been reported in pure cultures in synthetic Potato Dextrose Broth medium [3]. Esters are formed by the acylation of an alcohol by an acyl CoA. This reaction can be catalysed by alcohol acyltransferases [28]. In KlDh associations, no ester was produced. The aptitude of the Kl cultures for ester production therefore completely disappeared when associated with *D. hansenii*. Since alcohols are produced in high quantities in KlDh association, it can be assumed either that (i) the presence of *D. hansenii* inhibited the accumulation of acyl CoA in the Kl cultures, thus limiting ester formation or (ii) that esters are degraded by *D. hansenii* following their production by *K. lactis*. This yeast has been found to possess a significant esterolytic activity [30].

Despite the possible strain-dependence of some of our results and the limited number of microorganisms tested, this study showed that relatively complex interaction phenomena could occur during cheese ripening – e.g. antagonistic interactions, metabolic and enzymatic cooperation, and catabolic interactions. Therefore, this suggests that the understanding of interaction phenomena taking place within the cheese ecosystem is probably much more complex, and would require the use of more adequate molecular approaches to access the aptitude to develop functionalities – amino acid catabolism, catabolic activities and antimicrobial activities – within the microbial community of the ecosystem during ripening.

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