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Original article

Interactions between microorganisms in a simple ecosystem: yogurt bacteria as a study model

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Abstract – Yogurt is a simple ecosystem whose successful manufacture relies on interactions between two thermophilic lactic acid bacteria, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb. bulgaricus*). The present work studied the impact of co-culturing *S. thermophilus* with *Lb. bulgaricus* on bacterial growth in milk. To achieve this, we considered both sides of the bacterial association, by characterising proteolysis and quantifying formic acid in pure and mixed cultures of *S. thermophilus* and *Lb. bulgaricus*. In pure cultures, *S. thermophilus* CNRZ385 exhibited better growth than the two *Lb. bulgaricus* strains (1038 and CNRZ397). The *S. thermophilus/Lb. bulgaricus* association was positive for *S. thermophilus* growth with strain 1038, contrary to the association with strain 397. These effects were correlated with the different proteolytic capacities of the two *Lb. bulgaricus* strains. In contrast, the bacterial association had no significant effect on *Lb. bulgaricus* growth, possibly because of an insufficient production of formic acid by the *S. thermophilus* strain. We also showed that the *S. thermophilus/Lb. bulgarius* association affected the production of volatile molecules involved in flavour development.

***Streptococcus thermophilus* / *Lactobacillus delbrueckii* subsp. *bulgaricus* / milk co-cultures / proteolysis / formic acid / flavour**

Résumé – Interactions entre microorganismes dans un écosystème simple : les bactéries du yaourt comme modèle d'étude. Le yaourt constitue un écosystème simple dont la fabrication repose sur les interactions prenant place entre les deux espèces de bactéries lactiques *Streptococcus thermophilus* et *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb. bulgaricus).* Au cours de ce travail, nous avons étudié l'impact de la co-culture de *S. thermophilus* avec *Lb. bulgaricus* sur la croissance bactérienne dans le lait. Pour cela, nous nous sommes intéressés aux deux aspects de l'association, en caractérisant la protéolyse et en quantifiant l'acide formique dans des cultures pures et mixtes de *S. thermophilus* et *Lb. bulgaricus*. En culture pure, *S. thermophilus* CNRZ385 se développait mieux que les deux souches de *Lb. bulgaricus* (1038 et CNRZ397). L'association *S. thermophilus/Lb. bulgaricus* avait un effet bénéfique sur la croissance de *S. thermophilus* avec la souche 1038 contrairement à l'association avec la souche CNRZ397. Ces effets étaient corrélés à des capacités protéolytiques différentes des deux souches de *Lb. bulgaricus*. Par ailleurs, l'association bactérienne n'avait pas d'effet significatif sur la croissance de *Lb. bulgaricus* possiblement en raison d'une production insuffisante d'acide formique par la souche de *S. thermophilus*. Nous avons également montré que l'association *S. thermophilus/Lb. bulgaricus* affectait la production de molecules volatiles impliquées dans le développement de la flaveur.

***Streptococcus thermophilus* / *Lactobacillus delbrueckii* subsp. *bulgaricus* / co-culture en lait / protéolyse / acide formique / flaveur**

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1. INTRODUCTION

Lactic acid bacteria (LAB) are widely employed in the dairy industry for their milk acidification and flavour development properties. The thermophilic LAB, *S. thermophilus* and *Lb. bulgaricus*, are currently used as starters for the production of yogurt and Italian cheeses. For these applications, the ability for rapid growth of these bacteria is crucial to enabling intense and rapid acidification of milk.

LAB are fastidious micro-organisms and their growth is often restricted in milk because of its paucity in essential nutriments. Thus the success of milk fermentation relies most often upon the synergy between *S. thermophilus* and *Lb. bulgaricus*. Because both bacteria are able to grow alone in milk, this indirect positive interaction is called proto-cooperation [9]. This positive relationship often has a beneficial effect on bacterial growth and on the production of lactic acid and aroma compounds. Some of the effectors of this association have been identified and result from the metabolic activities of the two bacterial partners. Indeed, *S. thermophilus* produces pyruvic acid, formic acid and CO_2 (for reviews see [28, 33]) which stimulate the growth of *Lb. bulgaricus.* In turn, *Lb. bulgaricus* produces peptides and amino acids that stimulate the growth of *S. thermophilus* [1, 2, 12, 22, 23], because *S. thermophilus* is only weakly proteolytic when compared with *Lactobacillus*. On the other hand, although the effect of associating these LAB species is often positive, it can also be neutral or detrimental depending on the bacterial strains employed, the type of milk, the method used to heat the milk and the temperature of milk fermentation.

We have previously shown that cell-wall proteinases from *S. thermophilus* and *Lb. bulgaricus* do not play the same role in mixed cultures [4]. In the present study, and working with the same strains, we considered the effect of the addition of *Lb. bulgaricus* on *S. thermophilus* growth, using one strain of *S. thermophilus* and two strains of *Lb. bulgaricus* with different growth profiles in milk. The originality of this work resides in the simultaneous study of several essential factors involved in the association (proteolysis and formic acid production) and thus consideration of both sides of the association (the effect of each partner on the growth of the other). We also considered the effect of co-culture on the production of flavour compounds (volatile molecules and peptides).

2. MATERIALS AND METHODS

2.1. Bacterial strains, culture conditions and bacterial counting

The bacterial strains studied were: *S. thermophilus* CNRZ 385, *Lb. bulgaricus* CNRZ 397 and *Lb. bulgaricus* 1038 (a streptomycin-resistant mutant of strain CNRZ 208 = ATCC 11842) (Van de Guchte, personal communication) (for the reasons for these choices, see [4]).

S. thermophilus and *Lb. bulgaricus* strains were grown in three different media: reconstituted skim milk (Nilac Low Heat Milk powder, NIZO, Ede, the Netherlands) heated for 10 min at 95 °C, M17 medium [30] supplemented with 10 $g \cdot L^{-1}$ lactose, and MRS medium [6] supplemented with 20 $g \cdot L^{-1}$ lactose, acidified at pH 5.2, and further supplemented with streptomycin (2 $mg \cdot mL^{-1}$) when required.

Stock cultures of all *S. thermophilus* and *Lb. bulgaricus* strains were prepared as previously described [4].

Mixed cultures of *S. thermophilus* and *Lb. bulgaricus* strains were performed at 42 °C by inoculating skim milk with 5×10^6 $UFC \cdot mL^{-1}$ of the stock cultures of each strain. We chose a *S. thermophilus*:*Lb. bulgaricus* ratio of 1:1 because this had previously demonstrated satisfactory results concerning the texture and aroma development of yogurt [5, 15, 21]. Every 20 min, the pH of the culture was determined and bacteria were counted as described above. The total bacterial populations were estimated by

adding the data of counts for each bacterial species on a specific medium to other counts, as shown above. All cultures were repeated in quadruplicate and the final bacterial counts and pH measurements were means of the four values obtained.

2.2. Quantification of proteolysis

Proteolysis was characterised by quantifying free amino acids and peptides and identifying the peptides in milk supernatant cultures. The values corresponded to the means of the four values obtained during four independent cultures. Values for milk controls were subtracted from all quantifications obtained for the different samples. Negative values and positive values thus indicated consumption or production by comparison with milk, respectively.

The supernatants were recovered after precipitation with TCA (1.2%) by centrifugation (4500 × *g* for 20 min at 4 °C) of the bacterial milk cultures after 2, 3, 4 and 5 h of incubation in the case of pure cultures and after 5 h of incubation for mixed cultures. Twenty mL of the supernatants were concentrated by ultrafiltration through a 30 kg·mol^{-1} concentrator (Centriplus, Millipore, Bedford, USA) by centrifugation at 4500 × *g* for 1 h. Two to four mL were used for the quantification of proteolysis.

2.2.1. Quantification of free amino acids

The free amino acid content in concentrated supernatants was determined using an amino acid analyser (LC3000; Biotronik, Maintal, Germany). The levels of different amino acids were calculated by comparison with a standard mixture (amino acid standard H, Pierce, Rockford, USA) and expressed in nmol·g^{-1} for 10^7 UFC·mL^{-1}. In order to quantify total amino acids, the sum of all amino acid concentrations was calculated.

2.2.2. Quantification of peptides

One millilitre of the aforementioned concentrated supernatants was dried with a Speed-Vac system to reach a volume of <500 µL; when necessary, the volume was adjusted to 500 µL. Peptides were separated using RP-HPLC by the injection of 200 µL of each sample onto a C18 column (hypersil PEP100, 250 mm × 4.6 mm, 5 µm; Thermo Hypersil-Keystone, Bellefonte, USA). A trifluoroacetic acid (TFA)/acetonitrile (ACN) solvent system was used: solvent A = 0.115% TFA, and solvent B = 0.1% TFA/60% ACN. Elution was carried out using the following gradient: 100% solvent A for 10 min, 0–30% solvent B for 40 min, 95% solvent B for 5 min and 100% solvent A for 10 min, at a flow rate of 1 mL·min^{-1} and recording at 214 nm.

Peptide peaks were quantified by calculating their surface area, and expressed as a surface area of arbitrary units for 10^7 UFC·mL^{-1}. The quantification of total peptide peaks was achieved by adding together the surface areas of all peaks between peak 13 and peak 22, because peaks with lower retention times were not formed of peptides.

2.2.3. Peptide identification

The different peptides present in peaks were identified by their molecular weight using a mass spectrometer (Maldi-Tof, Voyager-DE STR, PerSeptive Biosystems, Houston, USA) and their identification confirmed using a pulse-liquid sequencer (model 477, Applied Biosystem, Forster City, USA).

2.3. Analysis of volatile compounds by solid-phase microextraction (SPME) and GC-MS

Volatile compound analyses were performed according to the method described by Kieronczyk et al. [14], with the following modifications. Five mL of milk culture were acidified to pH 1.0 and the column length was 60.0 m. Helium flow was maintained at 0.5 mL·min^{-1} for 10 min, and was then increased at a rate of 1 mL·min^{-1} to 1.4 mL·min^{-1} and kept constant for the

Table I. Final populations ($\times 10^8$ UFC·mL^{-1}) and pH of pure cultures (after 5 h of fermentation) and mixed cultures (after 4 h 40 min and 5 h 40 min for mixed cultures 385/1038 and 385/397, respectively). Values represent mean values from four independent experiments and standard deviations are indicated in brackets. Milk control values were subtracted from all values obtained for the different samples. Negative values and positive values thus indicate consumption or production, respectively, by comparison with milk. *St.*: *Streptococcus thermophilus*; and *Lb.*: *Lactobacillus delbrueckii* subsp. *bulgaricus*.

Cultures	Final pH	Final total populations	Final populations of *S. thermophilus*	Final populations of *Lb. bulgaricus*
St. 385	4.71 (±0.11)	–	7.53 (±0.53)	–
Lb. 397	5.14 (±0.051)	–	–	0.96 (±0.25)
Lb. 1038	5.49 (±0.053)	–	–	0.52 (±0.12)
St. 385/*Lb.* 397	4.80 (±0.18)	7.05 (±0.24)	6.10 (±0.8)	0.95 (±0.24)
St. 385/*Lb.* 1038	4.73 (±0.09)	13.90 (±2.26)	13.10 (±2.1)	0.79 (±0.13)

remainder of the time. The oven temperature was held at 40 °C for 7 min, increased to 150 °C by increments of 10 °C/min and then to 240 °C by the same increments. Separated compounds were detected and identified by their retention times and their mass spectra, compared with those in the NIST98 library (John Wiley & Sons Inc., New York, USA). Their concentration was calculated by the range calibration of specific ions: 29 for acetaldehyde, 86 for diacetyl (2,3 butanedione), 100 for 2,3 pentanedione, 94 for dimethyl disulfide (DMDS), 88 for acetoin (3 hydroxy 2 butanone) and 106 for benzaldehyde. Milk control values were subtracted from each of the quantities obtained for different samples (mean values from four repeated experiments).

2.4. Formic acid assay

Formic acid levels were determined in culture supernatants after 2, 3, 4 and 5 h of fermentation. Supernatants were concentrated using a Speed-Vac system, to between 5 and 12.5 times, using the Boehringer kit (Boehringer, Mannheim, Germany) and formic acid was measured according to the manufacturer's recommendations. The concentrations of formic acid were expressed as mg·L^{-1}, and the values of milk controls were subtracted from all the quantities obtained for the different samples (mean values from four repeated experiments).

3. RESULTS AND DISCUSSION

3.1. Effect of the *S. thermophilus* /*Lb. bulgaricus* association on bacterial growth in milk

3.1.1. Characterisation of growth in pure and mixed cultures

In pure cultures, the final, viable populations of *S. thermophilus* were larger than those obtained with the two *Lb. bulgaricus* strains (×7.8–14), inducing a better acidification of milk (lower pH) (Tab. I).

In *S. thermophilus* /*Lb. bulgaricus* mixed cultures, the growth of *S. thermophilus* could be divided into three phases, corresponding to the three phases of milk acidification (Fig. 1). During the first phase, *S. thermophilus* 385 grew exponentially, while the growth of *Lb. bulgaricus* was "suppressed", thus confirming that *S. thermophilus* was mainly responsible for this first acidification phase, as has previously been observed [20, 29]. The second phase corresponded to a pause in *S. thermophilus* growth, and concomitantly to a stabilisation of the pH, whereas *Lb. bulgaricus* growth

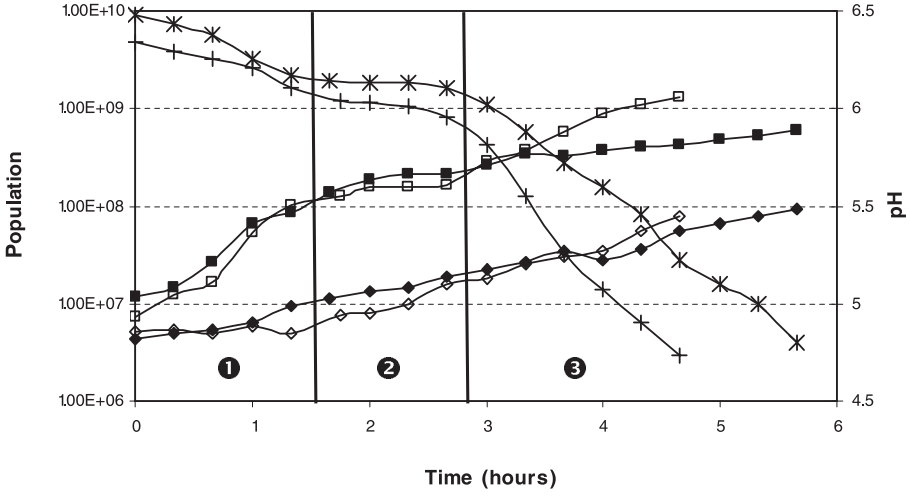

Figure 1. Growth and acidification kinetics of *S. thermophilus* (385) and *Lb. bulgaricus* (397 and 1038) in mixed cultures. ×, pH of 385/397 mixed culture; +, pH of 385/1038 mixed culture; ■, population of strain 385 in 385/397 mixed cultures; □, population of strain 385 in 385/1038 mixed cultures; ◆, population of strain 397 in 385/397 mixed cultures; and ◇, population of strain 1038 in 385/1038 mixed cultures. ❶, ❷, and ❸: phase number. Populations are expressed in UFC·mL^{-1} and represent mean values from four independent experiments.

was initiated. During the final phase, *Lb. bulgaricus* pursued its growth and *S. thermophilus* began a second growth phase. This "diauxic" growth of *S. thermophilus* in mixed cultures in milk has previously been reported in pure cultures [17].

3.1.2. Effect as a function of LAB species

Our results indicated that the association was more beneficial to *S. thermophilus* than to *Lb. bulgaricus*, because in mixed cultures, *Lactobacillus* populations did not differ significantly from those found in pure cultures (Tab. I).

This could possibly be attributed to the higher nutritional requirements of *Lactobacillus* by comparison with *S. thermophilus* [7, 17], particularly with respect to amino acids and formic acid (see below). On the other hand, *S. thermophilus* and *Lb. bulgaricus* may compete for the essential nutrients that are limiting in milk. Because *S. thermophilus* 385 grows faster in milk than *Lb. bulgaricus* 397 and 1038 (data not shown; and [18] for other strains), *S. thermophilus* may have a greater competitive advantage, as has previously been proposed for other bacteria [9]. Furthermore, *S. thermophilus* may have been advantaged by the culture temperature (in this case, 42 °C) which was closer to its optimal growth temperature (40–45 °C) than to the optimal temperature of *Lb. bulgaricus* (45–50 °C). Indeed, Tayeb et al. [29] previously reported a lower biomass production of *Lb. bulgaricus* when mixed cultures with *S. thermophilus* were performed at 37 °C compared with 42 °C; this was correlated to a reduction in growth rate.

3.1.3. Effect as a function of Lb. bulgaricus strains

With one *Lactobacillus* strain (397), the effect of the co-culture was slightly negative in terms of the final total populations (Tab. I). The populations obtained in mixed

Table II. Quantification of global proteolysis (total free amino acids and peptides) of proline (Pro), lysine (Lys) and valine (Val), and quantification of volatile molecules in pure and mixed cultures of *S. thermophilus* and *Lb. bulgaricus*. Total amino acid levels expressed in nmol·g^{-1} for 10^7 UFC·mL^{-1}, peptide levels in arbitrary units 10^7 UFC·mL^{-1}, Pro, Lys and Val levels in mg·L^{-1} for 10^7 UFC·mL^{-1}, acetaldehyde and acetoin in mg·mL^{-1} (ppm), and diacetyl in ng·L^{-1} (ppb). Values are the means obtained during four independent experiments and standard deviations are shown in brackets. Milk controls were subtracted from all values obtained for the different samples. Negative values and positive values thus indicate consumption or production, respectively, by comparison with milk. *St.*: *Streptococcus thermophilus*; and *Lb.*: *Lactobacillus delbrueckii* subsp. *bulgaricus*. A.A.: amino acid.

		Cultures				
		St. 385	*Lb.* 397	*Lb.* 1038	*St.* 385/*Lb.* 397	*St.* 385/*Lb.* 1038
Global proteolysis	Free A.A.	–5 (±0.8)	153 (±29)	263 (±22)	18 (±3)	–2 (±0.35)
	Peptides	7 (±3.3)	177 (±31)	259 (±52)	33 (±7.7)	10 (±1.3)
Free A.A.	Pro	0.2 (±0.03)	3.54 (±0.003)	6.09 (±0.56)	0.69 (±0.08)	0.24 (±0.035)
	Lys	–0.03 (±0.02)	2.6 (±0.04)	4.67 (±0.33)	–0.54 (±0.03)	–0.36 (±0.021)
	Val	–0.02 (±0.05)	2.15 (±0.03)	3.06 (±0.22)	0.18 (±0.04)	0.01 (±0.04)
Volatile molecules	Acetaldehyde	1022 (±105)	371 (±119)	303 ±32)	947 (±219)	564 (±146)
	Acetoin	85 (±7.6)	16 (±2.5)	2 (±0.08)	21 (±8.9)	46 (±8.8)
	Diacetyl	210 (±14)	46.7 (±15.2)	3.7 (±0.8)	0.4 (±0.57)	0.1 (±0.04)

cultures were in fact smaller (7.05×10^8 UFC·mL^{-1}) than the sum of the populations obtained in pure cultures (8.5×10^8 UFC·mL^{-1}) and were also achieved more slowly (5 h 40 min versus 4 h 40 min). In contrast, association with *Lactobacillus* strain 1038 was positive because of a two-fold increase in *S. thermophilus* populations in mixed cultures when compared with pure cultures (Tab. I), because of the better second growth phase of *S. thermophilus* (Fig. 1).

3.2. Proteolysis in pure and mixed cultures of *S. thermophilus* and *Lb. bulgaricus* in milk

We quantified free amino acids and peptides in the culture supernatants and determined their composition. These measurements reflected the balance between the production and consumption of these products by the bacteria. It is noteworthy that peptide identification was often complicated and sometimes impossible because of the complexity of the peaks, which contained up to 10 different peptides.

3.2.1. Proteolysis in pure cultures

Cultures containing the two strains of *Lb. bulgaricus* exhibited higher levels of both free amino acids and peptides when compared with *S. thermophilus* cultures (Tab. II). These stronger proteolytic activities of *Lactobacillus* compared with *S. thermophilus* were in line with the findings of previous studies where proteolysis had been evaluated globally by measuring tyrosine release [8, 21, 24].

Lb. bulgaricus strains produced almost all the amino acids (apart from Glu, Asn, and Cys), and proline, lysine and valine represented 42–43% of the total amino acids produced (Tab. II). In contrast, *S. thermophilus* cultures produced only proline

Table III. Identification and quantification of peptides in the pure and mixed cultures of *S. thermophilus* (385) and *Lb. bulgaricus* (397 and 1038). Values are the means obtained from four independent experiments and standard deviations are shown in brackets; they are expressed in arbitrary units 10^7 UFC·mL^{-1}. n.i.: not identified. *St.*: *Streptococcus thermophilus*; and *Lb.*: *Lactobacillus delbrueckii* subsp. *bulgaricus.*

Peak number	Sequence	Peak surface area in culture				
		St. 385	*Lb.* 397	*Lb.*1038	*St.* 385/*Lb.* 397	*St.* 385/*Lb.* 1038
13	α_{s1} 1-8 RPKHPIKH	18.5 (±2)	784 (±51)	1180 (±65)	247 (±20)	75 (±1)
	α_{s1} 1-9 RPKHPIKHQ					
14	α_{s1} 115-124 SAEERLHSMK	0	334 (±78)	705 (±130)	48 (±19)	25 (±4)
	β 97-106 KVKEAMAPKH					
15	β 97-107 KVKEAMAPKHK	0	0	0	15 (±1)	0
16	β 29-34 KIEKFQ	-	–12 (±3)	–49.6 (±13)	-	–2 (±1)
	β 29-35 KIEKFQS					
16A	n.i.	–1.2 (±0.2)	–15 (±4)	–50 (±23)	–7.4 (±0)	–2.2 (±0)
16B	α_{s1} 1-13 RPKHPIKHQGLPQ	6.4 (±2)	266 (±31)	171 (±48)	12.3 (±5)	2.1 (±0.8)
	κ 155-169 SPPEINTVQVTSTAV					
16C	n.i.	5.7 (±1)	0	0	0	0
17	β 7-27 NVPGEIVESLSSSEESITRIN	0	116 (±52.6)	0	15.9 (±1)	1.4 (±2)
	β 47-56 DKIHPKAQTQ					
	β 144-154 MHQPHQPLPPT					
	κ 6-17 EQPIRCEKDERF					
18	n.i.	11.8 (±1)	78.5 (±7)	57.9 (±9)	5.9 (± 0)	0.9 (±0.1)
19	n.i.	-	137 (±24)	25.6 (±2.7)	–6.8 (±3.2)	1.8 (±0.7)
21	α_{s1} 1-17 RPKHPIKHQGLPQEVLN	29.4 (±5.5)	0	0	0	1.7 (±0.5)
22	α_{s1} 193-199 KTTMPLN	0	55.8 (±7.8)	0	0	0
	α_{s1} 191-199 SEKTTMPLN					

(Tab. II), and most of the other amino acids were consumed. Furthermore, peptide peaks 13, 16B and 18 (Tab. III) were present at higher levels in cultures involving *Lactobacillus* strains than in those with *S. thermophilus* cultures.

However, peaks 14, 17 and 22 were specific to *Lactobacillus* strains, while peaks 16C and 21 were specific to *Streptococcus* (Tab. III).

The levels of amino acids (total and Pro, Lys and Val) and peptides were higher with strain 1038 than with strain 397 (Tab. II); most of the peptide peaks were present in both *Lactobacillus* cultures but in different quantities (Tab. III). Furthermore, peaks 17 and 22 were specific to cultures involving strain 397.

3.2.2. Proteolysis in mixed cultures

Quantities of free amino acids and peptides were much lower in mixed cultures than in pure cultures (Tab. II). This decrease probably resulted from consumption of these proteolytic products by *S. thermophilus,* as the latter broadly dominated in mixed cultures (Tab. I). Furthermore, this difference was more marked with strain 1038 of

Lb. bulgaricus than with strain 397, probably to some extent because larger populations of *S. thermophilus* were present in mixed cultures with strain 1038 than with those containing strain 397 (Tab. I).

Most *S. thermophilus* strains require Met, His, Pro and Glu [16] to ensure their growth; the first three were produced as free amino acids by *Lb. bulgaricus* strains 1038 and 397; glutamic acid (which is not produced by these strains) was possibly supplied in the form of peptides, since caseins are rich in glutamic acid and most of the peptides we identified contained Glu (Tab. III). In addition, valine (which is very efficient at stimulating *S. thermophilus)* (reviewed by Tamime and Robinson, [28]) was one of the three more abundant amino acids produced by our strains of *Lb. bulgaricus*. In contrast, the peptides and amino acids produced by the two *Lactobacillus* strains in pure cultures were probably assimilated to a great extent by *S. thermophilus* in mixed cultures.

The present results therefore suggest that the two *Lactobacillus* strains probably quantitatively and qualitatively sustain *S. thermophilus* growth through their proteolytic activities. These activities resulted, at least to some extent, from the cell-wall proteinase, PrtB, which plays an essential role in the growth of *S. thermophilus* 385 in mixed cultures in milk during the second growth phase [4].

In mixed cultures, amino acid and peptide production and consumption differed, depending on the strain of *Lb. bulgaricus* involved. Using strain 1038, amino acid and peptide consumption was globally higher than with strain 397 (Tabs. II and III), which was correlated with the larger final populations of *S. thermophilus* obtained (Tab. I). In addition, peaks 15 and 21 (Tab. III) were only present in mixed cultures involving strains 397 and 1038, respectively.

All these differences in the proteolytic capacity of *Lactobacillus* strains may contribute to the growth differences of *S. thermophilus* in mixed cultures and therefore explain the stimulation of *S. thermophilus* by one *Lactobacillus* strain but not by the other.

Furthermore, the lower levels of peptides determined in mixed cultures than in pure cultures may have a beneficial impact on yogurt flavour, as some peptides cause bitterness in yogurts [11]. During this study, this may have been the case with respect to peptides α_{s1} 1-8, α_{s1} 1-9, and α_{s1} 1-13 from peaks 13 and 16B (Tab. III) which, according to Ney's rule [19], have a potential for bitterness because of their hydrophobicity. On the other hand, the differences in peptide yields between mixed cultures may affect the health benefits of yogurt in terms of its content of bioactive peptides. Thus peptides α_{s1} 1-9, and α_{s1} 1-13, which exert an angiotensin-converting inhibitory effect [25] and thus may lower hypertension, were present at different levels, depending on the *Lactobacillus* strain (Tab. III).

3.3. Production of formic acid in pure and mixed cultures of *S. thermophilus* and *Lb. bulgaricus* in milk

As a precursor of purine, formic acid is required for the growth of *Lb. bulgaricus* [10, 27], probably because pyruvate-formate lyase is absent in *Lb. bulgaricus* [26]. It was therefore quantified in the different cultures, as it is also known to be produced by *S. thermophilus* [10, 20, 31].

Among the three strains tested, only *S. thermophilus* produced formic acid, whereas the levels of formic acid produced by the two *Lactobacillus* strains and milk did not differ significantly (0.08–0.3 $mg \cdot L^{-1}$). *S. thermophilus* started to produce formic acid to a significant extent after 4 h of fermentation (2.24 $mg \cdot L^{-1}$), the level reaching 4.03 $mg \cdot L^{-1}$ after 5 h.

In mixed cultures, the levels of formic acid fell, more markedly when strain 397 was involved (0.56 $mg \cdot L^{-1}$) than with

strain 1038 (1.18 mg·L^{-1}). These decreases in mixed cultures probably resulted from the consumption of formic acid by *Lb. bulgaricus*.

Formic acid is produced at a late stage by *S. thermophilus* 385 and in small quantities when compared with other strains (reviewed by Tamime and Robinson, [28]); this may have contributed to the lack of stimulation exerted by the co-culture on the growth of *Lb. bulgaricus*. Furthermore, the milk was not heated to high temperatures during this study (but at 95 °C for 10 min, as is applied during the manufacture of yogurt) and contained little formic acid (0.3 mg·L^{-1}) when compared with milks heated to high temperatures (≃ 120 °C) which contain 5–220 mg·L^{-1} formic acid [13, 20] and are thus more conducive to *Lb. bulgaricus* growth.

3.4. Effect of the *S. thermophilus/Lb. bulgaricus* association on the production of volatile molecules in milk

In pure cultures, *S. thermophilus* produced more acetaldehyde, acetoin and diacetyl than *Lactobacillus* strains (Tab. II), which contrasts with most reports on acetaldehyde [8, 28, 32]. This may have resulted from the larger final populations of *S. thermophilus* than of *Lb. bulgaricus* (Tab. I).

In mixed cultures, acetaldehyde and acetoin were more abundant than in *Lb. bulgaricus* pure cultures, whereas diacetyl concentrations were markedly lower (Tab. II).

It is noteworthy that levels of these molecules and other carbonyl compounds are not crucial as such to yogurt flavour, but the ratios between them endow yogurt with its typical and full flavour, as has been emphasised by several authors [3, 21].

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