Effect of *Lactococcus garvieae*, *Lactococcus lactis* and *Enterococcus faecalis* on the behaviour of *Staphylococcus aureus* in microfiltered milk

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Abstract

The effect of four strains of *Lactococcus garvieae*, three strains of *Lactococcus lactis* and one strain of *Enterococcus faecalis* on *Staphylococcus aureus* SA15 growth in microfiltered milk was evaluated. *Lactococcus* and *Enterococcus* strains were co-cultured with *S. aureus* in microfiltered milk and in medium buffered at pH 6.8. All *Lactococcus* and *Enterococcus* strains were able to inhibit *S. aureus* growth after 6 h of incubation. Inhibition by *L. lactis* and *E. faecalis* strains could be partially attributed to the decrease in pH below 6.0 as it has been observed in medium buffered at pH 6.8. *L. garvieae* strains were the most effective to inhibit *S. aureus* growth without acidification. Inhibition of *S. aureus* could not be attributed neither to production of lactate, acetate or nor to antistaphylococcal substance. Amino acids competition was not involved in the inhibition by *L. garvieae* as addition of valine, isoleucine, threonine, methionine and phenylalanine did not suppress the inhibition of *S. aureus*.

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1. Introduction

Safety of raw milk cheeses implies that the development of food borne pathogens be controlled, in compliance with European regulations. *Staphylococcus aureus* can be involved in *Toxi-Infections Alimentaires Collectives* (TIACs) involving milk and dairy products (De Buyser et al., 2001). In 2006, the European legislation laid down the obligation to determine the presence of enterotoxins in cheese if the level of *S. aureus* is over 10^5 CFU g⁻¹. Bacterial contamination of milk has been largely reduced by identifying the potential sources of *S. aureus*, leading to controlling mastitis in herds and applying hygienic practices (Sommerhauser et al., 2003; Chassagne et al., 2005). Nevertheless, the presence of bacterial species in milk cannot be avoided.

In spite of a low count in milk, its count during the first hours of cheesemaking may exceed that authorized by the legislation (Sesques, 1994; Meyrand et al., 1998; Lamprell, 2003). Therefore, *S. aureus* must be inhibited at a very early stage of the cheesemaking process. This can be achieved by strong acidification in cheese as *S. aureus* growth can stop in 6 h in non-cooked semi-hard cheeses with a pH value below 5.6 (Delbes et al., 2006). However, strong acidification may limit the growth and activities of other bacterial populations involved in the development of the sensorial properties of certain raw milk cheeses (Feeney et al., 2002; Callon et al., 2005).

S. aureus inhibition in culture media could also be achieved through bacteriocin production by *Lactobacillus plantarum* (Stecchini et al., 1991; Todorov et al., 1999; Hernandez et al., 2005) and *Lactobacillus delbrueckii* (Miteva et al., 1998). Nevertheless, the effectiveness of these bacteriocins in cheese is not well documented, even if inhibition by nisin produced by *Lactococcus lactis*, or

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enterocin produced by *Enterococcus faecalis* has been observed (Zottola et al., 1994; Hamama et al., 2002; Giraffa, 2003). The combination of high pressure treatments and bacteriocin-producing lactic acid bacteria (LAB) inhibited *S. aureus* growth in cheese (Arques et al., 2005). Such treatments may be difficult to apply in some cheese technologies, particularly for farm cheese production.

In purpose to use biopreservation for inhibiting *S. aureus* growth in raw-milk farm production, it is important to select starter cultures. Therefore, the aim of this study was to evaluate the effect of different strains of LAB commonly found in raw milk cheeses and belonging to *L. lactis*, *E. faecalis* and *Lactococcus garvieae* on *S. aureus* growth in microfiltered milk.

2. Material and methods

2.1. Bacterial strains and culture conditions

2.1.1. Bacterial strains

One strain of *S. aureus* SA15 isolated from raw milk was selected for this study. Four strains of *L. garvieae* (N201, Tan 1, Tan 2, and 1204), three of *L. lactis* subsp. *lactis* (N650, N658 and N688), and one strain of *E. faecalis* (N516) isolated from raw milk cheeses were selected to tested in co-cultured with *S. aureus* in microfiltered milk. The strains of LAB were identified by 16S ribosomal RNA gene sequencing as described by Callon et al. (2004) and their genomic diversity was assessed by REP-PCR (Jersek et al., 1999).

2.1.2. Inoculum preparation

2.1.2.1. Preparation of *S. aureus* inoculum. For the co-culture in microfiltered milk, *S. aureus* was pre-cultured in BHI (Biokar Diagnostic, Pantin, France) broth modified by adding 12 g l⁻¹ of skimmed milk powder (Lactalis Industry, Bourgbarré, France) to pre-adapt the strain to its future incubation in milk. For the co-culture in TS buffered medium, *S. aureus* was pre-cultured in BHI (Biokar Diagnostic, Pantin, France). The cultures were incubated at 37 °C during 18 h. Then the cultures were centrifuged to 9600 × *g* for 15 min at 4 °C (SIGMA 3MK, Bioblock Scientific, Illkirch, France) and the cell pellet was washed once in Ringer solution (Biokar Diagnostic, Pantin, France) before inoculation into commercial microfiltered milk or TS buffered medium at level 150 CFU ml⁻¹.

2.1.2.2. Preparation of LAB inoculum. For the co-culture of LAB and *S. aureus* in microfiltered milk, each strain was pre-cultured from a frozen cell suspension in 9 ml of skimmed milk (120 g of skimmed milk powder l⁻¹ (Lactalis Industry) distilled water were sterilized at 115 °C for 15 min. After 18 h of incubation at 30 °C, 1 ml of the culture containing 1 × 10¹⁰ CFU ml⁻¹ was inoculated into 100 ml of full (36 g fat l⁻¹) microfiltered milk (Marguerite) previously inoculated with *S. aureus* as described above.

L. garvieae N201, *L. lactis* N658 and *E. faecalis* N516 were pre-cultured in 9 ml of M17 broth for 18 h at 30 °C before their inoculation in TS buffered medium. Then the cultures were centrifuged to 9600 × *g* for 15 min at 4 °C (Sigma 3MK). The cell pellet was washed once in Ringer solution (Biokar) before inoculation at final concentration of 1 × 10⁸ CFU ml⁻¹ into TS buffered medium.

2.1.3. Culture media

S. aureus alone was cultivated in commercial full fat microfiltered milk (Marguerite, Villefranche sur Saône, ARNAS, France) at different initial pH values at 33 °C for 24 h. Milk was adjusted to pH 6.50, 6.25, 6.00, 5.78 and 5.60 with 90% lactic acid (Prolabo, Lyon, France), prior to inoculation.

The effect of the eight strains of LAB on *S. aureus* growth was tested in commercial full fat microfiltered milk (Marguerite, Villefranche sur Saône, ARNAS, France). In this milk was also studied the effect of addition of 10 μmol l⁻¹ of methionine, threonine, phenylalanine, isoleucine and valine (MERCK, Darmstadt, Germany) on *S. aureus* growth in co-culture with *L. garvieae* N201. These amino acids were selected as they were metabolized by *L. garvieae* N201 (see results in Table 2). They were prepared in distilled water and sterilized by filtration through a 0.45 μm-pore-size cellulose acetate filter (Minisart, Sartorius AG, Germany).

S. aureus was also co-cultivated with *L. garvieae* N201, *L. lactis* N658 or *E. faecalis* in TS medium containing 17 g l⁻¹ tryptone (Biokar Diagnostic, Pantin, France), 3 g l⁻¹ peptone (Biokar Diagnostic, Pantin, France), 6 g l⁻¹ yeast extract (Biokar Diagnostic, Pantin, France), 2.5 g l⁻¹ K₂HPO₄ (MERCK, Darmstadt, Germany), 5 g l⁻¹ NaCl (Prolabo, Lyon, France). It was buffered at pH 6.8 with phosphate buffer KH₂PO₄/K₂HPO₄ 0.1 mol (KH₂PO₄, Prolabo; K₂HPO₄, Merck) to avoid the effect of decreasing the pH value of the medium on the growth of *S. aureus*.

2.1.4. Conditions of incubation

The milk inoculated with *S. aureus* alone as well as all co-cultures in microfiltered milk and buffered medium were incubated in water baths at temperatures simulating the decrease in temperature recorded during the cheese making process of some semi-hard cheeses such as Saint-Nectaire cheese. The evolution of temperature during the incubation was automatically driven and recorded by the software of CINAC system (YSEBAERT, Frepillon, France). At 0 h, the temperature was 33 °C, and then gradually decreased to 30.5 at 3 h; 27.3 at 6 h, 24.6 at 9 h, 22.9 at 12 h, 21.0 at 18 h and to 19 °C after 24 h of incubation. pH values were recorded with CINAC (YSEBAERT). All the experiments were repeated three times for each strain of LAB.

2.2. *Staphylococcus aureus* and lactic acid bacteria counts

S. aureus and LAB strains were counted at 0, 3, 6 and 24 h. Milk and TS-buffered medium samples were homogenized with a stomacher Lab Blender (Seward Medical,

London, UK) for a time optimised at 4 min for dissociating all the cell aggregates. After appropriate dilution in Ringer solution, *S. aureus* was enumerated on Rabbit Plasma Fibrinogen Agar (RPFA) (De Buyser et al., 2003) incubated for 24 at 37 °C. LAB were enumerated on M17 medium (Biokar Diagnostic, Pantin, France) incubated for 48 h at 30 °C.

2.3. Chemical analyses

2.3.1. Lactic and acetic acid contents

L-Lactate, D-lactate and acetic acid contents in microfiltered milk and TS buffered medium were determined using the spectrometric method as recommended in the Kits EnzyPlus of Diffchamb (Kits EnzyPlus Diffchamb, SARL, Lyon, France). Results were expressed in g l^{-1} of microfiltered milk.

2.3.2. Amino acid analyses

To analyze amino acids in microfiltered milk cultures, the proteins were precipitated by adding four volumes of methanol to one volume of samples followed by overnight incubation on ice. Then the mixture was centrifuged and the supernatant was kept for amino acid analyses. Free amino acids in the medium were measured by HPLC (HP 1090, Hewlett-Packard, Waldbronn, Germany). The amino acids were automatically derived with OrthoPhtalic Aldehyde (OPA) (Sigma-Aldrich, Steinheim, Germany) and 9-fluorenylmethyl-chloroformate (FMOC-C1) (Sigma-Aldrich). The metabolites were separated on column Hypersil C18 (Thermo Hypersil, Cheshire, UK) at 35 °C by a linear gradient of acetate buffer (pH 7.2)-EDTA-triethyl aminetetrahydrofuranne and gradient of acetate buffer (pH 7.2)-methanol-acetonitrile.

Two detectors were used in parallel; the first one was a diode bar spectrophotometer at 338 nm for OPA derivatives and 262 nm for FMOC derivatives.

2.4. Antistaphylococcal properties of the supernatant

An agar well-diffusion assay, as described by Hernandez et al. (2005), was used for the detection of antistaphylococcal activity in the supernatants of co-cultures of *S. aureus* with *Lactococcus* or *Enterococcus* strains in TS buffered medium. The method was modified as following; M17 agar plates (1.5% agar) were overlaid with 10 ml of BHI agar (0.75% agar), inoculated with 100 μl of an overnight culture of *S. aureus*. The samples of 6 and 24 h old co-cultures of *Lactococcus* or *Enterococcus* strains with *S. aureus* in TS buffered medium were centrifuged to $9600 \times g$ for 15 min at 4 °C. The co-culture supernatants were concentrated to 1×10^{-1} of their original volume using an ultrafiltration spin column (Vivaspin, VIVASCIENCE, Hannover, Germany) with a 3000 Da pore size membrane. The concentrated supernatant was deposited in the agar plate wells. The zone of inhibition was visually examined.

2.5. Statistical analysis

pH values, lactate, acetate and amino acid contents in microfiltered milk at 3, 6 and 24 h of incubation, were analyzed by analysis of variance (ANOVA). When the differences were significant, a Newman Keuls test was performed. Statistical correlations between pH values and *S. aureus* counts in microfiltered milk were carried out by calculating Pearson's correlation coefficient. All the analysis was performed with the software StatSoft (StatSoft, Inc., Tulsa, OK, USA).

3. Results

3.1. Effect of initial pH values on *S. aureus* growth

The effect of initial pH value on *S. aureus* growth in microfiltered milk was evaluated (Fig. 1). The *S. aureus* count at 3, 6 and 24 h was similar for initial pH values between 6.0 and 6.65. After 6 h of incubation, the *S. aureus* counts were significantly lower in milk initially adjusted at pH 5.60 and 5.78 than those adjusted at pH values above 6.0. At 24 h, whatever the initial pH, there was not significant difference in *S. aureus* counts, even at pH 5.6.

3.2. Effect of lactic acid bacteria inoculation on *S. aureus* count

The effect of LAB strains on *S. aureus* counts in microfiltered milks is indicated in Table 1. Without LAB inoculation, *S. aureus* grew through the incubation period to $7.01 \log_{10} \text{CFU ml}^{-1}$ at 24 h. At 3 h, the level of *S. aureus* was significantly the highest in the medium inoculated with *S. aureus* alone. The lowest count similar to the level at 0 h was found in the media inoculated with the two *L. garvieae* strains (Tan1 or Tan2). At 6 h, the level of *S. aureus* was significantly lower in co-cultures with LAB strains. No growth occurred between 3 and 24 h in the media inoculated with *L. lactis* (N650, N658 and N688) or *E. faecalis* N516; whereas the *S. aureus* population increased by $3.5 \log_{10}$ in the media without inoculation and by less than $1.2 \log_{10}$ in those with *L. garvieae* strains.

There were significant differences in *S. aureus* counts after 24 h of incubation, depending on the LAB strains inoculated. At 24 h, the *S. aureus* counts in milks inoculated with *L. garvieae* strains (N201, Tan 1, Tan 2, and 1204) ($< 4.4 \log_{10}$) were higher than that in the medium with *L. lactis* (N650, N658 and N688) or *E. faecalis* N516 ($< 3.17 \log_{10}$) but significantly lower than that in the control without inoculation ($7.01 \log_{10}$).

At 6 h, the pH of milk inoculated with *L. lactis* or *E. faecalis* was significantly lower than that found in the two milk samples inoculated with *S. aureus* alone and with *S. aureus* and *L. garvieae* (Table 1). After 6 h of incubation pH values in milks inoculated with *L. garvieae* were lower than that inoculated with *S. aureus* alone, although the difference was not statistically significant. At 24 h, the pH

Fig. 1. Effect of pH values on *S. aureus* count in microfiltered milk after 3, 6 and 24 h of incubation at 33 °C. Values are the means of three experiments. At the same time, letters (a and b) indicate homogeneous statistical processing groups. Numbers with different letters are not equal; a and b are classified in decreasing order (a > b). For each time of incubation (0, 3, 6 and 24 h), means with different letters were significantly different by Newman–Keuls statistical test; the significant of the test (NS, non-significant; $P < 0.01$). pH values: 6.65; 6.5; 6.25; 6.0; 5.78; 5.60.

Table 1

S. aureus SA15 and lactic acid bacteria (LAB) counts with pH values following *S. aureus* SA15 and *Lactococcus* or *Enterococcus* strain co-culture in microfiltered milk

Time	<i>S. aureus</i>	Co-culture of <i>S. aureus</i> SA15 with different strains of LAB in microfiltered milk							
	SA15	N650	N658	N688	N516	N201	1204	Tan1	Tan2
<i>S. aureus</i> count									
0	2.22±0.04	2.17±0.12	2.19±0.12	2.15±0.07	2.14±0.23	2.19±0.18	2.14±0.14	2.17±0.06	2.15±0.13
3	3.48±0.28a	3.04±0.40ab	3.04±0.29ab	2.96±0.24ab	3.01±0.22ab	3.13±0.25ab	2.93±0.20ab	2.73±0.27b	2.72±0.15b
6	4.45±0.19a	3.20±0.23b	3.23±0.33b	3.16±0.37b	3.12±0.25b	3.58±0.18b	3.43±0.30b	3.07±0.31b	3.19±0.24b
24	7.01±0.04a	3.11±0.23c	3.06±0.17c	3.17±0.43c	3.18±0.29c	4.32±0.41b	3.85±0.36bc	3.94±0.72bc	3.66±0.36bc
Lactic acid bacteria counts									
0	0.00±0.00	8.09±0.12	8.11±0.08	8.17±0.22	8.20±0.20	8.05±0.08	8.14±0.13	8.12±0.12	8.15±0.08
3	0.00±0.00	8.96±0.21	8.89±0.43	9.00±0.20	9.01±0.23	8.69±0.36	8.62±0.38	8.57±0.32	8.70±0.27
6	0.00±0.00	9.28±0.19ab	9.42±0.16a	9.44±0.09a	9.70±0.28a	9.05±0.08ab	8.67±0.48b	9.10±0.36ab	8.98±0.28ab
24	0.00±0.00	9.90±0.46ab	9.52±0.28ab	10.07±0.4a	10.13±0.2a	9.38±0.5ab	9.04±0.25b	9.27±0.46ab	8.92±0.45b
pH values									
0	6.72±0.04	6.72±0.04	6.72±0.04	6.72±0.04	6.72±0.04	6.72±0.04	6.72±0.04	6.72±0.04	6.72±0.04
3	6.68±0.07a	6.17±0.07de	6.23 ±0.04cde	6.08±0.12e	6.16±0.08de	6.46±0.04b	6.46±0.03b	6.31±0.01cd	6.35±0.04bc
6	6.70±0.08a	5.60±0.27c	5.58±0.09c	5.26±0.38c	5.42±0.28c	6.41±0.05ab	6.41±0.02ab	6.24±0.03b	6.28±0.05ab
24	6.72±0.08a	4.37±0.09d	4.31±0.09d	4.36±0.13d	4.31±0.07d	6.28±0.04b	6.40±0.03b	5.91±0.13c	5.87±0.38c

The results of bacterial count are expressed as $\log_{10}$ CFU ml^{-1} of microfiltered milk. Values are the means of three experiments with each strain. In the same line, letters (a, b, c, d and e) indicate homogeneous statistical processing groups. Numbers with different letters are not equal; a, b, c, d and e are classified in decreasing order (a > b > c > d > e). For each time of incubation (0, 3, 6 and 24 h), means in the same line with different letters were significantly different by Newman–Keuls statistical test; the significant of the test (no letter, non-significant; $P < 0.05$).

value of the milk inoculated with *L. garvieae* was significantly lower than that found in milk with *S. aureus* alone.

3.3. Amino acid content and *S. aureus* count

Amino acids were quantified in the culture supernatants from microfiltered milk samples inoculated with *S. aureus* alone or with *L. garvieae* N201, *L. lactis* N658 or *E. faecalis* N516 (Table 2).

Arginine, cysteine and tryptophan were never detected in non-inoculated or inoculated milk at any time during incubation.

At 6 and 24 h, supernatants of co-cultures with *L. lactis* or *E. faecalis* strains were characterized by the highest levels of aspartic acid, glutamic acid, histidine, valine, methionine, isoleucine, phenylalanine, alanine, tyrosine, leucine, lysine, proline even if the level of valine, methionine and leucine tended to decrease between 6 and 24 h. The decrease in valine, methionine, alanine and leucine was particularly

Table 2

Concentration of amino acids ($\mu\text{mol l}^{-1}$) in microfiltered milk (0 h) or in the microfiltered milk inoculated with *S. aureus* SA15 alone or *S. aureus* SA15 with *L. garvieae* (N201), *L. lactis* subsp. *lactis* (N658), or *E. faecalis* (N516) at 3, 6 and 24 h

Time	0 h	3 h				6 h				24 h			
	Milk	<i>S. aureus</i> SA15	<i>SA15 in co-culture with</i>			<i>SA15 in co-culture with</i>				<i>SA15 in co-culture with</i>			
			<i>L. garvieae</i> N201	<i>L. lactis</i> N658	<i>E. faecalis</i> N516	<i>S. aureus</i> SA15	<i>L. garvieae</i> N201	<i>L. lactis</i> N658	<i>E. faecalis</i> N516	<i>S. aureus</i> SA15	<i>L. garvieae</i> N201	<i>L. lactis</i> N658	<i>E. faecalis</i> N516
Asp	15±2	11±2b	20±4b	63±2 a	59±11a	14±2b	25±3b	60±9a	64±13a	12±3d	25±5c	58±6a	45±3b
Glu	298±18	287±11	310±23	405±103	396±106	275±11b	289±11b	540±106a	595±97a	274±23c	282±22c	693±57a	590±72b
His	0±0	0±0b	0±0b	26±11a	4±8b	0±0b	0±0b	31±5a	6±11b	0±0c	3±6c	31±8a	20±4b
Gly	91±9	88±8	86±34	41±28	54±5	91±5a	62±13b	22±5c	61±17b	118±22a	43±11b	12±11b	44±16b
Val	17±2	18±1	26±2	64±48	53±31	17±0b	18±6b	99±26a	83±25a	17±1c	7±6d	55±3a	26±2b
Met	4±7	4±8	4±8	30±12	15±16	0±0b	0±0b	40±8a	20±18b	0±0b	0±0b	18±1a	0±0b
Ile	17±3	14±5	18±2	23±11	16±15	15±2b	10±9b	33±8a	28±5a	16±4ab	4±8b	21±7a	17±1ab
Phe	4±8	11±2b	14±1b	41±20a	43±8a	12±2c	12±2c	72±8a	56±9b	12±2c	3±6d	50±4a	28±3b
Thr	7±7	10±1	0±0	7±6	3±5	10±1a	0±0b	10±2a	4±8ab	3±5	0±0	10±1	10±9
Ser	19±3	19±4	27±6	49±39	31±18	19±3b	20±3b	47±15a	36±27ab	14±3	18±4	22±3	22±5
Ala	38±5	36±4	43±6	108±56	113±32	37±2b	35±7b	145±18a	156±74a	34±3	34±10	49±1	48±14
Tyr	0±0	0±0	0±0	79±56	22±39	0±0b	0±0b	148±17a	57±10b	0±0c	0±0c	114±5a	44±13b
Nva	58±5	61±5a	60±1a	61±11a	15±18b	64±11a	59±6a	61±4a	13±9b	66±7a	59±7a	61±6a	21±25b
Leu	12±2	14±1	21±2	103±104	94±54	16±4b	15±3b	203±29a	188±316a	14±2c	13±1c	124±9a	72±7b
Lys	0±0	0±0	0±0	130±132	109±96	0±0b	0±0b	265±2a	236±18a	0±0c	0±0c	268±18a	217±19b
Pro	0±0	0±0b	0±0b	364±151a	391±130a	0±0b	0±0b	552±152a	544±57a	0±0b	0±0b	492±143a	508±109a

Values are the mean of three experiments with each strain. In the same line for each incubation time, 3, 6 and 24 h, the letters (a, b, c and d) indicate homogeneous statistical processing groups for each amino acid for $P < 0.05$ or $P < 0.01$. Numbers with different letters are not equal; a, b, c and d are classified in decreasing order ($a > b > c > d$). For each time of incubation (3, 6 and 24 h), means in the same line with different letters were significantly different by Newman–Keuls statistical test; the significant of the test (no letter, non-significant). Limit of detection was $10 \mu\text{mol l}^{-1}$. Arginine, tryptophan and cysteine were never detected in microfiltered milk at 0 h or in inoculated milk with *S. aureus* alone or with LAB at any time during incubation. Asp, aspartic acid; Glu, glutamic acid; His, histidine; Gly, glycine; Val, valine; Met, methionine; Ile, isoleucine; Phe, phenylalanine; Thr, threonine; Ser, serine; Ala, alanine; Tyr, tyrosine; Nva, norvaline; Leu, leucine; Lys, lysine; Pro, proline.

marked between 6 and 24 h and was the strongest with *E. faecalis*. In contrast, the 6 and 24 h media inoculated with *S. aureus* alone or *S. aureus* and *L. garvieae* exhibited the lowest concentration of these amino acids. Histidine, tyrosine, lysine and proline were not detected in microfiltered milk at 0 h or during the incubation in the media inoculated with *S. aureus* alone. Glycine content decreased during incubation in media with *Lactococcus* or *Enterococcus* strains, markedly so with *L. lactis*. In medium inoculated with *L. garvieae*, valine, methionine, isoleucine and threonine contents tended to decrease throughout the incubation period, and were the lowest after 24 h. In medium inoculated with *S. aureus* alone, the amino acid contents of aspartic acid, glutamic acid, glycine, valine, isoleucine, serine, alanine, norvaline and leucine did not change a lot ($< 10 \mu\text{mol l}^{-1}$) throughout the incubation.

Adding $10 \mu\text{mol l}^{-1}$ of each amino acid, valine, methionine, isoleucine, phenylalanine and threonine to the microfiltered milk did not modify *S. aureus* or *L. garvieae* N201 growth and did not modify pH values. It did not suppress the inhibition by *L. garvieae* N201.

3.4. *S. aureus* count and the concentration of lactate and acetate in microfiltered milk

After 24 h of incubation, concentrations of D-lactate and of acetate were very low ($< 0.5 \text{ g l}^{-1}$) in microfiltered milk

inoculated with *S. aureus* and *L. lactis* N658 or *E. faecalis* N516. L-Lactate, D-lactate and acetate were not detected in microfiltered milk inoculated with *S. aureus* alone or in co-culture with *L. garvieae* N201. At 24 h, L-lactate was only measured in milk inoculated with strains of *L. lactis* or *E. faecalis* at concentrations 6.3 g l^{-1} (Table 3).

At 6 h, the *S. aureus* count was slightly correlated with milk pH values ($r = 0.29$; $p < 0.05$, $n = 27$). At 24 h, there was a positive correlation between the pH values and *S. aureus* count ($r = 0.74$; $p < 0.05$, $n = 27$).

3.5. Effect of *Lactococcus* or *Enterococcus* strains on *S. aureus* growth in buffered media

In TS medium buffered at pH 6.8, the differences in *S. aureus* count between the control and co-culture assays were not statistically significant 3 and 6 h after inoculation. The *S. aureus* count (around 4 log) was significantly lower only after 24 h of incubation in the medium inoculated with *Lactococcus* or *Enterococcus* strains than in that with *S. aureus* alone (5 log) (Table 4). The growth of *S. aureus* was lower in TS medium than in microfiltered milk (7 log at 24 h). The pH of all media remained stable at 6.8 throughout the incubation, with or without LAB. L-Lactate, D-lactate or acetate was not detected in the supernatants of cultures inoculated with *S. aureus* alone or with the LAB throughout the incubation.

Table 3

L-Acid lactic, D-acid lactic, acetic acid contents (g l^{-1}) in microfiltered milk inoculated with *S. aureus* SA15 alone and in co-culture with *L. garvieae* (N201), *L. lactis* subsp. *lactis* (N658), or *E. faecalis* (N516)

Time	L-Lactate			D-Lactate			Acetic acid		
	3 h	6 h	24 h	3 h	6 h	24 h	3 h	6 h	24 h
<i>S. aureus</i> SA15	0.01 ± 0.01b	0.02 ± 0.03b	0.01 ± 0.01b	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.01d	0.03 ± 0.01c	0.02 ± 0.01c
<i>S. aureus</i> SA15+ <i>L. garvieae</i> N201	0.39 ± 0.07b	0.49 ± 0.12b	0.90 ± 0.17b	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.01c	0.04 ± 0.02c	0.04 ± 0.00c
<i>S. aureus</i> SA15+ <i>L. lactis</i> N658	1.94 ± 0.89a	3.63 ± 0.60a	6.36 ± 2.05a	0.23 ± 0.01	0.19 ± 0.15	0.34 ± 0.19a	0.39 ± 0.03a	0.41 ± 0.02a	0.37 ± 0.03a
<i>S. aureus</i> SA15+ <i>E. faecalis</i> N516	1.31 ± 0.13a	4.42 ± 0.60a	6.37 ± 1.55a	0.21 ± 0.12	0.19 ± 0.08	0.12 ± 0.01b	0.12 ± 0.01b	0.14 ± 0.02b	0.17 ± 0.01b
P	**	**	**	NS	NS	*	**	**	**

Values are the mean of three experiments with each strain. In the same column, letters (a, b, c and d) indicate homogeneous statistical processing groups. Numbers with different letters are not equal; a, b, c and d are classified in decreasing order ($a > b > c > d$). For each time of incubation (3, 6 and 24 h), means in the same column with different letters were significantly different by Newman–Keuls statistical test; the significant of the test. NS, non-significant.

* $P < 0.05$.

** $P < 0.01$.

Table 4

Counts of *S. aureus* SA15, *L. garvieae* (N201), *L. lactis* subsp. *lactis* (N658), and *E. faecalis* (N516) in TS-buffered medium

Time (h)	<i>S. aureus</i> SA15 counts ($\log_{10}$ CFU ml^{-1})				Lactic acid bacteria counts ($\log_{10}$ CFU ml^{-1})			
	0	3	6	24	0	3	6	24
<i>S. aureus</i> SA15	2.25 ± 0.05	2.98 ± 0.30	3.44 ± 0.22	5.05 ± 0.27a	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
SA15+ <i>L. garvieae</i> N201	2.19 ± 0.12	2.84 ± 0.27	3.04 ± 0.30	3.78 ± 0.21b	8.12 ± 0.12	9.25 ± 0.57	9.28 ± 0.59	9.46 ± 0.39
SA15+ <i>L. lactis</i> N658	2.24 ± 0.09	2.88 ± 0.42	3.10 ± 0.35	3.77 ± 0.05b	8.14 ± 0.21	9.27 ± 0.43	9.33 ± 0.33	9.57 ± 0.38
SA15+ <i>E. faecalis</i> N516	2.22 ± 0.11	3.00 ± 0.11	3.35 ± 0.16	4.06 ± 0.17b	8.09 ± 0.19	9.22 ± 0.41	9.23 ± 0.21	9.29 ± 0.47

Values are the mean of three experiments with each strain. In the same column, letters (a and b) indicate homogeneous statistical processing groups; a and b are classified in decreasing order ($a > b$). For each time of incubation (3, 6 and 24 h), means in the same column with different letters were significantly different by Newman–Keuls statistical test; the significant of the test (no letter, non-significant, $P < 0.05$).

The concentrated supernatants of the co-culture of *L. garvieae* N201, *L. lactis* N658 and *E. faecalis* N516 with *S. aureus* in TS buffered medium after 6 and 24 h of incubation did not produce any inhibition zone against *S. aureus* tested on agar plate according to the method of Hernandez et al. (2005).

4. Discussion

S. aureus was inhibited by *L. lactis*, *E. faecalis* and *L. garvieae* from 6 h of incubation in microfiltered milk and only at 24 h in TS medium buffered at pH 6.8. pH values can be involved in the inhibition by *L. lactis* and *E. faecalis* as the pH drop at values inhibiting *S. aureus*. pH has been often described as an important factor to control *S. aureus* in cheese. Indeed, Meyrand et al. (1999) reported that *S. aureus* was inhibited in lactic goat cheeses with a pH 4.5 at the end of draining. In the same way, the growth of *S. aureus* in non-cooked semi-hard cheeses was dependant on the pH values (Delbes et al., 2006). Stecchini et al. (1991) indicated that the inhibitory effect of the starter culture on *S. aureus* growth was not only due to the decrease in pH.

Other factors can be involved in the inhibition as this occur at pH 6.8, especially with *L. garvieae*. Moreover,

L. garvieae produced slight quantity of lactate and no acetate.

In our study, the inhibition could not be due to the production of antistaphylococcal substances in supernatant. Strains of *Enterococcus* and *L. garvieae* were able to produce bacteriocin, respectively, enterocin (Giraffa, 2003; Leroy et al., 2003) and garviecin L1-5 (Villani et al., 2001). Nevertheless, as in our study, Ammor et al. (2006) failed to detect antimicrobial substances in culture supernatants inhibiting *S. aureus* growth.

The amino acid content was not a limiting factor for *S. aureus* growth. Some aminoacids (histidine, tyrosine, lysine, proline, arginine, tryptophan and cysteine) were in low concentration in milk and nevertheless the growth was better than in TS medium with high amount of free amino acids. Complementation of milk with aminoacids metabolized by *L. garvieae* did not suppress the inhibition.

Our result did not agree with the amino acid requirement described in the literature. These disagreements between our results and those from other studies could result from differences in culture media and incubation conditions. Lincoln et al. (1995) found that seven *S. aureus* strains required arginine, proline, cystine, valine, leucine and glycine for their growth but they performed their study in a chemically defined medium at 37 °C. The same result was

obtained by Onoue and Mori (1997) in a chemically defined medium. Keller et al. (1978) observed that *S. aureus* could utilize glutamate, proline, histidine, aspartate, alanine, threonine, serine or glycine as a major energy source.

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